STUDIES ON INDUCED MUTANTS OF *PIRICULARIA ORYZAE* AND ON THEIR PATHOGENICITY[1]

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(Received July 13, 1966)

Introduction

Piricularia oryzae Cav., a causal organism of the blast of rice plant, exhibits parasitic specialization in its hosts. Strains of the fungus, however, differ in virulence, and concomitantly, varieties of host plants differ in resistance. Differentiation into physiologic race on the basis of parasitism has been demonstrated. Presumably this situation may be clarified by genetic studies. Despite the degree of variability observed in the laboratory cultures of the organism, a program of induced mutation was initiated. The present paper describes the lethal and mutagenic effect of various kinds of mutagens on the fungus. And the auxotrophic and biochemical mutants were selected and their pathogenicity was examined. More detailed descriptions of the nutritional relation between host and pathogen will be given in later papers.

Materials and Methods

Culture and media: A highly virulent and stable strain of *Piricularia oryzae* No. 13, originally isolated at Laboratory of Plant Pathology, Taiwan Agricultural Research Institute was selected for treatment. The strain was maintained on complete medium (CM) on which they produced abundant conidia. The temperature of incubation used through the work was 26°C to 28°C.

The minimal medium (MM) used in this work was similar to that described by Tanaka (1963) with minor modifications. It had the following compositions: sucrose, 15.0 g; NH_4NO_3 1.0 g; KH_2PO_4 0.5 g; K_2HPO_4 0.5 g; $MgSO_4 \cdot 7H_2O$ 0.25 g; $CaCl_2 \cdot 6H_2O$ 0.05 g; $FeSO_4 \cdot 7H_2O$ 0.75 mg; $MnSO_4 \cdot 5H_2O$ 0.22 mg; $CuSO_4 \cdot 5H_2O$ 0.60 mg; $ZnCl_2$ 7.5 mg; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ 0.06 mg; H_3BO_3 0.09 mg; biotin 5.0 ug; thiamine 2.5 mg; H_2O 1000 g. The other screening media were prepared by variously supplementing the minimal medium. The "complete"

(1) Paper No. 50 of scientific Journal Series, Institute of Botany, Academia Sinica. This research was partly supported by National Council on Science Development.

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medium was composed as follows: minimal medium, one liter; "vitamin free" casein hydrolysate, 5 g; yeast extract, 2 g; yeast nucleic acid hydrolysate (Perkins 1949) 0.5 ml; vitamin solution, 5 ml.

The vitamin solution consisted of riboflavin, 50 mg; pyridoxine HCl, 60 mg; calcium pantothenate, 200 mg; p-aminobenzoic acid, 50 mg; nicotinic acid, 200 mg; choline chloride, 200 mg; inositol, 400 mg; biotin, 0.5 r; folic acid, 0.0045 mg; distilled water to make one liter.

For solid media 17 g/1 of DIFCO bacto-agar was used. All media were adjusted to pH 6.0 to 7.0.

Ultraviolet radiation: Aqueous conidial suspensions were prepared from 10-day-old cultures. Conidiophores and other debris were removed by filtering several times through sterile cotton wool. The concentration of conidia was adjusted to about 10^4–10^5 conidia per ml., by haemacytometer counts. The source of ultraviolet was a mercury vapor quartz tube, gride type, that emitted a high percentage of radiation at 2537 Å with an intensity of about 155 microwatts per sq. cm at distance of 18 inches. Immediately after treatment the dishes were covered and placed in the dark to prevent photo-reaction. To start with, survival curves were determined. Subsequently, treatments were applied that killed 95–99% of the conidia in order to produce auxotrophs.

Treatment by chemical mutagens: Five different chemical mutagens were tried. There were: ethyl methane sulfonate, hydroxylamine, N-methyl-N′-nitro-N-nitrosoquanidine (MNG), sulfonilamide, and DL-ethionine. Conidial suspensions were prepared from 10-day-old cultures. Suspensions were filtered twice through sterile cotton wool. The conidia were centrifuged, washed twice with 0.2 M acetate buffer at pH 5 and finally resuspended in the same buffer. Mutagens were freshly dissolved in the same acetate buffer to a concentration twice that used for treatment. After 10 minutes they were diluted to the final concentration by mixing with an equal amount of conidial suspension. The mixtures were incubated in a water bath at 30°C. At time intervals thereafter, samples of the mixture were washed and plated on complete agar medium. Those plates were incubated at 28°C for three days.

Screening for mutants: Most of the screening for biochemical mutants was done on solid media. After the treatment with mutagens, survivors were placed in petri dishes with complete medium. After 3–4 days of incubation the resulting colonies were transfered to complete medium and transfers were made later to minimal medium. Transfers showing good differential growth on the CM and MM were tested futher in the following five solid media: complete, minimal, minimal plus "vitamin free" casein hydrolysate, minimal plus yeast nucleic acid hydrolysate, and minimal plus vitamin solution. Subsequent tests on the individual components of the supplement of the appropriate

medium indicated the mutant's specific requirement. An intermediate step in the screening for the deficient mutants, the method described by Lederberg (1950) was used.

Determination of pathogenicity. Two procedures were used to detected the infectivity of mutants. In one, the conidia were plated on misato medium (Misato, 1957) or mixed with barley media (Yamanaka 1961) for the formation of conidia. Conidia were harvested and suspended in water. The conidial suspensions were directly sprayed on 10 day old rice seedlings of 13 differential hosts. In the other procedure, the conidial suspensions were directly injected into the young leaf inside the sheath with a syringe until the conidial suspension exuded from the tip of shoot (Kuribayashi 1953). Lesions appeared when the young leaf unfolded. The infectivity was examined after one week.

Results

Conidia responsed to various kind of mutagens: The lethal and mutagenic effect of various chemical mutagens and ultraviolet irradiation were examined. The effects were measured by the rate of killing of the conidia and by the percentages of mutants induced, based, on the number of conidia plated and the number surviving. All colonies which did not grow on minimal medium were considered as a possible auxotrophic mutants. The results are shown in Table 1.

Table 1. *Survival of conidia of P. oryzae following treatment with mutagens*

Mutagens	Dosage of mutagens	Number of survivors	Percentage of survival
Ethyl methane sulfonate	40 ug/ml	1,448	33.7%
	60	1,168	25.2
Hydroxylamine	10	1,142	24.8
	100	916	19.8
MNG	4	170	3.7
	20	0	0
	40	0	0
Sulfonilamide	40	684	14.8
	60	574	12.7
	100	478	10.3
DL-ethionine	40	1,000	21.7
	60	780	17.0
	100	542	11.7
UV irradiation	2 minutes	467	10.1
	4	156	3.4
	6	18	0.4

Original conidial concentration 4.6×10^3 cells/ml. The reaction mixtures, containing conidia and chemical mutagens, were incubated at 25°C for 12 hrs.

Among these mutagens, ultraviolet irradiation and MNG treatment produced a strong lethal effect on the conida. The number of survivors was very low after treatment with UV and MNG. On the contrary, ethyl methane sulfonate, hydroxylamine, sulfonilamide and DL-ethionine were less effective in inducing death of conidia, even at higher concentrations and longer periods of treatment. The rate of survivors was checked for their mutagenic effect on auxotrophic mutation. No auxotrophic mutants were obtained from treatment by ethyl methane sulfonate, hydroxylamine, sulfonilamide and DL-ethionine. Few auxotrophic mutants were obtained from MNG treatment and UV irradiation. MNG appeared to be the more promising mutagen. Therefore it was chosen for further experimentation. Several concentrations of MNG were tested and 200 ug/ml appeared to be most suitable. The preparation of conidial suspensions and the treatment of conidia with MNG were described before. After treatment 10 ml samples were removed at 10 minutes intervals. The conidia were centrifuged down, resuspended with distilled water and plated on complete medium. The results are shown in Table 2.

Table 2. *Mutagenic effect of MNG on conidia of P. oryzae*

Time (minutes)	No. of surviving cells/ml	% of survivors	No. of auxotrophic mutants	% of auxotrophic mutants
0	4,632			
10	189	4.08	15	7.9
20	97	2.10	15	14.6
30	62	1.30	10	16.0
40	47	1.00	6	12.7
50	33	0.80	8	24.0
60	25	0.50	5	20.0
70	4	0.08	2	50.0
80	0	0	—	—

Type of mutants: All of the biochemical mutants completely lost their ability to grow on minimal medium and all showed single deficiencies. Mutants requiring histidine, lysine, asparagine, glutamic acid, methionine, arginine, serine, and isoleusine were isolated.

Several strains from the same population of survivors of mutagenic treatment required the same amino acid for growth. Four argininеless, three asparagineless, two lysineless, two histidineless, one methionineless, one glutamic acidless, one serineless, one isoleusineless and one xanthosineless were obtained. The four mutants A_{80}, B_{56}, U_1, I_{10} which required arginine were

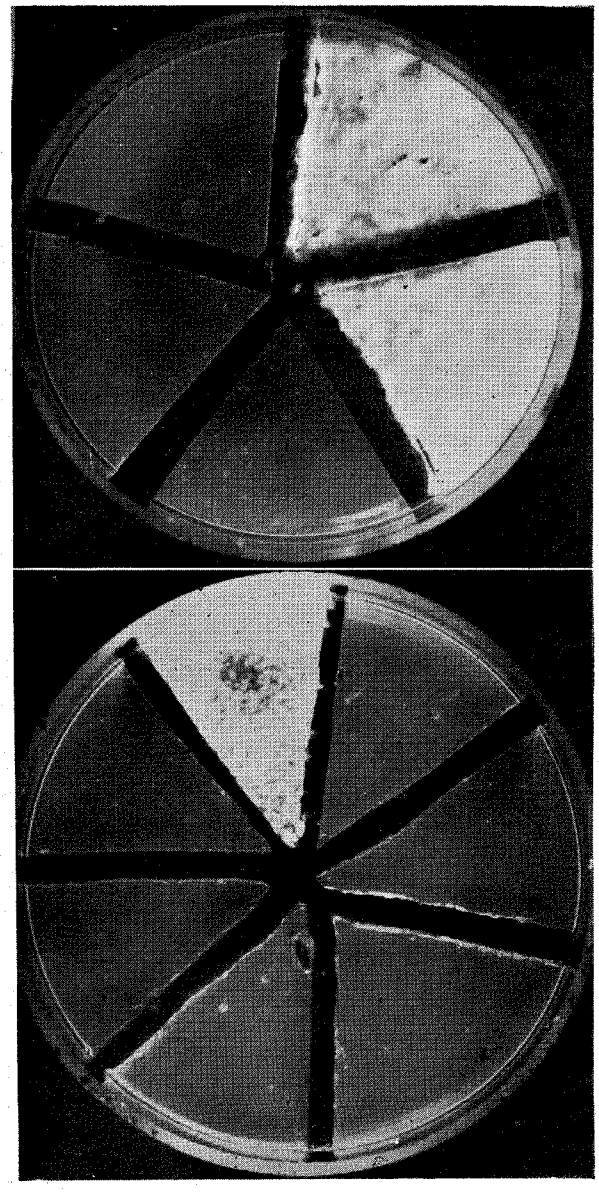

Fig. 1 Auxonographic plates for an amino acid required by a strain of *P. oryzae*. Left, growth response for glutamic acid. Right, growth response to complete medium and casein hydrolysate.

tested with a number of substances which had been shown to be involved in the synthesis of arginine or were closely related to it, namely proline, ornithine, citrulline, arginine. A_{80}, I_{10} and U_1 responded only to arginine, and B_{56} responded to both arginine and citrulline. Two lysineless mutants, B_{29} and B_{91} were tested with lysine and diamino pimelic acid. Both of them responded only to lysine.

The color of the colony and the ability to sporulate were also observed among the biochemical mutants. The color of the colony of mutants A_{18}, A_{80}, B_{91} and T_{11} was darker than that of the wild type, and that of B_{29}, B_{56}, on the other hand was lighter. Mutants A_{18}, B_{29}, A_{80} and B_{56}, lost the ability of sporulation on complete medium and only a very few conidia were detected on the Misato medium which was most suitable for sporulation of wild type

Table 3. *Mutants and their nutritional requirement and P. oryzae on 13 differential host of rice plant*

Nutrient required	Strains	Pathogenicity on 13 differential host												
		1	2	3	4	5	6	7	8	9	10	11	12	13
Arginine	I_{10}	O	O	R	O	S	R	S	O	R	R	O	R	O
	U_1	R	R	O	R	R	O	O	R	O	R	O	R	O
	A_{80}	R	O	O	R	R	R	R	R	R	O	O	R	R
	B_{56}	R	R	R	R	R	O	O	O	R	R	R	R	R
Histidine	A_{40}	S	R	R	R	S	S	S	S	S	R	R	R	S
	A_{60}	O	O	O	O	R	R	S	R	R	O	R	R	O
Asparagine	B_{37}	R	R	R	R	R	S	R	R	R	R	R	R	R
	M_{59}	S	S	R	S	S	S	S	S	S	R	R	R	S
	K_{10}	O	O	O	R	S	R	S	R	S	S	R	S	S
Lysine	B_{29}	R	R	O	R	O	R	S	R	R	R	R	R	S
	B_{91}	O	R	R	R	R	R	S	R	S	R	R	R	R
Methionine	A_{18}	R	O	O	O	S	S	R	S	S	R	R	R	R
Glutamic acid	U_2	R	R	S	R	S	S	S	S	S	R	R	R	R
Serine	I_{28}	—	—	—	—	—	—	—	—	—	—	—	—	—
Isoleusine	P_2	O	O	O	O	S	O	S	R	R	O	O	R	O
Xanthosine	T_{11}	O	O	O	R	R	O	O	R	R	R	O	R	O
Parent strain No. 13		S	S	S	S	S	S	S	S	S	S	R	S	S

R=resistant type: Lesions were restricted in small area; size not large than 1–1.5 mm. in diameter; dark-brown in color.
S=susceptible type: Expanding lesions; water soaked appearence; spindle shaped.
O=no symptom:
Differential host: (1) Taichung 171, (2) Taichung line 33, (3) Chianung-yu 280, (4) Taichung 65, (5) Kos-Chio-Lin-Chou, (6) Kachsiung-Ta-Li-Chen-Yu, (7) Taichung-Ti-Chio-Wu-Chien, (8) Kung-Shan-Wu-Shen-Ken, (9) Cutsugulcul, (10) Natnla (11) Kanto 51, (12) Norin 21, (13) Sensho.

and other mutants. The conidia of all these auxotrophic strains germinated in the absence of their respective nutritional requirements. The growth of mutants B_{29} and B_{56} was slower than that of wild type.

The pathogenicity of parent and mutants of *P. oryzae:* The parent strain and mutants were tested for their pathogenicity on 13 differential hosts which were used to distinguish the physiologic races of *P. oryzae* originally. These are: Taichung 171, Taichung line 33, Chianung-yu 280, Taichung 65, Kos-Chio-Lin-Chou, Kachsiung-Ta-Li-Chen-Yu, Teichung-Ti-Chio-Wu-Chien, Kung-Shan-Wu-Shin-Ken, Cutaugulcul, Natala, Kanto 51, Norin 21, Sensho. As it can be seen in Table 3, there were altogether sixteen mutants and were grouped into 9 kinds of nutritional requirement, apparently the pathogenicity of these auxotroph were quite different from that of the parent strain. Most of the biochemical mutants showed reduced pathogenicity. They did not form any typical spindle-shaped lesion on the leaf of the host. On the other hand, showing only small restricted spots of dark brown indicating the intrusion of the parasite were shown in majority cases found. Some mutants lost their pathogenicity completely to infect some varieties of rice plant. However other mutants still preserved their strong pathogenicity.

Disscusion

Ultraviolet irradiation is used for inducing mutations of *P. oryzae.* It is found that most of the U. V. induced mutants are variable and unsuitable as genetic markers. Nutritionally exacting mutants are sought, but only few are found. There are only two mutants, U_1 and U_2, obtained from ultraviolet irradiation treatment of approximately 10^{10} conidia. A large number of auxotrophic mutants are obtained but when they are tested for specific nutritional requirements, they revert back to prototrophy. Several chemical mutagens are tested and MNG appears to be the most promising. Several auxotrophs are obtained with this mutagen. It is surprising that several auxotrophs with the same nutritional requirement, however, very rarely, this is found, probably each auxotroph comes from an independent mutation, and the mutational sites are perhaps different for those having the same requirement. Mutants requiring arginine, lysine, asparagine and histidine are most frequently found. Possibly genic sites of asparagine, arginine, lysine, histidine are more mutable with MNG than those of other nutritional requirements. A further explanation may be that although diverse mutations are induced, however only those which are readily dissociated form a heterokaryotic condition are detected.

Many isolates appear to be less pathogenic than their parents. Some of the weakly pathogenic mutants, however, do not sporulate.

Virulence of biochemical mutants of other pathogenic organisms would vary according to the particular nutritional deficiencies. Among plant pathogens, several studies have been reported (Boxton 1956, Kline *et al* 1957, Tinlin 1963 etc.). Generally pathogens may be devided into two groups. 1) Comparatively large number of biochemical mutants would have their pathogenicity reduced and 2) Small number or none of the auxotrophes would have their pathogenicity reduced or lost. According to Yamasaki *et al* (1964) *P. oryzae* seems to belong to the latter. In our experiment, however 13 varieties of rice plant are inoculated and found that most of the biochemical mutants have their pathogenicity reduced. The difference may be caused by the used of different parent strain and differential hosts. The biochemical mutants of *P. oryzae* exhibit a pattern of virulence and avirulence for varieties of rice plant. This pattern has been interpreted as an expression of the relationship between the demand for required nutrients by the parasite and the supply of these nutrients by the host, either at the site of inoculation or of localization. The conidia of these mutants can germinate in distilled water, impling that they do not require on exogenous supply of the nutrient which must be supplied for growth. It means that the demand for requied nutrients by the parasite and the supply of these nutrients by the host are not at the site of inoculation and may be at the site of localization. Since most of mutants can form small restricted lesions on their hosts showing that they retain the ability of penetration into the host cell but only lose the ability to grow in the host. It should be noted that the growth of the mutants will be affected by an inadequate supply of the required nutrients available from the host cell. The resistance or susceptibility of varieties to specific biochemical mutants may reflect specifically different concentration of the required nutrients in the host.

The use of induced biochemical mutants enables the production of experimental models for control of pathogenicity. Garber (1954) reported that specific mutants of *Erwinina aroideae* were avirulent for certain host species but virulent for others. Garber *et al* (1956) encountered variable responses by many mutants *E. aroideae* and hypothesized that this was due to possible differences in the supply to the mutant of the required subtances by different varieties of the hosts. Further studies are in progress to determine whether differences of pathogenicity of those mutants may be due at least in part, to differences in their nutritional requirements which are met in varying degrees by different host varieties.

Summary

Biochemical mutants were induced in a line of *Piricularia oryzae* by treat-

ment with various kinds of mutagens. Several mutants were obtained from the survivors of ultraviolet irradiation by a modified total-isolation technique. Among the mutagens tested, N-methyl-N′-nitro-N-nitrosoquanidine seemed to be the best mutagen for inducing biochemical mutants of this orgsnism.

Sixteen nutritional exacting mutants were obtained. They required methionine, lysine, histidine, arginine, asparagine, glutamic acid, leusine, isoleusine and xanthosine respectively. Some of the mutants were morphologically distinct from their parent. Some of them even lost the ability of sporulation.

These mutants were tested for their pathogenicity on 13 differential hosts. Most of mutants had their pathogenicity reduced.

誘導水稻稻熱病病原菌的突變與其病原性之研究

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本實驗用各種不同之誘變劑（mutagens）誘導水稻稻熱病病原菌突變，並自突變菌株中選擇營養變異株，所使用之誘變劑有 ethyl methane sulfonate, hydroxylamine, N-methyl-N′-nitro-N-nitrosoquanidine, DL-ethionine, sulfonilamide, 和紫外線。其中以 N-methyl-N′-nitro-N-nitrosoquanidine 及紫外光線最好，經此兩種誘變劑處理後得16營養變異株，它們單獨各別需要 methionine, lysine, histidine, arginine, asparagine, glutamic acid, leusine, isoleusine 和 xanthosine。此等突變種中有一部分失去其產生孢子之能力，有的其菌落形態與親本不同。將此等營養變異株接種於13種辨別寄主上發現大部分之突變種其病原性均有衰退之現象。

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