Effect of phenazine against rice leaf blight bacterium, and its biochemical mechanism.*

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In the previous paper¹⁴⁾, it was reported that *Xanthomonas oryzae* lacks the abilities of utilizing various type of inorganic sulfur sources especially under nicotinic acid amide deficient condition and that thus the bacterium requires sulfur containing amino acids for its rapid growth in the minimum basal medium, and it was also described that the growth of the bacterium quantitatively depends on the amount of L-cystine in such a medium.

Employing the chemically defined medium containing L-cystine, which was given in the previous paper¹⁴⁾, in which the growth environment seems to be similar to that in the host plant at least as for the growth limiting factor, the antibacterial activities of various compounds including antibiotics and synthetics against *Xanthomonas oryzae* have been assayed. During the screening program, the strong bacteriostatic activity of phenazine against the bacterium was noted.

This paper deals the *in vitro* and *in vivo* studies on phenazine as a new disinfectant against *Xanthomonas oryzae* and its biochemical aspect on the action mechanism upon the bacterium.

Materials and methods

The chemically defined medium used for the turbidimetric dilution assay was previously described containing 4 μ g/ml L-cystine in the minimum basal medium¹⁴⁾. The minimum basal medium contained 0.5 % of sucrose, 0.115 % of monosodium glutamate, 0.3 % of ammonium hydrogen phosphate, 0.2 % of potassium dihydrogen phosphate, 0.1 % of magnesium chloride ($6\text{H}_2\text{O}$), 0.001 % ferrous sulfate ($7\text{H}_2\text{O}$) and 0.001 % of manganese sulfate ($4\text{H}_2\text{O}$) at pH 6.5-7.0.

The growth per cent was measured by a conventional spectrophotometer determining the optical density at 660 $m\mu$ as previously described¹⁴⁾. The antibacterial activities of various compounds were expressed by the inhibitory concentration (μ g/ml) on the basis of 50 % growth inhibition against *Xanthomonas oryzae* N-5824.

The rice plant tests in the phytotrone were performed employing the single needle inoculation method^{5, 8, 13)}. The rice plants of 6 leaves age were used. The protective activities of various compounds against the bacterial leaf blight disease were expressed in the term of disease inhibitory value (per cent) on the basis of the none treated control as zero. The strain of *Xanthomonas oryzae* used in this experiment was H-5809.

The most of antibiotics used in the experiments were taken from our collection. The biochemical reagents used were obtained from the commercial sources.

The oxygen uptake due to the aerobic oxidation of each respiratory substrate was determined manometrically using Warburg's respirometer at 30°C. The reaction mixture in the vessel contained 1.0 ml of the starved cell suspension in the side arm, 0.8 ml of isotonic NaCl M/10 phosphate buffer solution at pH 7.0, 0.9 ml of substrate solution (0.1 M), 0.1 ml of methanol for

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control run or 0.1 ml of phenazine methanol solution for the inhibition experiments, and 0.2 ml of 20 % KOH separated in the center well. In the case of the direct reading of total oxygen uptake due to the complete oxidation upon a few micro moles of substrate, 0.1 ml of water was added in lieu of 0.1 ml of methanol in the above mixture.

The anaerobic dehydrogenation upon each respiratory substrate was spectrophotometrically determined by the formation of formazan pigment from 2,3,5-triphenyl-2,1,3,4-tetrazolium using the optical density at 525 m μ after two hours incubation at 30°C. The reaction mixture contained 1.0 ml of the dialyzed cell free extracts (the press-sap from French's press at 15 tons/cm² at 5°C and subsequent centrifugation at around 12,000 xG), 0.5 ml of 0.2 % the tetrazolium solution, 0.5 ml of 0.005 % methyleneblue solution, 0.9 ml of substrate solution (sodium salt), 2.0 ml of isotonic NaCl M/10 phosphate buffer solution and 0.1 ml methanol or phenazine methanol solution⁹. No enzymic reduction of methanol was observed.

The time course survey on the redox-potential at a given pH was performed employing Thunberg-Borosok tube devised with platinum electrode and saturated type calomel electrode as a partner cell at 28°C. The compensation method was used to determine the electro-motive force using Shimazu saturated type standard cell as a reference. The reaction mixture contained 1.0 ml of the cell free extracts, 1.0 ml of M/10 pH 7.0 phosphate buffer solution, 0.9 ml of the substrate solution (sodium succinate), 0.9 ml of water and 10 μ g/0.2 ml of phenazine methanol solution. In the control run, 0.2 ml of methanol or water was used in lieu of the phenazine methanol solution. None enzymic reduction of phenazine was performed titrating 18 mg phenazine in 10 ml of 95 % methanol by N/10 aqueous solution of NaSH at constant pH 7.0-7.2.

The solubilized succinate oxidase system of the bacterium was prepared according to the method described by Matsunaka⁹. The reduction of the cytochrome system was observed by the difference spectrum using Hitachi recording spectrophotometer at 28°C. The reaction mixture in this experiment contained 4 ml of the solubilized succinate oxidase system, 0.1 ml of 0.5 M sodium succinate aqueous solution and 0.1 ml of methanol or phenazine 12 μ g/0.1 ml of methanol solution. Water in lieu of 0.1 ml of 0.5 M succinate solution and methanol in lieu of 0.1 ml of the phenazine methanol solution in the above mixture was subjected at reference position. No inhibitory effect upon the reduction of the cytochrome system was observed by the addition of this small amount of methanol. In another control run, phenazine 12 μ g/0.1 ml methanol solution in lieu of 0.1 ml methanol and water in lieu of 0.5 M sodium succinate solution in the above mixture was used.

Results and discussion

(1) The *in vitro* and *in vivo* antibacterial activities of phenazine comparing with the other antibacterial agents.

The following Table 1 summarized the results of both the bacteriostatic activity of phenazine by the turbidimetric assay comparing with other compounds including inhibitors upon the function of thiol group or upon the metabolism of sulfur containing amino acids, and the inhibitory values of those compounds obtained by the rice plant pot tests.

The peculiar bacteriostatic characteristic of phenazine comparing with chloramphenicol and lenamycin (synm : cellocidin) was given in Fig. 1 showing the more inclined relation between the growth and the dose of phenazine than the later two. This tendency seems to imply the merit of phenazine in the bacteriostatic activities especially under the lower dose below about 1.0 μ g/ml.

From the above findings, the difference of two inhibitory values in the rice plant pot test

Table 1. The concentration of each compound for 50 % growth inhibition upon *Xanthomonas oryzae* and the inhibitory value against the leaf blight disease of rice plant.

Materials	Inhibitory concentration ($\mu\text{g/ml}$) at 50% inhibition in the minimum basal medium plus L-cystine.	Inhibitory value % obtained by the pot test.	
		Dose (ppm)	Value (%)
O-iodosobenzoic acid	6.3	150	40
formamidine disulfide hydrochloride	73.0	150	23
2-mercaptobenzothiazole	17.0	150	15
furylmercaptooxadiazole	18.0	150	27
2-thiouracil	20.0	150	18
sodium iodoacetate	3.2	150	12
p-chloroacetophenone	>100.0	150	23
p-chloromercuribenzoic acid	6.3	150	0
phenylmercuric acetate	0.4	17	29
DL-ethionine	50.0	150	44
streptomycin sesquisulfate	20.0	100	39
chloramphenicol	2.5	100	82
lenamycin(synm : cellocidin) ⁽¹⁰⁾⁽¹¹⁾	1.25	100	56
phenazine	0.8	150	83*
phenazine methosulfate	1.56	150	0

* An application with wettable powder.

Fig. 1 The relation between the concentration of active agent and the growth per cent.

between phenazine and its monomethyl ammonium salt type derivative, phenazine methosulfate, suggested that two nitrogen atoms in phenazine nucleus should be free from any alkyl substituents implying that the *in vivo* bacteriostatic activity might be attributed by the proper two tertiary amine type nitrogen atoms, although, as for the details of the different mechanisms operated for the *in vivo* activities, it is uncertain whether the specific responses of host for the two compounds might selectively separated the two related derivatives. Moreover, by the other *in vitro* studies which were carried out against other bacterial pathogens belong to *Xanthomonas*, such as *X. citri*,

X. phaseoli and *X. pruni*, no bacteriostatic activities were observed against *X. phaseoli* and *X. pruni* excepting a moderate action against *X. citri*.

The compounds which showed the reverse action upon the bacteriostatic activity of phenazine against the bacterium were sought employing the minimum basal medium containing 10 $\mu\text{g/ml}$ L-cystine. Equal to 20 times moles of cysteine, glutathione, lipoic acid, riboflavine, vitamin K₁ and pyridoxal phosphate on the basis of 1 $\mu\text{g/ml}$ phenazine added to the medium were used for the experiments. Among those compounds tested, only 20 times moles of cysteine on the basis of a mole of phenazine showed the tendency to reverse the bacteriostatic action of phenazine. From the above observations, it might be supposed that one site of the action mechanisms of phenazine upon the bacterium seemed to be related to the metabolic reactions or functions of sulfur containing amino acids which are essentially required for the rapid growth of the bacterium. However, the detail mechanism of the action of phenazine is not yet clear whether phenazine plays a direct action upon a certain metabolic reaction and/or a function concerning sulfur containing amino acids in the bacterium or the indirect action including the inhibition against the respiratory¹²⁾, energy generating²⁾ and/or the nucleotide-protein synthesizing system⁴⁾.

Depending on their structural modifications, phenazine derivatives are said to accelerate or inhibit various dehydrogenases upon hexosemonophosphate, lactate, α -hydroxyglutamate and succinate^{1,17,3)}. Their ability to do this depends on the reversibility of their oxidation reduction at values of pH and Eh encountered biologically¹⁵⁾. This is the most clear understanding of the biological action of phenazines.

According to the above knowledges, first of all, the effect of phenazine on the respiratory system of the bacterium was investigated.

(2) Effects of phenazine on the respiratory system of *Xanthomonas oryzae*.

As for the background of the respiratory experiments, the oxidative utilization of various respiratory substrates by the bacterium was investigated. The results were summarized in Fig. 2.

The behaviors regarding the utilizations of the 3 kinds of carbohydrates such as D-glucose, D-fructose and glycerol for the growth of the bacterium in separated experiments, which were determined by the turbidimetric measurement above described, showed the same tendency to the behaviors of the oxidative utilizations of the carbohydrates by the bacterium, which were shown in Fig. 2. Moreover, it was ascertained that the bacterium oxidatively utilizes the all members of tricarboxylic acid (TCA) cycle reaction excepting that oxaloacetate remains inert, implying that the bacterium operates usual TCA cycle reaction.

Fig. 2. The utilizations of various respiratory substrates by *Xanthomonas oryzae*.

1. D-glucose (A). 2. D-fructose (A).
3. glycerol (A). 4. pyruvate (A).
5. acetate (A). 6. citrate (A).
7. isocitrate (A). 8. isocitrate (AN).
9. α -KGA (A). 10. α -KGA (AN).
11. succinate (A). 12. succinate (AN).
13. fumarate (A). 14. malate (A).
15. malate (AN). 16. oxaloacetate (A).
17. glyoxylate (A).

* Per cent oxidation of each substrate on the basis of the theoretical oxygen uptake due to the complete oxidation via EM pathway and/or TCA cycle reactions, under aerobic condition.

** Formazan produced ($\mu\text{g/5 ml}$ reaction mixture) under anaerobic condition.

The inhibitory effects of phenazine upon respiration of the bacterium, under both aerobic and anaerobic condition, were investigated. The results were shown in Table 2. Besides the inhibitory effects, the acceleration of the endogenous respiration especially under anaerobic condition was often observed when phenazine was added to the reaction mixture without the addition of any substrates.

The above results with the preceding considerations seemed to suggest that phenazine inhibited the respiration with the incorporation of itself into an intermediate position of the respiratory system of the bacterium. Thus, the evidences for such an incorporation to form a rate regulating system in the respiratory system was sought.

When phenazine was incubated with the cell free extracts above described under the presence of sodium succinate, a clear inflection was shown in the course of the decreasing redox potential under anaerobic condition in Thunberg-Borsook tube devised with platinum electrode as shown in Fig. 3.

The inflection point appeared usually at around +164 mV at pH 7.0 in the incubation experi-

ments either with or without sodium succinate as the respiratory substrate. Although the respiratory substrate in the later case was yet uncertain, this inflection seemed to imply that phenazine is reduced when it is incubated with the cell free extracts. In fact, the inflection point showed a quite good coincidence with that of the redox titration curve of phenazine with sodium hydrogen sulfide. Moreover, in this pH of the reaction mixture, the reduction may proceed under two electron step reaction⁷⁾ to form dihydrophenazine which will receive the subsequent reversible oxidation by the higher potential redox system.

Using the cell free extracts or the intact cell suspension in M/10 pH 7.0 phosphate buffer solution, the potential-time course studies by Thunberg-Borsook tube gave the following electromotive spectrum regarding the redox system of *X. oryzae* showing that phenazine was incorporated into a intermediate position of the respiratory system of the bacterium. From this finding, a substantial difference concerning the potentials E_0 (pH 7.0) between phenazine methosulfate was

Table 2. The effects of phenazine upon the respiration of *X. oryzae*.

Respiratory substrate	Per cent inhibition (%) under the presence of 2.5 μ g/ml phenazine.	
	Aerobic	Anaerobic
D-glucose	20	—
acetate	27	—
isocitrate	—	27
Alpha-Ketoglutarate	28	35
succinate	41	50
malate	31	46

Fig. 3. The enzymic reduction of phenazine by the cell free extract of *X. oryzae*.

Sodium succinate was used as the substrate.

recognized suggesting that the two compounds should be located in the different position of the respiratory system of the bacterium corresponding with the different *in vitro* activities already shown in Table 1. However, regarding the detail mechanisms operated for the *in vivo* activities of the two related compounds, the difference was yet uncertain and was remained for future studies.

Further more, as shown in the following Fig. 5, the experiments employing the solubilized succinate oxidase system of the bacterium⁶⁾ also showed that the addition of phenazine caused the delaying of the reduction of the cytochrome system, when the reduction of the cytochrome system was observed spectrometrically determining the difference spectrum between with and without the presence of the substrate.

Thus, the above standard monometric, photometric, potentiometric and spectroscopic experimental evidences regarding the effects of phenazine on the respiratory system of

Xanthomonas oryzae, implies that one site of the action mechanism of phenazine against the bacterium is the incorporation and conjugation of phenazine into the bacterial respiratory system

Fig. 4. The electro-motive spectrum of the respiratory system of *X. oryzae*.

Fig. 5. The effect of phenazine upon the enzymic reduction of cytochrome system of *X. oryzae*.

to form the rate limiting redox system in it.

Summary

(1) Employing the chemically defined medium containing L-cystine, in which the growth environmental condition seems to be similar to that in the host plant at least as for the growth limiting factor, phenazine was found as a peculiar bacteriostatic agent having the wide range of partial growth inhibitory activity against *Xanthomonas oryzae*.

(2) Regarding rice plant pot tests against the leaf blight disease in our phytotrone, phenazine showed a fairly high inhibitory value, dose at 200 ppm was ca. 93 % and dose at 150 ppm was ca. 83 %.

(3) One aspect on the action mechanism of phenazine against the bacterium was elucidated, that is, phenazine seemed to be incorporated into the respiratory system of the bacterium and it formed the respiratory regulating system to suppress the rate of the respiration.

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和文摘要

イネ白葉枯病防除剤としてのフェナジンとその作用機構

関 沢 泰 治**・渡 辺 哲 郎**・小 田 信**

シスチンを含有する合成培地を使用して多種の薬物のイネ白葉枯病菌に対する効果を比濁法で試験した結果、フェナジンが広い濃度範囲において特異な静菌作用を示すことを見出した。単針接種法によるポット試験においてもフェナジンは高い防除価を示すことを認めた。フェナジンは最少静菌濃度附近において糖源、TCA サイクル員などの呼吸基質のイネ白葉枯病菌による酸化を好氣的・嫌氣的両条件で抑制し、菌の無細胞抽出液を白金電極付ツンベルグ管中でフェナジン存在下に保温するとフェナジン—ジヒドロフェナジンによると見られる緩衝電位が記録される。また可溶性コハク酸酸化酵素系ではフェナジンの添加により本菌のチトクロム系の還元が抑制されることなどが明らかになった。すなわちフェナジンはイネ白葉枯病菌の呼吸系の中に取込まれて呼吸を抑制する系を新たに形成することが静菌作用を示す一つの原因であると考えた。

(明治製菓中研)

文 献 紹 介

Rhizopus 菌 2 種の胞子嚢胞子の成熟と発芽の電子顕微鏡的研究

Hawker, L. E. and P. M. Abbott: An electron microscope study of maturation and germination of sporangiospores of two species of *Rhizopus*. J. gen. Microbiol. 32 (2) : 295-298, 1963.

本論文は *R. sexualis* および *R. nigricans* の胞子嚢胞子の成熟およびその発芽の微細構造を電子顕微鏡を用いて観察した結果で、観察材料を過マンガン酸カリおよびオスミック酸過マンガン酸カリで固定し、araldite に包埋している。

これらの菌の未熟胞子嚢胞子の胞子膜は比較的薄く、1層からなっているが、成熟胞子の特徴的な縦の隆起となると思われる肥厚はすでに見られた。この肥厚部はオスミック酸固定材料で黒つぶくあらわれ、脂質と思われる。原形質膜は薄く、胞子膜に密着し、内細胞質網状構造は少数の小胞 (cisternae) とともに内細胞質中に散在していた。典型的な核、ミトコンドリアおよび脂肪質の貯蔵栄養小球体も存在していた。未熟胞子中には新しく分裂したと思われる2〜4コの核が中心附近にみられる。胞子は、成熟すれば、レモン型になる。隆起膜は非常に厚く

(0.3〜0.6 μ)、脂質に包まれ、規則的に配列された成分からなっている。成熟胞子の核数は普通4コで、赤道附近にあるようである。胞子膜の肥厚を除いて、最も著しい変化はミトコンドリアの形および大きさの増加にある。

発芽に際して、最初に認められる変化は縦に配列された成分からなる内膜の形成である。これはもとの胞子膜とは著しく異なっている。発芽初期に胞子は大きくなり、引伸ばされる。ミトコンドリア数は増加するが、それは多分休眠胞子中のわん曲した大きなミトコンドリアの分裂によるものと思われる。多くの胞子では、核やミトコンドリアがある部分に密集していたが、これは発芽管が出現する部分のようである。発芽するとき、もとの胞子膜は非常に引伸ばされ、壊れて、新しく形成された弾力ある内膜に包まれた発芽管が出現する。胞子の内容物である核およびミトコンドリアの両方が長く伸び流れの方向に位置し、発芽管へと流れ込む。発芽管の先端にミトコンドリアがあり、核は先端より少し離れて存在していた。胞子中の液胞は発芽管より離れた部分に発達した。

(福島雅夫)