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POSSIBLE MECHANISM OF SYMPTOM INHIBITION OF BACTERIAL BLIGHT OF RICE BY AN ENDOPHYTIC BACTERIUM ISOLATED FROM RICE[1,2]

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Abstract

Mixed inoculation of *Xanthomonas oryzae* with individuals of a group of endophytic bacteria in rice and *Xanthomonas oryzae* inhibited symptom development most efficiently as compared to mixed inoculation with other groups of endophytic bacteria or with saprophytic antagonists of *Xanthomonas*. Representative isolates were selected from each group in our studies. Significant symptom inhibition was found only when mixed inoculation or simultanenous inoculation were carried out at the same site. The endophytic bacteria must be inoculated first in the later case. The time interval between the two inoculations must not exceed six hours. *In vivo* survival studies showed that in leaves mixedly inoculated with 10R (symptom inhibiting endophytic bacteria) or with *Bacillus subilis* N210 (antagonistic saprophytic bacteria), *X. oryzae* attained a high population later than inoculation of *X. oryzae* alone or mixed with 6S which does not have symptom inhibition ability. Endophytic bacteria multiplies well in rice whereas the saprophytic bacterium does not. 10R was found to produce a bacteriostatic activity against *Xanthomonas* in vitro. It is suggested that in mixed inoculation of *X. oryzae*, the inhibitory activity produced by 10R impedes initial multiplication of *X. oryzae* in rice; thereby inhibiting symptom development.

Introduction

Interactions of microorganisms in nature and in vitro are frequently observed. Interference with the initiation of successful infection has been reported when testing mixed inocula of closely related forms of viral, bacterial or fungal pathogens; or virulent and avirulent organisms, or saprophytes and pathogens (Goodmans 1965; Shinde 1974). Hsieh and Buddenhagen (1974) reported that the suppression of leaf blight occured when equal or higher concentration of *Erwinia herbicola* was inoculated with *Xanthomonas oryzae*.

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They suggested *E. herbicola* may shift the pH to unfavorable levels and nutrients become limiting to *X. oryzae* or that a phytoalexin reaction is involved. Reddy and Kauffmann (1974) also found a reduction of disease when mixed inocula of avirulent and virulent strains of *X. oryzae* were applied to BJI rice variety, they explained the phenomenon by means of competition for infection and multiplication sites or the induction of a phytoalexin-like reaction. Erskine and Lopatecki (1975) also investigated the interactions between *E. amylovora* and related saprophytic bacteria both *in vitro* and *in vivo* condition, they found in their system, inhibition was not due to pH. They pointed to the possibility of production of an inhibitory substance by the saprophyte. Huang and Chang (1976) isolated, categorized and screened a number of endophytic bacteria from rice and they found a group of bacteria exhibited pronounced symptom inhibition ability. In this studies, 10R, a white gram-negative endophytic bacterium was selected as the representative of this group of bacteria. Using this system, further investigation of the nature of the inhibitory effect and its mechanism was carried out.

Materials and methods

Bacteria

Xanthomonas oryzae (isolate 604) was used in most experiments. 604S, a streptomycin mutant originating from 604 was used in population studies and where specified. 604S was indistinguishable from the parent isolated in virulence and in growth properties. Endophytic bacteria in rice were isolated from root and stem. Four representative isolaes were selected. Among them, 10R exhibits symptom inhibition ability; but the other three isolates (12S, 6S, 27R) do not (Huang and Chang, 1976). *Bacillus subtilis* N210 which is antagonistic to *X. oryzae* was isolated from soil (Huang and Chang 1975). *Escherichia coli* and the other plant pathogentic bacteria such as, *Agrobacterium tumefasciens* and *Pseudomonas syringae* were provided by our laboratory.

Culture

All bacteria were grown on potato sucrose (PS) media. Streptomycin mutant was grown on PS media supplemented with 0.5% streptomycin sulfate (w/v). Bacteria used for experiments were grown on slants for 24 hr and were washed from slant surface with sterile distilled water. Bacterial suspensions were adjusted to the required concentration turbidimetrically by using a spectrophotometer (Gilford 2004S) at 525 nm. *In vivo* studies all of the inocula were adjusted to concentration of 10^8 cells/ml.

Inoculation

Leaves of rice plant cultivar IR8 grown in a greenhouse were inoculated by a single needle prick method at maximum tillering stage. Inoculated plants were kept in a greenhouse at 28 to 35°C. Observations for appearance of symptoms were made daily starting 4 days after inoculation.

In vitro assays of bacteriostatic activity

Double agar layer method was used for assaying the presence of bacteriostatic activity. The bacteria to be assayed was first plated. The microorganism to be tested for production of the antibiotic was spooted in the center by using a transfer loop. Concentration of 10^8 cells/ml was used. Culture filtrate was assayed by the filter paper disc method (0.1 μl aliquots were used).

Extraction of bacteriostatic activity

Three-day-old 10R culture was centrifuged at 8,000 g for 5 min. Bacterial pellet was discarded. Supernatant was sterilized by passing through a 0.45 μ millipore filter (Selectron). The culture filtrate was lyophilized to dryness and resuspended in minimal amount of sterile distilled water. The resuspended filtrate was then assayed for activity.

Preliminary partial characterization of bacteriostatic substance

The extracted bacteriostatic substance was passed through a Sephadex G-25 column, and eluted with 0.02 M tris-HCl buffer at pH 7.5. Blue dextran was used to determine the void volume. Bromophenol blue was used as a marker for low molecular weight substances. All dyes were prerun before the sample was applied. Each fraction 2.5 ml was assayed for bacteriostatic activity. Heat stability was tested by heating the concentrated culture filtrate at 60°C for 10 min. Pronase sensitivity was tested by incubating equal volume of concentrated culture filtrate and pronase (1 mg/ml) for 30 min at room temperature.

Results

Nature of the inhibition effect

Effect of inoculation site and time on symptom inhibition was studied (Table 1). When 10R was first inoculated to rice leaves by the single needle prick method, followed immediately by inoculation of 604 at the same site; the percentage of infection was considerably reduced as compared to 604 inoculated alone (% infection was rated as the number of plants showing symptoms out of total number of plants inoculated). Percent of infection of simultaneous inoculations was lower than that of mixed inoculation. This was probably

due to the spatial interference of 10R, 604 entry into the intercellular spaces became more difficult. Simultaneous inoculations at different sites were ineffective. To see whether symptom inhibition is due to induced resistance in the plant, 604 was inoculated at same or different site at 72 hr after 10R inoculation. No inhibition was observed in both cases. The nature of the effect was further examined by a series of time intervals and order of

Table 1. *Effect of site and time of the inoculation of 10R on the infection of 604*

Inoculum		Site of Inoculation	Time Interval in hours	% infection(2)
604		—	0	100
604+10R		—	0	68
10R	604	same site	0	37
		same site	72	100
		different site(1)	0	97
		different site	72	100
H_2O	604	same site	72	100
		different site	72	100

(1) Second inoculation site is 1 cm from the first.
(2) Infection rate 10 days after inoculation, % infection rated as number of plants showing symptoms out of total number of plants inoculated.

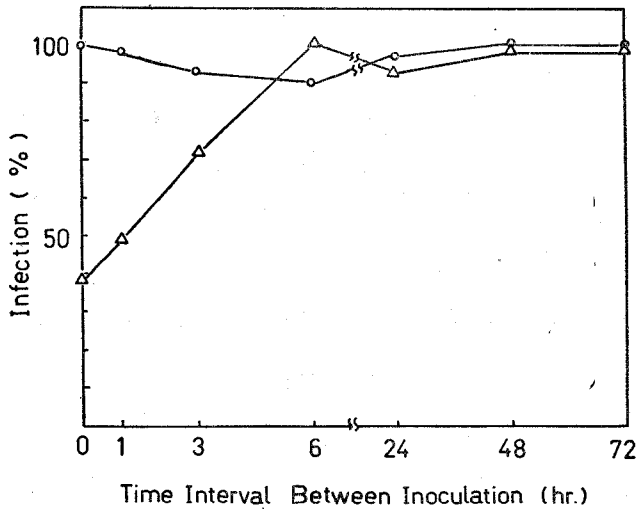

Fig. 1. Percent of infection of *X. oryzae* and 10R at different order and time intervals. All inoculation was done at the same site. (○——○) *X. oryzae* inoculation was followed by 10R inoculation at the time interval indicated. (△——△) 10R inoculation was followed by *X. oryzae* inoculation at the time interval indicated. Infection was rated 10 days after inoculation. Percent infection was rated as number of plants showing symptoms out of total number of plants inoculated.

inoculation experiments (Fig. 1). 604 was first inoculated at different time intervals after first inoculation, 10R was then inoculated at the same site. No reduction in infection was observed in all cases. When the order of inoculation was reversed, with 10R inoculation followed by 604; significant reduction of disease was observed at 0 time, 1 hr and 3 hr. Percent of infection increased as time interval increased between the two inoculations. When the time interval exceeded 6 hr, disease development was not reduced.

In vivo population studies

604S alone, mixed inocula of 604S with 10R or N210 or 6S were inoculated to surface sterilized IR8 rice leaves. Isolation and population changes of each bacterium within the leaf tissue was estimated at periodical intervals after inoculations (Fig. 2). Symptom development was also rated. Isolations and population estimations were made by grinding up surface sterilized leaf samples and serial dilution plating. Leaf samples consisted of 3 cm of leaf length on each side of the needle prick site. For mixed inoculated samples, isolation was made on streptomycin media to estimate 604S population and on regular media for nonpathogenic bacteria. Population of 604S inoculated alone increased rapidly to a maximum of 10^6 cells/ml per 6 cm leaf length in 9 days, after which no further multiplication occurred. The population of 604S in leaves mixedly inoculated with N210 increased slowly and gradually, reaching 10^6 cells/ml/per 6 cm leaf length by 12 days. N210 did not multiply during the first 2 days and its population increased slowly and gradually, reaching a

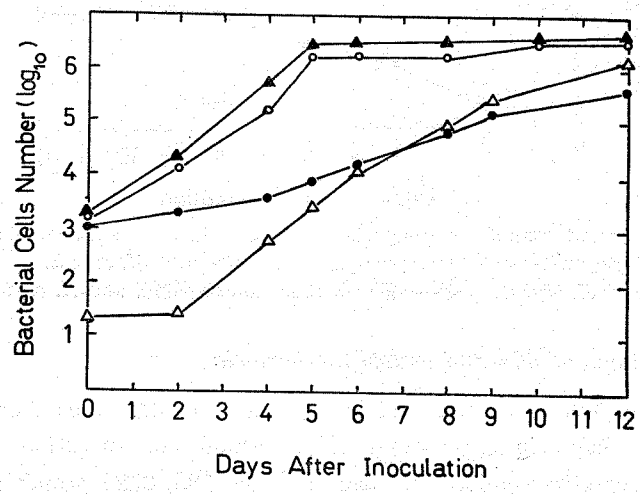

Fig. 2. Population changes of *X. oryzae* in rice leaves mixed inoculated with other bacteria after inoculations. (▲——▲) *X. oryzae* alone. (○——○) *X. oryzae* and 6S. (△——△) *X. oryzae* and 10R. (●——●) *X. oryzae* and *Bacillus subtilis* N210.

maximum of 10^2 cells/ml/per 6 cm leaf length in 8 days. In leaves mixedly inoculated with 604S and 10R, the population of 604S at 0 time was pronouncedly lower than the others. There was a lag phase of 2 days, after which it multiplied rapidly; reaching 5×10^5 cells/ml/per 6 cm leaf length by 12 days. Symptom appearance seemed to be somewhat related to 604S population. Symptom appeared when 604S population reached 10^5 cells/ml per 6 cm leaf samples. Single inoculation of 604S or mixed with 6S showed 86% and 78% infection respectively in 4–6 days. Hundred percent infection was attained in 8–9 days. In mixed inoculations of 604 and N210, 89% infection was found in 10 days. Disease severity was rated as follow: wilted plants as severe, lesion size greater than 2 cm was rated as moderate and lesions smaller than 2 cm as mild. In normal inoculation of 604S, 25% of inoculated plants showed severe infection, 50% showed moderate infection and 25% showed mild infection at 12 days. In mixed inoculation with 10R, 52% infection was observed at 10 days. At 12 days, 12.5% of infected plants showed severe symptoms, 12.5% showed moderate symptoms and 75% showed mild symptoms.

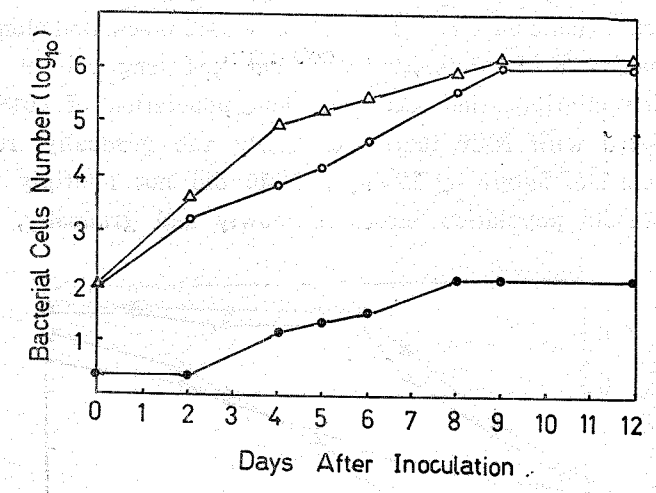

Fig. 3. Population changes of nonpathogenic bacteria in rice leaves mixed with *X. oryzae* after inoculations. (▲——▲) 10R and *X. oryzae*. (○——○) 6S and *X. oryzae*. (●——●) *Bacillus subtilis* N210. and *X. oryzae*.

In vitro interactions of 604 and endophytic bacteria

Both 604 and 10R were streaked in separate parallel lines 1 cm apart on PS plates. Both bacteria grew vigorously. Equal concentration of 604 was mixed with 10R or other endophytic bacteria (6S, 12S, 27R) which do not have symptom inhibition ability. The mixtures were then plated on PS media after serial dilutions. Abundant 604 colonies were recovered in mixture with other endophytic bacteria, but no 604 colonies were recovered in mixture with 10R.

The other endophytic bacteria and 10R were grown separately in shake culture for 3 days at 28°C. Filter sterilized culture filtrates were obtained and equal concentration of 604 was grown in the different filtrates. No significant difference in 604 population was detected in the different culture filtrates. 604 was singly and mixed inoculated with 10R or 6S in shake culture at 28°C. Population changes of each bacterium in each case were estimated by dilution plating periodically. 604 population attained 10^8 cells/ml at different time in the different cultures: 18 hr in single culture of 604 and 6S, 48 hr in mixed culture of 604 and 10R.

Detection of bacteriostatic activity from 10R

Different concentration of 604 were plated on PS media using the double agar layer method. A loop of 10R suspensions (10^8 cells/ml) was spotted in the center of each plate. The same procedure was repeated with the other endophytic bacteria. A zone of inhibition of width of 5 mm was detected on plates with 604 cell density lower than 5×10^5 cells/ml. No zone was found with the other endophytic bacteria. However the zone disappeared in 2 days as 604 grew towards 10R in the center. The spectrum of this bacteriostatic activity was examined (Table 2). The activity was detected against all the *Xanthomonas* tested and *P. syringae*, no activity was found against *E. coli*, *A. tumefasciencs* and the other endophytic bacteria tested.

Table 2. *Bacteriostatic activity spectrum of 10R*

Bacteria(1)	Group	Zone of Inhibition
Escherichia coli	—	—
Agrobacterium tumefasciens	Plant Pathogen	—
Pseudomonas syringae	Plant Pathogen	+
Xanthomonas oryzae (604)	Xanthomonas	+
X. citri	Xanthomonas	+
X. pruni	Xanthomonas	+
X. manihotis	Xanthomonas	+
27R	Endophytic bacteria from rice	—
12S	Endophytic bacteria from rice	—
6S	Endophytic bacteria from rice	—

(1) The bacteria tested was first plated by the double layer method, 10R was then spotted in the center.

Preliminary studies on the bacteriostatic substance from 10R

Zone of inhibition was found when concentrated 10R culture filtrate was assayed for activity. The active filtrate was passed through a Sephadex-G-25

column. Activity was detected in the fraction corresponding to void volume and the fractions corresponding to and immediately after the bromophenol blue fraction. The concentrated culture filtrate was also tested for heat stability and pronase sensitivity. Activity was lost upon heat 60°C, but not when pronase treated.

Discussion

Our studies on the nature of the symptom inhibition effect indicate that time of entry and proximity of the two interacting microorganisms, 604 and 10R, are limiting factors for inhibition of disease development since time interval between inoculations exceeding 6 hr or inoculation at different sites did not inhibit symptoms. Furthermore, the order of inoculation experiments showed that if 604 entered the tissue before 10R; or that 10R has migrated some distance from the inoculation site and 604's entry was not hindered, rapid disease development was not prevented. In another experiment, 604 was isolated 9 cm above the inoculation site, but 10R was not (unpublished data). Once 604 moves away from the endophytic bacteria, it multiplies freely and then symptoms develop. In mixed inoculation, the inhibition was present at 0 time and its effect decreased rapidly with time. Therefore induced resistance or production of phytoalexin is unlikely. Moreover 10R is endophytic in rice, it probably would not be able to survive in the tissue if a host defense mechnism is elicited.

Symptom development seems to correlate with population of 604 in rice leaves. 604 in singly inoculated leaves attained a population level of 10^5 cells/ml in 4 days and symptoms also appear in 4–5 days. Similar survival curve was obtained in leaves mixed inoculated with 604 and 6S, therefore competition for nutrient is not a factor in disease reduction. However, in leaves mixedly inoculated with 604 and 10R or N210, 604 population attained a high level much later. 604 in leaves mixed inoculated with N210 multiplied slowly and gradually. Mode of action of the antibiotic produced by N210 is unknown, but its effect apparently is slow. 604 population was much lower at 10 time in leaves inoculated with a 1:1 mixture of 604 and 10R, although inoculum concentration of all bacteria were adjusted to 10^8 cells/ml. Therefore an inhibitory substance against 604 must be present at 0 time. Its activity persisted for 2 days since there was a lag in 604 multiplication, after which the population increased rapidly. N210 is saprophytic, it does not grow well in rice leaves whereas endophytic 6S and 10R multiply rapidly. The inhibitory substance not only restrain 604 multiplication, in addition it probably plays another role in symptom inhibition because leaves mixedly inoculated with 10R exhibited more mild symptoms than leaves mixedly inoculated with N210. Also disease symptom

appeared 3 days later in the former case even though similar 604 population level was attained in 9 days.

In vitro studies support the premise that an inhibitory substance is produced by 10R. When 604 was plated with 10R, no 604 colonies can be recovered, but not when plated with other bacteria with no symptom inhibition ability. *In vitro* population studies also revealed that 604 multiplication was more affected by the presence of 10R than 6S. Bacteriostatic activity was detected when high concentration of 10R was spotted in the midst of 604 bacterial lawn of cell density lower than 5×10^5 cells/ml. The activity detected is weak and is present in low concentration, concentrated culture filtrate was required for its detection. It does diffuse a great distance. This explains why no antagonism was observed in parallel streak culture of the organisms. Preliminary studies on the activity showed that it is specific against *Xanthomonas* and *P. syringae.* This agrees with our preliminary host specificity test, 10R also inhibits symptom of *X. citri* in citrus and *X. manihotis* in cassava when mixedly inoculated. The activity has a molecular weight over 5,000 since it was eluted from G-25 Sephadex column at void volume. The inhibitory substances eluted at low molecular weight fractions was probably a salt effect since the culture filtrate was highly concentrated. However the possibility of more than one bacteriostatic activity produced by 10R cannot be completely abandoned. The activity of bacteriostatic substance is heat labile and pronase insensitive.

Other possible mechanisms for symptom suppression have also been considered. Some workers have found that the symptom inhibiting organism changes the pH, the environment thus becomes unfavorable for growth of the pathogen (Goodman, 1965). However, we did not detect any significant change in pH in liquid media incubated with 10R (unpublished data). Spatial interference as proposes by Lippincott and Lippincott (1969) is not the case in the system since UV treated or heat killed 10R did not suppress symptoms when mixedly inoculated with 604 (Huang and Chang, 1976). The possibility of gene transfer seems unlikely in this case. Using sucrose density gradient centrifugation, 604 can be separated from 10R after mixing for 2 hours. The bacteria were then inoculated to rice leaves separately. No suppression of symptoms was observed (unpublished data).

It is therefore concluded that inhibition of symptom in mixed inoculation of *X. oryzae* (604) and 10R is due to impediment of initial multiplication of the pathogen by the inhibitory activity produced by 10R. However, preliminary attempts to assay for the activity *in vivo* has been unsuccessful. Demonstration and further investigation of the mode of action of the inhibitory activity of 10R *in vivo* remains to be carried out. Understanding this mechansim of symptom suppression might give light to biological control of bacterial blight

of rice.

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水稻體中非病原性細菌抑制水稻白葉枯病病徵的可能機構

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將水稻白葉枯病病原細菌（*Xanthomonas oryzae*）和水稻體內非病原性細菌混合接種，能抑制病徵的發展。爲了解此抑制病徵的性質，曾選擇10R，6S及 *Bacillus subtilis* N210三個各具不同特性的菌株做爲研究的材料。10R 能在水稻體中生存，同時又能產生抗菌物質。6S能在水稻體中生存但不產生抗菌物質。*Bacillus subtilis* N210卽不能在水稻體中生存，但能產生抗菌物質。當水稻白葉枯病病原菌以1：1的比率接種，在水稻葉上，結果發現祇有10R能抑制病徵，而6S及 *Bacillus subtilis* N210 卽沒有此種能力。6S可能與病原菌競爭空間但無法使病原菌致死。*Bacillus subtilis* N210 雖在初步能使病原菌死亡，但等到一部分病原菌侵入寄主後就無法與其競爭。而10R因能與病原菌同時侵入水稻體中又能產生抗菌物質故能有效的殺死病原菌使病徵的表現停頓或緩慢下來。