NUTRITIONAL ASPECT OF HOST-PARASITE RELATIONSHIP IN THE RICE BLAST FUNGUS[1]

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Introduction

Previous studies have shown that the biochemical mutants of *Piricularia oryzae* exhibit a pattern of virulence and avirulence for varieties of rice plant (Kuo *et al* 1967). This pattern may be considered as an expression of relationship between the demand for required nutrients by pathogens and the supply of these nutrients by the host either at the site of inoculation or location (Tuveson and Garber 1959, Garber *et al* 1956 and Garber 1954). The absence of such a pattern was also reported for biochemical mutants of *Venturia inaequalis* (Cke) Wint., infecting varieties of a susceptiable host species (Boone *et al* 1957). According to Garber's nutrition-inhibition hypothesis (Garber 1956) of pathogenicity, the unavailability of one or more nutrients in the host environment required by the parasite for proliferation or metabolism, results in avirulence. Unavailability of nutrient may result from a low concentration of these nutrients in utilizable form or the presence of antagonists or inhibitors which interfers with the uptake or utilization of nutrients.

The purpose of the present study is to estimate how a resistant host differs from a susceptible one in the concentration of specific amino acids required by the pathogens, or the avirulence of biochemical mutants is due to their inability to grow in the host due principally to the deficiency of the pertinent amino acid. In the present work, three biochemical mutants of *P. oryzae* and six differential rice varieties are selected to be used in this experiment. The amount of specific amino acids required for the proper growth of mutants as well as the concentration of the specific amino acids in the host varieties are quantitatively compared.

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Materials and Methods

Mutants employed: Three biochemical mutants of *P. oryzae* used in this study were induced from the treatment of N-methyl-N'-nitro-N-nitrosoquanidine. They required glutamic acid, histidine and arginine, respectively.

Growth response test with different concentration of required substances: The complete medium (CM) and the minimum medium (MM) used were similar to our previous report (Kuo *et al* 1967). For the determination of the grwoth of biochemical mutants at different concentrations of the required substances, the flasks containing liquid MM with 10^{-5} *M* up to 10^{-2} *M* of the required substance were inoculated with a piece of mycelial mat of mutants which was 4 mm in diameter. The flasks were then shaken at approximately 28°C for 5 days. The growth was estimated by measurement of the dry weight of mycelial growth. (Mycelia were dried at 65°C for 48 hrs.)

Differential host varieties used: Six rice varieties, which showed different response to the biochemical mutants were used as differentials. They were: Taichung 171, Chia-Nung-Yu 280, Taichung 65, Kao-Chio-Liu-Chou, Kung-Shan-Wu-Shen-Ken and Kanto 51. They were grown in a green house. When they were about forty-day old, the leaf sheaths of the main stem were harvested. The top section about 2 cm in length was discarded and the next section about 10 cm in length was selected. These selected leaf sheaths were used for the determination of pathogenicity, the analysis of the required amino acids, and the extracts from which were also used to test the growth of the pathogen.

Pathogenicity test: Sakamoto's inner leaf sheath inoculation method (Sakamoto 1949) was adapted. Inocula for the test were aqueous suspension obtained from Yamanaka's barley medium method (Yamanaka 1961). Conidia were washed several times before inoculation. The concentration of conidia was about 1000 per ml. The leaf sheath sections were soaked in distilled water for 2 hours before inoculation. Inner surface of detached leaf-sheath was inoculated with conidial suspension, then these leaf sheaths were placed in Petri dishes which were saturated with water. The open side of leaf sheath was always placed upward. In testing the pathogenicity and nutrient requirement of pathogen, the final concentration of 10^{-3} *M* of the required nutrient was directly added into the conidial suspension. After 40-hour inoculation period at 28°C, the inner epidermal cells of leaf sheath were striped and mounted on a slide. The degree of infection was examined under the microscope. The "disease rating" was calculated by the method described by Takahashi (1951). In the case of susceptible varieties the mycelial development of the fungus in the epidermal cells appeared to be of vigorous growth. Whereas in the case of resistant varieties, no mycelial development could be observed. Only there

was a partial or entire browning of the cell membrane as well as the presence of brown colored granules in the cytoplasm of epidermal cells which were situated under the fungus appressoria.

Growth response tests with leaf sheath sap: The leaf sheaths, harvested as described in the preceeding paragraph, were heated to boiling for 20 minutes and then they were crushed with Warring blander. Distilled water was added at the rate of 100ml to 3 g of fresh tissue. The resulting suspension was cleaned by centrifugation and the supernatent liquid was adjusted to pH 6.0, in which ten grams per liter of sucrose was added, then dispensed in 25 ml amount in 125-ml Erlemeyer flasks. Two different liquid media were used in the growth-response test for each mutants. They were: 1), 20 ml leaf sheath sap plus 5 ml distilled water, 2), 20 ml leaf sheath sap plus 5 ml of concentrated solution of the required substance. The media were autoclaved for 15 minutes at 15 lb., then the supplementary solutions were added. The supplementary solutions were adjusted to pH 6.0 and sterilized by passing through bacterium-proof filter. The final concentration of the amino acids added as a supplement was 0.15%. The flasks were inoculated with a piece of mycelial mat, then they were shaken at 28°C for 5 days. The growth was estimated by measurement of the dry weight of the mycelial growth.

Amino acid content in leaf sheath: The following schedule was used for the analysis of free amino acid content in the host tissue. Twenty grams of fresh leaf sheaths were harvested as being described already. The amino acids were extracted with 300 ml of 80% ethanol for 10 minutes in a Warring blander. The extracts were filtered by passing through Whatman No. 1 filter paper and concentrated under vacuum below 40°C. After ethanol was evaporated the precipitates were removed by filteration with Celite No. 545. The aqueous fractions were desalted with Dowex 50W-X8 ion-exchange resin according to the procedure of Thompson *et al* (1959). The amino acids were eluted with 100 ml 2N ammonium hydroxide. The eluates were dried under vacuum below 40°C. The dried amino acids were recovered with 4 ml 0.001N HCl containing 10% isopropanol, so that 1 ml represented 5 g of leaf sheath (fresh weight). All paper chromatograms used for quantitative determinations were made on Whatman No. 1 filter paper. Two dimensional technique (descending) was used. The solvent systems employed were n-butanol: acetic acid:water (4:1:1 v/v/v) followed with m-cresol:phenol:borate buffer 0.1M pH 9.1 (25:25:7 w/w/v) in the second dimension. Chromatograms were run approximately 18-20 hr in each direction at room temperature and dried overnight at the same temperature after each run. Chromatograms were sprayed with 60 ml freshly prepared 0.5% ninhydrin in acetone. The color was developed by heating at 65°C for 30 min. All spots were marked and cut out and eluted with 4 ml 75%

ethanol containing 0.2 mg of Cu_2 $SO_4 \cdot 5H_2O$. All the elutates were read in Bechman Model DU Spectrophotometer at 540 mμ. Standerd curved were prepared under the same condition for each amino acid. Sample reading was converted into absolute amount of μg and calculated to μg/g of fresh weight.

Results

Pathogenicity of mutants with and without external supplement: The six rice varieties used as differentials were inoculated with: 1) original strain without any amino acid supplement, and 2) three mutants with and without amino acid supplement. As indicated in Table 2, all the varieties except Kanto 51 were susceptible to the original strain. When specific amino acids were not given, the pathogenicity of biochemical mutant was markedly different from that of the original strain. With regard to the mutant requiring glutamic acid, their mycelial penetration into the epidermal cells were observed in Taichung 171, Kung-Shan-Wu-Shen-Ken and Chia-Nung-Yu 280, but the mycelial development in the epidermal cells was poorer than when the original strain was used. The penetration of the germination tube of histidine requiring mutants could be seen in the epidermal cells of Chia-Nung-Yu 280, and Kung-Shan-Wu-Shen-Ken, but the mycelial development was very poor. The mutant requiring arginine was avirulent to all of these rice varieties. The conidia of this mutant could germinte well but no penetration of the mycelia into the epidermal cells ever occurred. If the loss of pathogenicity of biochemical mutants was related to the unavailability of the required nutrients (Garber 1956b), it should be possible to restore their pathogenicity by supplying the nutrient at the site of inoculation. When exogenous required nutrients were supplied as supplement (Table 1), the pathogenicity of glutamic acid requiring mutant was restored

Table 1. *Restoration of pathogenicity of nutritionally deficient mutants of* ***P. oryzae*** *by supplement with specific required amino acids to six differential host varieties.*

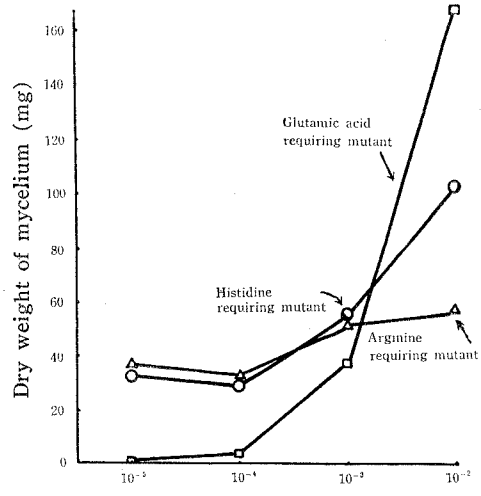
Strains / Supplement / Differential hosts used	Glutamic acid requiring		Histidine requiring		Arginine requiring		Original
	Without	With	Without	With	Without	With	Without
Taichung 171	*2.1	4.3	0.5	0.5	0.5	0.5	3.1
Chia-Nung-Yu 280	2.2	3.3	1.0	1.0	0.5	0.5	7.8
Taichung 65	0.5	0.5	0.5	1.5	0.5	0.5	3.0
Kao-Chio-Liu-Chou	0.5	1.7	0.7	1.0	0.5	0.5	3.7
Kung-Shan-Wu-Shen-Ken	2.0	4.0	1.0	1.1	0.5	0.5	4.0
Kanto 51	0.5	0.5	0.5	1.1	0.5	0.5	1.7

* disase rating is based on the Takahashi's method (Takahashi 1951).
0.5-1 = resistant; 1-2 = moderately resistant; larger then 2 = susceptible.

only to varieties in which penetration already occurred. (varieties Taichung 171. Chia-Nung-Yu 280, Kung-Shan-Wu-Shen-Ken). The mycelial development of the mutant in the epidermal cells of Taichung 171, Chia-Nung-Yu 280 and Kung-Shan-Wu-Shen-Ken was almost the same as that of the original strain and with the same disease rating after supplementation. However, with Taichung 65, Kao-Chio-Liu-Chou and Kanto 51 in which no penetration occurred, there was no mycelial development. Thus, the disease rating remained unchanged. The mutants requiring histidine and arginine remained avirulent even after a large amount of required amino acids was provided as supplement.

Growth response to different concentrations of required substance: Each of the three induced mutant strains was tested as to their growth response to the specific amino acid which they required. The minimum quantity of a specific amino acid supplement needed by respective mutants were estimated from the relationship between the concentration of the supplement and the growth of mutants. As shown in Fig. 1, the three mutants tested significantly differed in the minimum requirement for specific amino acid as well as in the response-curve to the concentration of specific amino acid. In the glutamic

Fig. 1. Growth-response of amino acid requiring mutants of *P. oryzae* at different concentrations of glutamic acid. histidine and arginine.



acid requiring mutant no growth was observed when the concentration of supplemented glutamic acid was below 10^{-4} *M*. Growth occurred when the concentration of glutamic acid was 10^{-3} *M* and was greatly enhanced at 10^{-2} *M* concentration. In general, this mutant gave a sensitive response to the concentration of glutamic acid. In the histidine requiring mutant, growth occurred at a low

concentration (10^{-5} *M*) of histidine and gradually promoted when higher concentration of histidine was added, but the response curve was not so sharp as the curve for glutamic acid requiring mutant. Likewise, the arginine requiring mutant could grow at low concentration (10^{-5} *M*) of arginine, but the growth was not promoted much with higher concentrations.

Growth of biochemical mutants in leaf sheath extracts: Three biochemical mutants were inoculated into aequeous extracts of leaf sheath of six rice varieties. The results are given in Table 2. There was some difference found in the growth of the 3 mutants in the 6 host varieties. However, the difference did not show that there was any correlation in existence between the nutrient requirement of the mutants and their pathogenicity (Table 1, Table 2). It would be reminded here that the extracts were obtained after being heated. Perhaps the contents might be denatured. When the extracts were supplemented with specific amino acid, the growth was increased as a general rule indicating the absence of inhibitors.

Table 2. *Growth of biochemical mutants in leaf sheath saps from differential hosts supplemented with and without requiring amino acids.*

Strains / Supplement / Differential hosts used	Glutamic acid requiring		Histidine requiring		Arginine requiring	
	Without	With	Without	With	Without	With
Taichung 171	*67	148	59	93	69	139
Chia-Nung-Yu 280	94	166	40	78	67	116
Taichung 65	108	161	34	51	87	130
Kao-Chio-Liu-Chou	62	159	64	74	77	126
Kung-Shan-Wu-Shen-Ken	55	122	89	147	86	128
Kanto 51	60	117	139	167	63	127

* dry weight of mycelial growth in mg.

Pathogenicity of mutants and the content of required substance in the host: The pathogenicity of the biochemical mutants as compared with the free amino acid content of each host variety is summerized in Table 3. The host varieties differed in the concentration of these pertinent amino acids in the leaf sheath. In general glutamic acid showed about 10 times higher concentration than histidine and about 50 times higher concentration than arginine. Varieties such as Chia-Nung-Yu 280 and Kao-Chio-Liu-Chou had a high glutamic acid content but Kung-Shan-Wu-Shen-Ken was with a low content. The histidine contents were low in Taichung 171, Chia-Nung-Yu 280, Kao-Chio-Liu-Chou, Kung-Shan-Wu-Shen-Ken and Kanto 51 except Taichung 65 which was more than two fold. Regarding arginine content Taichung 65 and Kanto 51 had a higher value than

Table 3. *The free amino acids content and response of six different rice varieties to inoculation with three specifice amino adci requiring mutants of* ***P. oryzae.***

Differential hosts	Content of glutmic acid	Response to glutamic acid requiring mutant	Content of histidine	Response to histidine requiring mutant	Content of arginine	Response to arginine requiring mutant	Response to original strain
Taichung 171	740(1)	S(2)	49	R	11	R	S
Chia-Nung-Yu 280	908	S	66	mR	16	R	S
Taichung 65	710	R	156	R	31	R	S
Kao-Chio-Liu -Chou	850	R	54	R	13	R	S
Kung-Shan-Wu -Shen-Ken	476	S	58	mR	5	R	S
Kanto 51	520	R	77	R	22	R	mR

(1) Content of amino acid was expressed by μg/g of fresh weight.
(2) R= Resistant; mR= Moderately resistant; S= susceptile.

Kung-Shan-Wu-Shen-Ken which had the lowest value. With regard to the concentration of free amino acids, though they differed greatly among the host varieties, however, they did not seem to indicate that the pathogenicity of the mutant would correlate with the concentration of the specific amino acid in the host varieties. Only in Chia-Nung-Yu 280 with a higher glutamic acid content it was found to be susceptible to glutamic acid requiring mutant. On the other hand, Kao-Chio-Liu-Chou which contained glutamic acid at a high concentration was resistant while Kung-Shan-Wu-Shen-Ken containing the lowest amount of glutamic acid was susceptible. With the arginine requiring mutant, though the host varieties markedly differed in arginine content, all of them were resistant to this mutant. With the histidine requiring mutant, Taichung 65 with 156 μg/g (fresh weight) of histidine, the highest of all the varieties tested was resistant while Chia-Nung-Yu 280 and Kung-Shan-Wu-Shen-Ken with 66–58 μg/g were moderately resistant. Apparently, the penetration of the germination tube could be observed in Chia-Nung-Yu 280, Kao-Chio-Liu-Chou and Kung-Shan-Wu-Shen-Ken, but the mycelial development was very poor in the host cells.

Discussion

According to the nutritional-inhibition hypothesis, the avirulence of pathogens with known nutritional deficiences reflects the unavailability of required compounds in the host environment at the site of inoculation or localization. Since the conidia of *P. oryzae* germinate on distilled water, it is reasonable to assume that the conidia have a sufficient supply of endogenous substances for germina-

tion. It may be assumed that the epidermal cell does not provide any substances for growth. Consequently it may be concluded that the loss of pathogenicity of mutants is related to the unavailability of the required compounds in the inner epidermal cell of leaf sheath. The host varieties were found to differ in the concentration of specific amino acids. It may be expected that a host containing a required amino acid at a high concentration is susceptible to the mutant requiring this very specific substance. Unfortunately it is not ture for all of the mutants used in this experiment even though the concentration of the specific amino acids in some of the hosts appear to be high enough for the growth of the mutants.

Furthermore, the growth respenses of these biochemical mutants to different concentrations of the required amino acids show that the minimum concentration of the required substances, arginine and histidine for the growth of arginine and histidine requiring mutants can be very low. With the increase of arginine and histidine concentration, the growth of both mutants is promoted. However, the response is not very sharp. On the other hand, the minimum concentration of glutamic acid necessary for the growth of glutamic acid requiring mutant is much higher and the growth response is sensitive to the increased concentration of glutamic acid. This difference is important for the restoration of pathogenicity by supplying the required amino acid as supplement. By supplying the require substance from a source outside the host, pathogenicity can be restored for some host varieties in case when glutamic acid requiring mutant is under consideration.

Pathogenesis necessitates a chain of events to happen in the causalty of disease. For *P. oryzae* it may include the affinity between the host and pathogen, invasion of the pathogen into the host cell, and finally the disease development. The absence of any of these steps would lead to the failure of infection and avirulence. The nutritional aspect of host-parasite relation of *P. oryzae* may be important in the step of disease development as demonstrated by the glutamic acid requiring mutant in certain host varieties. However, the avirulenece of arginine and histidine requiring mutants is due perhaps to mutations affecting their virulence and not to their inability to grow in the host.

Possibly there is a mechanism of resistance which would inhibit the uptake or utilization of nutrients by the mutants. However, most of the host varieties used for testing the pathogenicity of the mutants are susceptible to the original strain of pathogen and the growth of these mutants in the leaf sheath extracts supplied with their required substances is very good. Therefore inhibition factors can not play an important role as the cause of avirulence of the mutants. Since nutritional deficiency can not explain all the data found in our experiment, there must be involvement of many other factors in the host-

parasite relationship of *P. oryzae*. Togashi *et al* (1960) demonstrated that the application of piricularin, a metabolic product of the fungus, reduces the resistance of host cell to the growing hyphae. Ohata *et al* (1963) reported that the hypersensitive reaction of the host is a main cause of resistance in *P. oryzae*. Other factore such as antagonisms between metabolites, alterations in the physicochemical functions of cell may be also involved.

Summary

The minimum requirement for specific substances of strains of *Piricularia oryzae* and their respons to different concentration of specific substaces were found to be markedly different among three mutant strains observed. The glutamic acid requiring mutant showed a sensitive response to the concentration of glutamic acid. Whereas arginine and histidine requiring mutants could grow at a low concentration of arginine and histidine, but the gorwth was not promoted much by high concentrations of arginine and histidine supplied.

In leaf sheath extracts, the growth of all three mutants was poor. When nutrients required by the mutants were added, all mutants showed considerably better growth in the supplemented media. There was no correlation between the growth responses to leaf sap of the mutants and their pathogenicity.

When the exogenous required nutrient were supplied, the pathogenicity of glutamic acid requiring mutant was restored in some of the hosts used, but the mutant requiring histidine and arginine remained avirulent even after a large amount of required amino acids was provided.

The resistance or susceptibility of six rice varieties to three amino acid requiring mutants of *P. oryzae* and the content of free amino acid required by the mutants in these rice varieties were compared. The pathogenicity of these mutants for the six rice varieties did not correlate with the concentration of specific required amino acid in these rice varieties.

水稻稻熱病寄主及病原菌間營養問題之研究

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利用各別要求 glutamic acid, histidine 及 arginine 之水稻稻熱病病原菌之變異株及六種寄生性辨別用之水稻品種研究病原菌及寄主間之營養問題。要求 histidine 及 arginine 之變異株其對此等營養之要求極低，祇要有微量就足够其生長。增加此等營養分之供應未必能增加此變異株之生長，因此水稻體內含此等營養分高之品種未必爲感病性，相反者未必爲抗病性。要求 glutamic acid 之變異株其對 glutamic acid 之要求與上述二變異株不相同。其生長與所供應之 glutamic acid 之濃度成正比。供應 glutamic acid 能增加其病原

性。但不同品種水稻體內所含之 glutamic acid 均很高，如依濕重計算水稻體內所含之此等營養分均足够供給此變異株之最低生長，故寄主體內之此等營養分不是決定病原菌病原性之主要因子。

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