ANTIFUNGAL ACTIVITIES OF THE PHYLLOSPHERE ORGANISMS

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ABSTRACT

While studying the activities of nitrogen fixing organisms thriving on leaf surfaces of rice and jute plants, a number of bacterial isolates showing antifungal properties were encountered. Species of *Penicillium*, *Mucor* and *Rhizoctonia* are more affected than those of *Macrophomina*, *Fusarium*, *Aspergillus* and *Colletotrichum*. Cultures, upto five days old, effectively produce the active principles which diffuse into the medium but are very unstable and heat labile. Medium composition decisively controls the production of the active substances.

INTRODUCTION

AN ever increasing concern on the leaf surface organisms for their potentiality to fix nitrogen from the atmosphere led to study the effect of nitrogen fixing bacterial flora on the phyllosphere of some selected crops. The natural microbial inhabitants of the leaves have at least triple beneficial role on the host plant. Several experimental findings[1–5] indicate that phyllosphere nitrogen fixers may play a role in the nitrogenous nutrition of higher plants. In addition, these bacteria also yield growth regulating substances[6] and some antimicrobial substances. In the present paper the latter aspect is reported.

In general the inhibition of microbial invaders on leaf surfaces by a natural process may be due to any one or more of the following reasons: direct parasitism of a group to the invading ones, formation of antibiotic substances and toxic metabolites, release of acid substances making an unfavourable pH for growth, competition for nutrients or stimulation of the host's defence mechanisms. In the isolation procedures, fungal inhibition zones on potato dextrose agar (PDA) plates, due to the phyllosphere bacteria, were often noticed and this led to investigate the possible production of antifungal substances by a group of bacteria. Incidentally such isolates were all nitrogen fixers as were evident from their ability to grow in nitrogen-free medium and their capacity to increase nitrogen content of the broth medium as estimated by Microkjeldahl method. Production of antifungal antibiotic substances by leaf surface bacteria are not well reported[7]. However those which have been reported are mostly *Pseudomonas* sp. or *Bacillus* sp., none of which is a nitrogen fixer. Only Newhook[8] reported antibiotic producing property of *Bacillus*, *Pseudomonas* and *Chromobacterium* against *Botrytis cinera*.

Leben and Daft[9] were successful in isolating an antifungal peptide antibiotic from *P. antimycetica*. Filipek and Powell[10] also extracted some antibiotics from *P. morsprunorum*. More recently Lakshmi-Kumari *et al.*[11] reported production of antifungal antibiotic active against *Fusarium moniliforme* by *Azotobacter* sp. and earlier Mishustin *et al.*[12] also showed that this genus is capable of producing an antifungal antibiotic. Swinburne *et al.*[13] obtained at least two antifungal and eight antibacterial antibiotics from a strain of *B. subtilis* isolated from apple leaf scar tissue. From the above investigations it is clear that no nitrogen fixing organism, indigenous to the leaf surface and capable of inhibiting the growth of fungi has ever been isolated.

MATERIALS AND METHODS

Mature leaves of various crop plants were collected from the fields and brought to the laboratory inside sterile alkathene bags within two hours of collection. Organisms were isolated both by leaf impression and by leaf washing methods[14]. Plating was made on Burk's agar for the isolation of nitrogen fixers. Incubation was carried out at 30° C for 4 days. For screening of antibiotic producers, the inoculum of the isolated nitrogen fixers were obtained by growing them in potato-dextrose (PD) broth. Spores of test fungi were incorporated in molten PDA and plated. After solidification the plates were streaked with the test organism and incubated at 30° C for 5 days. Altogether four inhibition-zone-forming isolates were chosen for further studies. Spectrum of activities against various fungi was studied by incorporating different fungal conidia on PDA plates and subsequent cross-streaking.

To find out a relation between efficiency of antifungal activity and age of the inhibiting organisms, the isolates were grown in nitrogen-free Burk's broth medium upto 8 days period. Inoculum was obtained each day from the broth upto eight consecutive days. Streaking was made on PDA plates previously incorporated with conidia of *P. notatum*. The zones

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of inhibition were measured after 7 days of incubation.

To study whether the antibiotic principles produced are released into the medium, the isolates were grown in nitrogen-free Burk's broth and the growths were filtered through G_5 sintered glass bacterial filters. The culture filtrate was tested by placing it in shallow wells cut on PDA plates preincorporated with *Penicillium notatum* NCIM 749, along with the bacterial cells left on filter beds and the original cultures as controls.

Diffusion properties of the antibiotic principles were examined with slices of agar blocks cut off from the inhibitory zones developed on 24 hour old plates of *P. notatum* due to the test organisms. These slices were placed further on PDA plates preinoculated with *P. notatum*. In another procedure PDA plates supporting a good growth of antibiotic producers were scrapped off of the bacterium and then inverted on the upper lid with 2 to 3 ml chloroform and left as such for 30 minutes. Subsequently the plates were made free of chloroform by evaporating chloroform at 45° C under air current. These plates were now inoculated with the test fungi and incubated for 7 days.

A relationship between the medium composition and the antibiotic producing capability was also studied by inoculating nutrient agar, malt agar, PDA and Czapeck Dox agar with *P. notatum* and then cross-streaked with the bacterial isolates. For determining thermal stability 48 hours old bacterial cultures were treated in a water bath at 30°, 40°, 50° and 60° for 1 hour and then cross-streaked on PDA plates containing *P. notatum*.

Results

Two efficient nitrogen fixers isolated from rice plants (identified as *Azotobacter* sp. and *Beijerinkia* sp.) and one each from jute (*Micrococcus luteus*) and sugarcane (*Flavobacterium* sp.) were selected for further studies. Spectrum of activity was studied against 26 species of fungi of which *P. notatum* and *Mucor hiemalis* were inhibited by all the strains tested. *Rhizoctonia*, *Macrophomina*, *Fusarium*, *Aspergillus* and *Colletotrichum* were less affected. The isolates from rice showed a broader spectrum of activity while other isolates showed restricted activity. Of the 26 species tested 17 species were not affected and remaining 9 were inhibited to various degrees as shown in Table I. Those which were not affected are *Alternaria*

Table I

List of fungi inhibited by bacterial isolates showing degrees of inhibition

Test organism	Days	Zones of inhibition (in mm) due to the bacterial isolates			
		REN_2	PEN_2	JN_2	SN_2
Penicillium notatum NCIM 749	2	20	18	26	16
	7	20	18	26	16
Aspergillus niger	2	8	4	4	—
	7	8	4	4	—
Mucor hiemalis NCIM 872	2	12	8	8	4
	7	12	8	8	4
Helminthosporium sativum	2	4	—	—	—
	7	4	—	—	—
Fusarium oxysporum	2	8	—	—	—
	7	8	—	—	—
Rhizoctonia bataticola	2	12	8	4	—
	7	12	—	—	—
Alternaria crassa	2	8	4	—	—
	7	8	—	—	—
Macrophomina phaseoli	2	4	4	—	—
	7	4	4	—	—
Colletotrichum lunata	2	4	2	—	—
	7	4	2	—	—

— : no inhibition; REN_2 : Efficient nitrogen fixers from rice leaves; PEN_2 : Poor nitrogen fixers from rice leaves; JN_2 : Nitrogen fixers from jute leaves; SN_2 : Nitrogen fixers from sugarcane leaves.
Medium used was PDA.

solani, *Helminthosporium oryzae*, *Fusarium udum*, *Aspergillus flavus*, *A. flavipes*, *Absidia* sp., *Sordaria* sp., *Neurospora crassa*, *Candida albicans*, *Rhizoctonia solani*, *R. bataticola*, *Alternaria brassicola*, *Ustilaginoidae virens*, *Microsporum canis*, *M. albicans* and *Trichophyton cutisum*. Table II shows that with the increasing age of the cultures, the antifungal activities were reduced, and finally almost inactive after 7 days but if these cultures were transferred to a fresh medium then again the activities were resumed. Gradual decrease in such antibiotic response may be explained by the fact that the number of actively metabolising cells were reduced with aging of the culture. When a senescent culture was transferred to a fresh medium the cells were rejuvenated. Each rejuvenated cell could have the potentiality to produce the antibiotic principles. It is obvious from Table III that the maximum activity lies with the culture as a whole but when the organisms are collected from the filter bed and streaked, the activity is reduced while in the filtrate there is no activity. However, the original culture and the filtrate showed no difference in their pH value. Agar slices cut off from inhibition zones and placed on fresh plates with fungal conidia demonstrated little activity. Similarly after killing the bacterial cells with chloroform and subsequent spreading with sensitive fungal inoculum there was no inhibition of growth. Of the 4 media tested it was observed that PDA and Czapeck Dox agar favoured the formation of antifungal substances while in malt and nutrient agar no active substance was produced (Table IV). Pretreatment of the cultures at various temperatures showed a gradual decrease in activity with increasing temperature, being completely lost at 60° C (Table V).

TABLE II

Inhibitory activity and its dependence on the age of the isolates

Test organism : *Penicillium notatum* NCIM 749 grown on PDA

Age of the cultures (hr)	Inhibition zones (mm) due to bacterial culture			
	REN_2	PEN_2	JN_2	SN_2
24	22	18	26	16
48	20	16	24	12
72	16	12	20	10
96	12	10	16	10
120	12	4	16	8
144	8	4	12	4
168	6	2	6	4
196	0	0	0	0

For abbreviations refer to Table I.

TABLE III

Distribution pattern of the active principle of the culture

Test organism: *Penicillium notatum* NCIM 749 grown on PDA

Source of the material used for streaking	Zones of inhbition (in mm) from the bacterial isolates			
	REN_2	PEN_2	JN_2	SN_2
Original bacterial culture	21	18	25	16
From filter bed	16	11	18	12
From filtrate	—	—	—	—

—: No activity.

For abbreviations refer to Table I.

TABLE IV

Dependence of antibiosis on media composition

Test organism : *Penicillium notatum* NCIM 749

Medium used	Inhibition zones (mm) for the different bacterial isolates			
	REN_2	PEN_2	JN_2	SN_2
Potato-dextrose Agar	20	16	24	16
Czapeck-Dox Agar	26	24	8	8
Malt Agar	—	—	—	—

— : No activity.

For abbreviation refer to Table I.

TABLE V

Thermal stability of the antibiotic principles

Test organism : *Penicillium notatum* NCIM 749

Bacterial cultures	Zones of inhibition (mm) due to streaking of culture preheated at temperature (° C)			
	30°	40°	50°	60°
REN_2	20	16	8	—
PEN_2	18	12	4	—
JN_2	24	18	4	—
SN_2	16	10	2	—

— : No activity.

Discussion

An involvement of antibiosis between plant pathogen and the saprophytic microflora on leaf surface is well documented[15, 16]. Often it has been experimentally shown[16] that disease severity is greatly increased when a pathogen is reintroduced into its presterilised infection site like leaves, indicating that the saprophytic micro-organisms inhabiting the surfaces of the plants may serve as a biological buffer zone, thus preventing the pathogen from infecting the host. But many such studies were concerned with antibacterial substances whereas studies on antifungal activities are relatively rare. The scarcity of examples of biological control of fungal pathogens of aerial plant parts is possibly due to the emphasis placed on the control of air-borne pathogens by fungicides[17]. However the increasing awareness of the side effects of the fungicides on ecosystem and growing interest in pesticide-free agricultural products suggest that the biological control and fungicidal treatments should be considered in terms of the most effective methods of disease control in relation to the ecological damage which may result. Reports of bacteriocin production for biological control by Kerr and Htay[18] and findings of Michael and Nelson[19] of some antifungal activity involving a pseudomonad in carnations are there. But in our study the organisms tested are found to be simultaneously nitrogen fixers and producers of antifungal principles, although the spectrum is low. A single organism with such dual role would be highly desirable for agricultural application. From our experimental findings it seems that the substances produced are unstable and very little quantity is diffused into the medium but it is quite likely that in their natural habitat they are capable of inhibiting spread of fungal organisms which may compete for nutrients on the leaf surface[20].

By producing antifungal substances, they create a situation in which competition with fungi may be reduced thus resulting in a congenial condition for greater availability of nutrients from the host leaves to the nitrogen fixers.

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