SENSITIVITY DISTRIBUTION OF PHYTOPATHOGENIC BACTERIA AND FUNGI TO ANTIBIOTICS

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The minimal inhibitory concentrations (MIC) of various antibiotics and fungicides for *Erwinia carotovora, Pseudomonas coronafaciens* var. *atropurpurea, P. lachrymans, Alternaria mali, A. kikuchiana, Pyricularia oryzae, Botrytis* sp. and *Sclerotinia* sp. isolated from diseased plants in various localities of Japan were examined to enable the isolates to be grouped into sensitive and resistant strains. To minimize the effects of various variable conditions, MIC of isolates were pooled for either 2 or 3 years and were plotted in a single figure. The grouping values were determined on the basis of MIC values of the antibiotics and agricultural chemicals on phytopathogenic bacteria and fungi under investigations. The relationships between grouping values for isolates of bacteria and fungi and the control of disease on the plants correlated to each other were studied.

The resistance of organisms to various chemicals has been increasing over a long period of time causing serious problems in the field of antibacterial drugs and insecticides. Recently, it has been reported that some phytopathogenic bacteria and fungi resistant to antibiotics and synthetic fungicides were isolated from diseased plants in the field[1,2], and that the distribution of resistant strains has been rapidly increasing each years.

Minimal inhibitory concentrations (MIC) of various antibiotics and chemicals against phytopathogenic bacteria and fungi isolated from diseased plants from 1971 through 1976 were studied. A method for establishing the appropriate MIC values for grouping the isolates into sensitive and resistant strains is described. In the present study, the following method for the determination was adopted in order to determine MIC value for grouping the isolates into sensitive and resistant groups. MIC of the isolates were pooled for either 2 or 3 years and were plotted in a single figure. The MIC value of the lowest point of the curve between peaks was then used as the grouping value for grouping the isolates into sensitive and resistant strains. The relationship between the MIC values of the isolates of some bacteria and fungi and control of disease on the plant was investigated.

Materials and Methods

Strains of Phytopathogenic Bacteria and Fungi

The isolates of phytopathogenic bacteria and fungi were obtained from diseased plants collected in various localities of Japan. Figs. 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, and 14 show the number of isolates of *Erwinia carotovora, Pseudomonas coronafaciens* var. *atropurpurea, P. lachrymans, Alternaria mali, A. kikuchiana, Pyricularia oryzae, Botrytis* sp. and *Sclerotinia* sp., respectively.

Antibiotics and Fungicides

Antibiotics and fungicides used in this were: Blasticidin S(BcS), kasugamycin (KsM), polyoxin B (PoB), streptomycin (SM), tetrachloroisophthalonitrile (TPN), methyl-2-benzimidazolecarbamate (MBC), N-(3,5-dichlorophenyl)-1,2-dimethylcyclopropanedicarboximide (DDC), 3-hydroxy-5-methylisoxazole (HMZ) and $CuSO_4$.

Method of Sensitivity Testing (MIC)

MIC values for each antibiotic and fungicide were determined by streak culture on agar using two-fold serial dilution of the antibiotics and fungicides. As was described in the previous paper,[3] MIC values of antibiotic with phytopathogenic bacteria were determined on KING B medium by streak cultures on agar using overnight cultures for the streaks and incubating then for 24 hours at 28°C.

To obtain the MIC of antibiotics and fungicides with phytopathogenic fungi, the polyoxin B-potency-test medium[4] was used, *i.e.* for *A. mali, A. kikuchiana, Botrytis* sp. and *Sclerotinia* sp. Rice plant juice medium[5] was used for *P. oryzae*. For the determination of MIC values, mycelial fragments of a test isolate was inoculated on agar and kept for 48 hours at 26~28°C.

Methods of Determinating the Control of Plant Disease

To examine the control of *E. carotovora* by SM, detached leaves of Chinese cabbage were used. Overnight cultures of *E. carotovora* were suspended in sterile distilled water at about 10^8 cells/ml. The leaves were dipped in SM solution for 1 hour before inoculation. The detached leaf of Chinese cabbage were inoculated by stabbing the leaf with a dissecting needle dipped into the bacterial suspensions. Inoculated plant materials were then incubated in a moist chamber at 30°C. Rotting was observed daily up to 3 days after inoculation.

The detached leaf method was also used, to examine the control of *A. kikuchiana* on leaves of Nijisseiki pear by PoB.[4]

To examine the control effect by KsM and BcS on *P. oryzae* in rice seedling the pot test described[5] were used.

Results

Determination of MIC value to Differentiate Isolates into Sensitive and Resistant Strains

In order to minimize experimental errors due to variations in technique of the investigators, the relative frequency distribution was examined. The MIC value of control isolates for sensitive and resistant strains of *P. lachrymans* and *A. mali* were used. The MIC values for each 50 isolates reisolated from the parent isolates on agar medium were determined. Figs. 1 and 2 show the relative frequency distribution of MIC values for antibiotics in the control isolates of both *P. lachrymans* and *A. mali*. Due to differences of experimental conditions employed, considerable variations of MIC values were observed in most antibiotics studied.

Differentiation of Sensitive and Resistant Strains by the Grouping Value

(1) *Erwinia carotovora*

The MIC values of isolates of *E. carotovora* were pooled for 3 years. Fig. 3 shows the sensitivity

Fig. 1. Relative frequency distribution of MIC of *Pseudomonas lachrymans* to streptomycin.

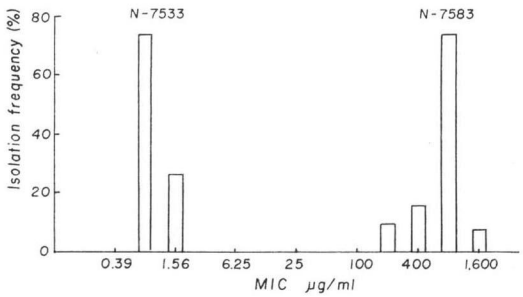
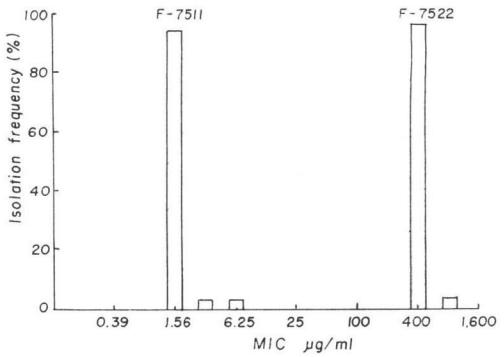


Fig. 2. Relative frequency distribution of MIC of *Alternaria mali* to polyoxin B.



distribution of SM and KsM. If we determine the lowest point of the curve as the MIC value of SM for grouping of isolates into sensitive and resistant groups, the grouping value should be 50 μg/ml. A similar experiment was carried out with KsM, and the results clearly demonstrate that all isolates were insensitive to KsM.

Fig. 3. Sensitivity distribution of *Erwinia carotovora* to streptomycin and kasugamycin.

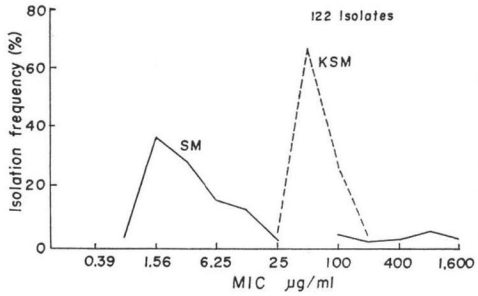


Fig. 4. Sensitivity distribution of *Pseudomonas coronafaciens* var. *atropurpurea* to streptomycin and kasugamycin.

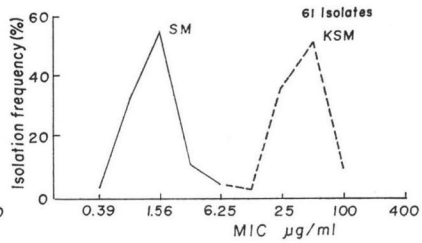


Fig. 5. Sensitivity distribution of *Pseudomonas lachrymans* to streptomycin and kasugamycin.

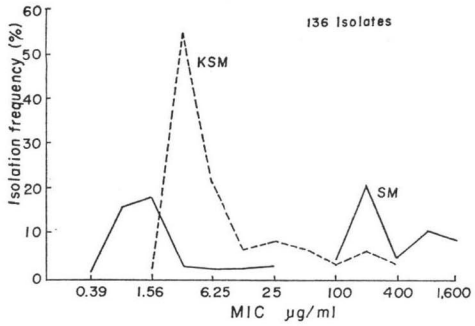


Fig. 6. Sensitivity distribution of *Alternaria mali* to polyoxin B and blasticidin S.

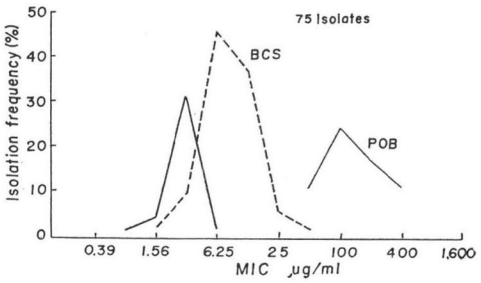


Fig. 7. Sensitivity distribution of *Alternaria kikuchiana* to polyoxin B.

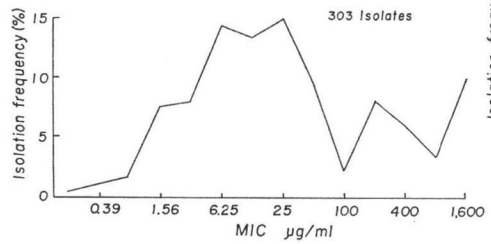
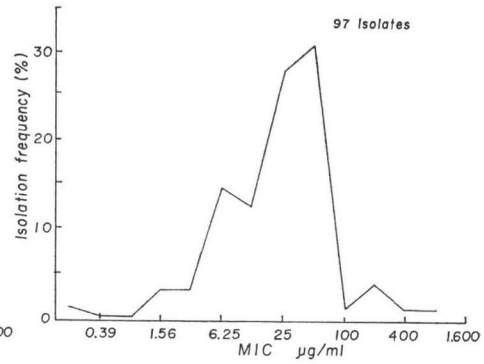


Fig. 8. Sensitivity distribution of *Alternaria kikuchiana* to blasticidin S.



(2) *Pseudomonas coronafaciens* var. *atropurpurea*

The MIC values of isolates of *P. coronafaciens* var. *atropurpurea* were pooled for 2 years. Fig. 4 shows each sensitivity distribution of SM and KsM. The figure clearly demonstrates that all isolates were sensitive to SM and KsM.

Fig. 9. Sensitivity distribution of *Pyricularia oryzae* to kasugamycin.

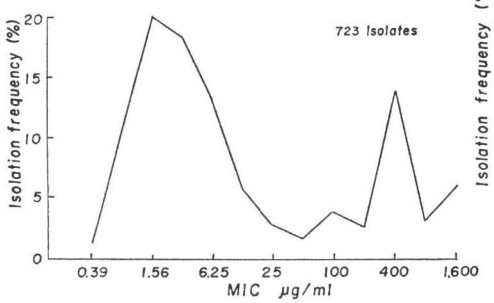


Fig. 10. Sensitivity distribution of *Pyricularia oryzae* to blasticidin S.

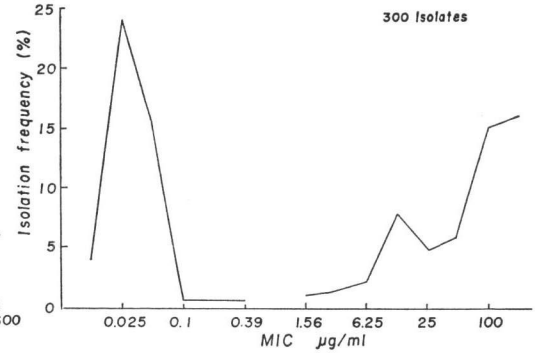


Fig. 11. Sensitivity distribution of *Botrytis* sp. to polyoxin B and MBC.

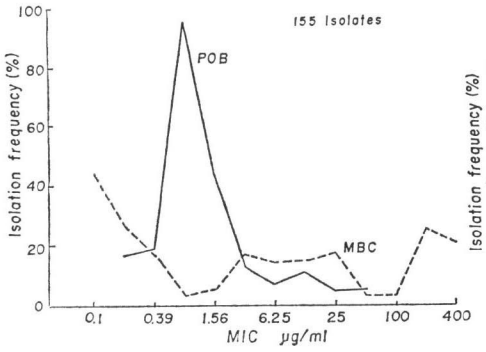


Fig. 12. Sensitivity distribution of *Botrytis* sp. to TPN, DDC and $CuSO_4$.

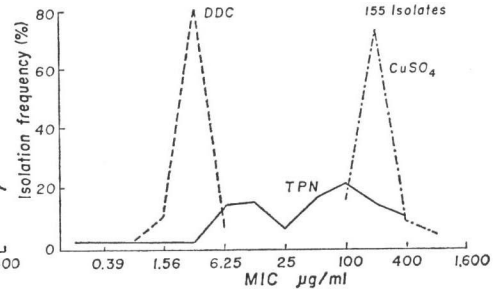


Fig. 13. Sensitivity distribution of *Sclerotinia* sp. to polyoxin B, MBC and HMZ.

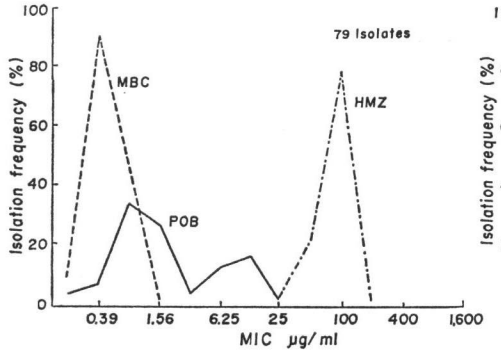
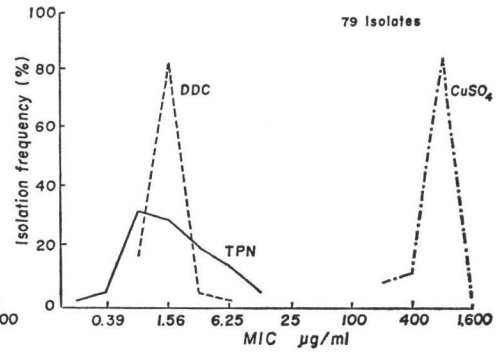


Fig. 14. Sensitivity distribution of *Sclerotinia* sp. to TPN, DDC and $CuSO_4$.



(3) *Pseudomonas lachrymans*

The MIC values of isolates of *P. lachrymans* were pooled for 3 years. Fig. 5 shows each sensitivity distribution of SM and KsM. The grouping value for SM should be 50 μg/ml. However, as shown in Fig. 5, it is difficult to determine the grouping value for KsM.

(4) *Alternaria mali*

The MIC values of isolates of *A. mali* were pooled for 2 years. Fig. 6 shows each sensitivity distribution of PoB and BcS. For determination of the MIC values of antibiotics for grouping of isolates into sensitive and resistant groups, the grouping value for PoB should be 50 μg/ml. For MIC of BcS, the grouping value should be 100 μg/ml.

(5) *Alternaria kikuchiana*

The MIC values of isolates of *A. kikuchiana* were pooled for 3 years. Figs. 7 and 8 show each sensitivity distribution of PoB and BcS. To determine the grouping value by pooling the MIC value

Table 1. Relation between MIC values of streptomycin in isolates of *Erwinia carotovora* and therapeutic effect on bacterial soft rot by the antibiotic on leaf of Chinese cabbage.

MIC of streptomycin (μg/ml)	No. of isolates	Diameters of local lesions tested*	
		Sensitive	Resistant
1,600	1	0	1
800	2	0	2
100	1	0	1
12.5	1	1	0
6.25	3	3	0
3.12	2	2	0
1.56	5	5	0

* Percent decrease in diameter of local lesion per inoculation by application of the antibiotic.

Table 2. Relation between MIC values of polyoxin B in isolates of *Alternaria kikuchiana* and therapeutic effect on black spot by the antibiotic on leaf of Nijisseiki pear.

MIC of polyoxin B (μg/ml)	No. of isolates	Diameters of local lesion tested*	
		Sensitive	Resistant
800	2	0	2
200	7	1	6
100	3	1	2
50	3	3	0
25	3	3	0

* Percent decrease in diameter of local lesion per inoculation by the antibiotic.

Table 3. Relation between MIC values of kasugamycin in isolates of *Pyricularia oryzae* and therapeutic effect on rice blast by the antibiotic on the rice seedling.

MIC of kasugamycin (μg/ml)	No. of isolates	Therapeutic effect of rice blast*	
		Sensitive	Resistant
>200	14	0	14
200	2	0	2
100	1	0	1
50	3	1	2
25	1	1	0
12.5	1	1	0
1.56	2	2	0
0.78	2	2	0

* Percent decrease in number of lesion per leaf by application of the antibiotic.

Table 4. Relation between MIC values of blasticidin S in isolates of *Pyricularia oryzae* and therapeutic effect on rice blast by the antibiotic on the rice seedling.

MIC of blasticidin S (μg/ml)	No. of isolates	Therapeutic effect of rice blast*	
		Sensitive	Resistant
100	1	0	1
25	1	0	1
12.5	2	0	2
<0.19	2	2	0

* Percent decrease in number of lesion per leaf by application of the antibiotic.

in isolates, MIC of PoB and BcS should be 100 μg/ml and 100 μg/ml respectively.

(6) *Pyricularia oryzae*

The MIC values of isolates of *P. oryzae* were pooled for 3 years. Figs. 9 and 10 show each sensitivity distribution of KsM and BcS. The grouping value for KsM and BcS should be 50 μg/ml and 1.56 μg/ml respectively.

(7) *Botrytis* sp.

The MIC values of isolates of *Botrytis* sp. were pooled for 2 years. Figs. 11 and 12 show each sensitivity distribution of PoB, MBC, TPN, DDC and $CuSO_4$. The determination of the grouping value for MBC should be either 0.78 μg/ml or 1.56 μg/ml and for TPN should be 25 μg/ml. As shown in Fig. 11, thus it is difficult to determine the grouping value for PoB. As shown in Fig. 12, these figures clearly demonstrate that all isolates were sensitive to DDC and $CuSO_4$.

(8) *Sclerotinia* sp.

The MIC values of isolates of *Sclerotinia* sp. were pooled for 2 years. Figs. 13 and 14 show each sensitivity distribution of PoB, MBC, TPN, DDC, HMZ and $CuSO_4$. These figures clearly demonstrate that all isolates were sensitive to PoB, MBC, TPN, DDC, HMZ and $CuSO_4$.

Relationship between MIC Values of Isolates and Control of Disease

The relationships between MIC values in isolates of *E. carotovora*, *A. kikuchiana* and *P. oryzae* and the control of disease on the plants was investigated. The results are shown in Tables 1, 2, 3 and 4.

In *E. carotovora*, 100 μg/ml-class of MIC values for SM was resistant as shown in the Table 1. In *A. kikuchiana*, 100 μg/ml-class and 200 μg/ml-class of MIC values for PoB were sensitive or resistant as shown in Table 2.

In *P. oryzae*, 50 μg/ml-class of MIC values for KsM were sensitive or resistant as shown in Table 3, and 12.5 μg/ml-class of MIC values for BcS was resistant as shown in Table 4.

Discussion

The distribution of MIC values in isolates of *E. carotovora*, *P. coronafaciens* var. *atropurpurea*, *P. lachrymans*, *A. mali*, *A. kikuchiana*, *P. oryzae*, *Botrytis* sp. and *Sclerotinia* sp. isolated from diseased plants in various localities of Japan was examined.

In the present study, relative frequency distributions of MIC values in isolates of both *P. lachrymans* and *A. mali* were tested. The result was found similar to the result of sensitivity of *Staphylococcus aureus* and *Escherichia coli* to antibiotics[6] and considerable variations of the MIC values were observed in most antibiotics studied.

OTAYA[6] reported that the moving pooling method for 2 or 3 years is ideal to get the grouping value for the grouping of isolates into sensitive and resistant groups in the field of antibacterial drugs. Therefore, the grouping value for the grouping of isolates into sensitive and resistant groups were determined by pooling the MIC values in isolates of phytopathogenic bacteria and fungi for 2 or 3 years.

In *E. carotovora*, sensitivity distribution to SM gave a three-peak curve and its distribution was found to differentiate the isolates into sensitive, intermediate and resistant strains.

In *P. coronafaciens* var. *atropurpurea*, the sensitivity distribution to SM and KsM gave a normal distribution curve suggesting that *P. coronafaciens* var. *atropurpurea* had little or no contact with antibiotics and agricultural chemicals in the field.

In *P. lachrymans*, sensitivity distribution to SM gave a three-peak curve. The isolates were classified into sensitive, intermediate and resistant groups.

In *A. mali*, sensitivity distributions to PoB and BcS gave a two-peak curve and a normal distribution curve respectively. The isolates were grouped into PoB sensitive and resistant strains.

In *A. kikuchiana*, sensitivity distribution to PoB gave a three-peak curve and the isolates were differentiated into sensitive, intermediate and resistant strains. BcS gave a two-peak curve and the isolates were differentiated into sensitive and resistant strains.

In *P. oryzae*, each sensitivity distribution to KsM and BcS gave a three-peak curve. The isolates were differentiated into sensitive, intermediate and resistant strains.

In *Botrytis* sp., sensitivity distribution of MBC gave a three-peak curve and isolates were differentiated into sensitive, intermediate and resistant strains. TPN gave a two-peak curve and the isolates were differentiated into sensitive and resistant strains. However, sensitivity distribution of PoB, DDC and $CuSO_4$ gave normal distribution curves.

In *Sclerotinia* sp., sensitivity distribution to PoB gave a two-peak curve and the isolates were differentiated into highly sensitive and sensitive strains. MBC, TPN, DDC, HMZ and $CuSO_4$ gave normal distribution curves.

Subsequently, the relationships were examined between MIC value in isolates and control of disease on the plant in which *E. carotovora* to SM, *A. kikuchiana* to PoB and *P. oryzae* to KsM and BcS. The results clearly demonstrate that the grouping values by MIC and control of disease on the plant correlated to each other.

This study might not be determinant as a certain grouping value for all antibiotics and fungicides and differentiate the isolates from diseased plants into sensitive and intermediate and resistant strains. However, the grouping values were applied to all types of phytopathogenic bacteria and fungi isolated from diseased plants and the determination of application dosage of antibiotics and fungicides was discussed in relation to the control effect by agricultural chemicals on phytopathogenic pathogens in the field.

The grouping value determined in this study might not be applicable as the concentration of antibiotics and fungicides to be used in the field. However, together with a consideration of other characteristic, method of application and pharmacokinetic properties of the agricultural chemicals, the grouping value might contribute important information for the application of the antibiotics and fungicides in the field.

The MIC values of various antibiotics and fungicides for phytopathogenic bacteria and fungi isolated from diseased plants were determined to differentiate the isolates into sensitive and resistant strains. The grouping values were determined on the basis of MIC values.

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