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INTERCROSS FERTILITY BETWEEN *HELMINTHOSPORIUM ORYZAE, H. ZIZANIAE* AND AN UNIDENTIFIED *HELMINTHOSPORIUM* SP. ON *ZIZANIA AQUATICA*[1,2]

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Abstract

The delimitation of three species of *Helminthosporium* i. e., *H. oryzae, H. zizaniae* and a species of *Helminthosporium* on *Zizania aquatica* was investigated by means of intercross fertility of monosporic cultures. *H. oryzae* and *H. zizaniae* were cross-fertile. *H.* sp on *Z. aquatica* was also cross-fertile with *H. oryzae* and *H. zizaniae.* Based on the genetic point of view and on the biological concept of species, the *Helminthosporium* cultures investigated are the same species, i. e. *H. oryzae.*

Introduction

Perithecium formation of *Helminthosporium oryzae* in the laboratory conditions so far was only reported by Ito and Kuribayshi in 1927. No other report from the main Asian rice grown areas on the perfect state of this fungus has been published since then as this author is aware. Tullis (Dickson, 1956) collected the perfect state under field conditions in the United States. Dickson found the perfect state in old straw and stubble in southern Mexico in 1953. The perfect state of other species of *Helminthosporium* has been extensively investigated in laboratory conditions (Ito & Kuribayashi, 1931; Luttrell, 1958; Nelson, 1957; 1964; Tinline, 1959).

H. zizaniae is the species established by Nishikado in 1928 which causes brown leaf spot on *Zizania latifolia.* The symptoms caused by *H. zizaniae* on *Z. latifolia* was exactly similar to those on rice leaves caused by *H. oryzae.* Nishikado (1928) showed that *H. zizaniae* also attacked *Oryza sativa,* but he established it as a new species based mainly on the shape of basal part of conidia and the hilum and the dimensions of conidia. Yamamoto *et al.* (1956)

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made a comparative investigation on the relationship between *H. zizaniae* and *H. oryzae* particularly on the pathogenicity of these two species of *Helminthosporium* on *Z. latifolia* and *O. sativa*. They also compared the dimensions of conidia of these *H. spp.* Based on their investigations they came to a conclusion that *H. zizaniae* might be classified as a race of *H. oryzae*.

The facts to be reported here were the results of the perithecium formation of *H. oryzae, H. zizaniae* and two isolates of *H. sp.* isolated from wild rice (*Zizania aquatica*) brown leaf spot in northern Minnesota, USA. The interspecific crosses between these *H.* sp. isolates with *H. oryzae* and *H. zizaniae* were also made to investigate their phylogenic relationship.

Materials and Methods

All isolates of *H. oryzae, H. zizaniae* and *H.* sp. on wild rice were obtained by means of single spore isolation including conidia and ascospores. They were cultured either on potato sucrose agar or on Czapek's agar. Methods of experiments to produce perithecia were adopted from those employed by Nelson (1957) and Luttrell (1958). Two small pieces of mycelial agar cut from random isolated strains were placed on opposite ends of the section of corn leaf which was placed on the center of a petri dish containing Sach's medium. The sections of corn leaves, approximately 2 cm^2 were sterilized by autoclaving for 20 min at 15 pounds. The petri dishes were then incubated at 24°C for one to two weeks before examination.

Recently Fukuki and Aragaki (1973) reported that dialyzing membrane was a better substitute for the perithecium formation than corn leaf section on *Cochliobolus heterstrophus*. Thus dialyzing membrane was also used to compare its effectiveness. Cellophane paper was also employed because it costs far less than dialyzing membrane. Dialyzing membrane and cellophane paper were sterilized before they were placed on the surface of Sach's agar medium.

Results

Helminthosporium oryzae

In preliminary tests, monoconidium isolates were crossed randomly on sterilized corn leaf sections and incubated at 24°C. Within one week black dot perithecia formed on corn leaf surface. In the beginning of the perithecium formation on leaf surface or autoclaved corn kernals a tuft of upward conidiophores and conidia developed on small perithecial initials. The perithecial initials enlarged gradually, at the same time the upward growing hyphae (which developed into conidiophores) disappeared. Most of the mature perithecia were global, pseudoparenchmatous, black, and formed an ostiolar beak. Asci are cylindrical to long, fusiform, slightly curved, and contain mostly

four to six ascospores. Ascospores are filiform and formed in a close helix in the ascus (Fig. 1).

Helminthosporium zizaniae

Single conidial isolates from Nankang, Taipei, and Wu-Feng in Taichung county were crossed randomly on sterilized corn leaf sections and incubated at 24°C for two weeks. Perithecia formed on corn leaf sections, the shape and size of perithecia, asci and ascospores are similar to those perithecia of *Cochliobolus miyabeanus* Ito et Kuribayashi.

*Matings between **H. oryzae** and **H. zizaniae***

The pathogenicity of *H. oryzae* and *H. zizaniae* on *O. sativa* and *Z. latifolia* was identical despite of heterologous or homologous inoculation (Yamamoto *et al.*, 1956; and Chang, 1974). It has been assumed that these species are actually the same species but could be different races. Cross-fertility or cross-sterility might provide a conclusive evidence to trace the phylogenic relation between these two species (according to Nishikado's identification and classification).

In preliminary experiments, random crosses were made between isolates of *H. oryzae* and *H. zizaniae* obtained from Taipei and Wu-Feng, the numbers of isolates used in preliminary crosses were limited. An isolate of *H. oryzae* from Taipei crossed with an isolate of *H. zizaniae* formed abundant perithecia on corn leaf sections. The results indicated that the *H. oryzae* and *H. zizaniae* were cross-fertile; when two compatible isolates meet on suitable substrates the perithecia develop.

Further mating experiments were carried out to confirm the cross-fertility between *H. oryzae* and *H. zizaniae.* Single spore cultures of *H. zizaniae* were isolated from Kuo-Sheng (國姓), Pu-Li (埔里) and Hou-Li (後里). Three isolates designated as K–1, K–2, and K–3 were isolated from Kuo-Sheng; four isolates designated as H–1, H–2, H–3, and H–4 were isolated from Hou-Li; and nine isolates designated as P–1, P–2, P–3, P–4, P–5, P–6, P–7, P–8 and P–9 were isolated from Pu-li. Those isolates were crossed separately with an isolate of *H. oryzae* designated as H–o–1 and an isolate of *H. zizaniae* designated as H–z–1. Isolates H–o–1 and H–z–1 were cross-fertile each other and formed abundant perithecia on suitable substrates, such as autoclaved corn leaf sections on Sach's agar medium which was used in these experiments.

The results shown in Table 1 confirm that *H. oryzae* and *H. zizaniae* were cross-fertile. Isolate H–o–1 of *H. oryzae* crossed with those isolates obtained at Pu-Li were fertile except P–1, however, H–o–1 were cross-sterile with those isolates obtained at Hou-li and Kuo-Sheng. Isolate H–z–1 was cross-fertile with those isolated from Kuo-Sheng, Hou-Li and P–1, and was cross-sterile with the rest of the isolates. This result by no means indicates the geographic distribution of incompatible strains because the numbers of isolates used in

Table 1. *Interspecific crosses of* ***H. oryzae*** *and* ***H. zizaniae***

Isolates of *H. zizaniae*	Mating reaction in crosses*	
	H. oryzae (H-o-1)	*H. zizaniae* (H-z-1)
K-1	−	+
K-2	−	+
K-3	−	+
H-1	−	+
H-2	−	+
H-3	−	+
H-4	−	+
P-1	−	+
P-2	+	−
P-3	+	−
P-4	+	−
P-5	+	−
P-6	+	−
P-7	+	−
P-8	+	−
P-9	+	−

* '+' indicates mature perithecia produced; '−' indicates no perithecia produced.

this study were very limited. It was highly possible that the isolates obtained at Kuo-Sheng and Hou-Li possess same mating factor, and were picked up to cross with H-o-1 strain of *H. oryzae*. Same situation could happen that the isolates obtained from Pu-Li with same mating factor except P-1 were picked up to cross with H-o-1. No intention was made here to investigate the geographic distribution of mating types of *H. oryzae* and *H. zizaniae*.

Potential of perithecium formation of F_2 ascospore self-crosses

This experiment was to determine whether the potential of perithecium formation in the progeny secured from cross between an isolate of *H. oryzae* (H-o-1) and an isolate of *H. zizaniae* (H-z-1) is persistent. F_2 single ascospore cultures BD-2 and BD-20 were crossed with BD-1, BD-2, BD-3, BD-4, ... BD-19, and BD-20. In preliminary tests it was found that the single ascospore cultures BD-2 and BD-20 were cross-fertile. The BD series cultures are the progeny of the cross of cultures of single ascospores of B and D. B and D are obtained from the single ascospore cultures which are the offspring of the cross between Isolates H-o-1 and H-z-1.

The results shown in Table 2 indicate that the potential of self-cross of F_2 ascospore cultures was as strong as their parents. Within these self crosses there were indeed showed some variation in potential of perithecium formation. BD-20 single ascospore culture crossed with BD-1, BD-7 and BD-10 showed

strong potential in perithecium formation, same results also showed in the crosses BD-2 with BD-3; BD-5; BD-6; BD-12; BD-16; BD-17; and BD-18.

Table 2. *Cross-fertility among F_2 ascosporic cultures of H. oryzae and H. zizaniae*

Isolates of monoascosporic cultures	Mating reaction in crosses*	
	BD-2	BD-20
BD-1	−	+
BD-2	−	+
BD-3	+	−
BD-4	−	+
BD-5	+	−
BD-6	+	−
BD-7	−	+
BD-8	+	−
BD-9	−	+
BD-10	−	+
BD-11	−	+
BD-12	+	−
BD-13	−	+
BD-14	−	+
BD-15	+	−
BD-16	+	−
BD-17	+	−
BD-18	+	−
BD-19	+	−
BD-20	+	−

* '+' denotes perithecia produced; '−' denotes no perithecia produced.

*Matings between **H. oryzae** and **H. zizaniae** with H. sp. isolated from brown leaf spot of **Zizania aquatica***

For last few years *Z. aquatica* has been extensively cultivated in northern Minnesota. After two years continued cultivation, brown leaf spot and internode rot have become one of the limiting factors on the cultivation of *Z. aquatica* in that part of country. The causal agent was suggested to be *H. oryzae*. However, no cross inoculation tests on *O. sativa* have been made so far as this author is understood. Based on the morphology of conidium, it seems to be doubtless that the causal agent of brown leaf spot on *Z. aquatica* is *H. oryzae*. When the detached leaf sections of *Z. latifolia* and *O. sativa* were inoculated by this pathogenic helminthosporium fungus, typical brown leaf spot appeared. Seven single conidium cultures of *H.* sp., i.e., G-1, G-2, G-3, G-4, G-5, G-6, and G-7 obtained from a culture supplied by Mr. Kroll of the Department of Plant Pathology, University of Minnesota and seven single

conidium cultures of *H.* sp, i.e., M-1, M-2, M-3, M-4, M-5, M-6, and M-7 isolated from a diseased leaf specimen were crossed with isolates H-o-1 and H-z-1. The results are shown in Table 3. All 14 single spore cultures from *Z. aquatica* were cross-fertile with isolate H-o-1 and were cross-sterile with H-z-1. Because isolates H-o-1 and H-z-1 were cross-fertile, therefore 14 single spore cultures must be with same mating factor, i.e., they possess either plus or minus factor. From the results a conclusion could be made that the causal agent of brown leaf spot on *Z. aquatica* is *H. oryzae.*

Table 3. *Cross-fertility of* ***H. sp*** *on* ***Z. aquatica*** *with* ***H. oryzae*** *and* ***H. zizaniae***

Isolates of *H.* sp on *Z. aquatica*	Mating reaction in crosses*	
	H. oryzae (H-o-1)	*H. zizaniae* (H-z-1)
M-1	+	–
M-2	+	–
M-3	+	–
M-4	+	–
M-5	+	–
M-6	+	–
M-7	+	–
G-1	+	–
G-2	+	–
G-3	+	–
G-4	+	–
G-5	+	–
G-6	+	–
G-7	+	–

* '+' denotes perithecia produced; '–' denotes no perithecia produced.

Perithecium formation ***Helminthosporium oryzae*** *on cellophane paper and dialyzing membrane*

Fukuki and Aragaki (1973) reported that dialyzing membrane served better than corn leaf sections as supporting substrate for perithecium formation in *C. heterostrophus* on Sach's medium. Dialyzing membrane is relatively expensive here in Taiwan, a substitute material is desired when corn leaves are unavailable. Cellophane paper, a not expensive and easily secured material was served to test its suitability for perithecium formation. Experimental results showed that perithecia formation in *C. miyabeanus* on cellophane paper was as good as on dialyzing membrane. It is speculated that many membrane materials, even tissue paper, can be served as a substrate on Sach's agar medium for perithecium formation in *C. miyabeanus.* However, no perithecia formed on

medium surface which was not covered by membrane materials. The explanation for perithecium formation only on membrane materials, such as corn leaves, dialyzing membrane, cellophane paper and paper is lacking.

Discussion

The perfect state of *H. oryzae* was first obtained by Ito and Kuribayashi in 1927 under laboratory conditions. Thereafter no report has been published on this subject, except Dickson and Tullis have found in the field conditions in southern Mexico and in the United States respectively. The present experimental results demonstrated that two mating factors, plus (+) and minus (−) were widely existed in Taiwan. This author employed various supporting substrates such as sterilized corn leaf section, dialyzing membrane, cellophane paper and corn kernels have demonstrated that all of them proved to be suitable for perithecium formation when they were placed on Sach's agar medium.

Regarding to the significance of the perfect state of this fungus in causing the epidemic in Taiwan, it is remained to be solved because no perfect state of this fungus has been found in Taiwan or other Asian countries as far as this author is aware. It is still not clear that ascigerous state of this fungus exists in nature under the yet unknown circumstances.

Ascigerous state of *H. zizaniae* was first produced in laboratory conditions by this author. No separate nomenclature will be given because *H. zizaniae* has been suggested as a race of *H. oryzae* based on its pathogenicity on *O. sativum* (Yamamoto *et al.*, 1956; Chang, 1974) and its cross-fertility with *H. oryzae* in mating experiments undertaken in the present investigation. The morphology of ascigerous states of both *H. oryzae* and *H. zizaniae* is identical.

Two isolates of *H.* sp. which cause brown leaf spot on *Z. aquatica* in northern Minnesota were also cross-fertile with an isolate of *H. oryzae* (H-o-1) in this investigation. This conclusive evidence indicates that the isolates secured from brown leaf spot on *Z. aquatica* are *H. oryzae.*

Intercross fertility among the species of *H. oryzae, H. zizaniae* and *H.* sp. on *Z. aquatica* in northern Minnesota provides a conclusive evidence that these species of *Helminthosporium* should be classified in one species, i.e., *H. oryzae* based on the concept of biological species. Mayr (1970) defined that species '...are actually as potentially interbreeding populations which are reproductively isolated from other such groups'. This concept of species has rarely been applied to delimit species in Mycota because it is impractical in some groups of fungi (Talbot, 1971). However, in the groups of fungi their intercross-fertility can be practically employed, then the biological concept of species should have its prestige to be applied in the classification of Mycota and in clarification of their phylogenic relationship.

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Literature Cited

CHANG, H. 1974. Unpublished data.

DICKSON, J. 1956. Diseases of field crops. 2nd ed. 517. Mcgraw-Hill. New York.

FUKUKI, K. A., and ARAGAKI, M. 1973. Perithecial formation by ***Cochliobolus heterostrophus*** on dialyzing membrane. Mycologia **65**: 705-709.

ITO., S., and KURIBAYASHI, K. 1927. Production of the ascigerous state in culture of ***Helminthosporium oryzae***. Ann. Phytopath. Soc. **2**: 1-18.

ITO, S., and KURIBAYASHI, K. 1931. The ascigerous forms of some graminicolus species of ***Helminthosporium*** in Japan. Jour. Facul. Agr., Hokkaido Imp. Univ., Sapporo, Japan. **29**: 85-123.

LUTTRELL, E. S. 1958. The perfect stage of ***Helminthosporium turcicum***. Phytopathology **58**: 281-289.

MAYR, E. 1970. Population, species, and evolution. 453 pp. The Belknap Press of Harvard University Press. Mass.

NELSON, R. 1957. Heterothallism in ***Helminthosporium maydis***. Phytopathology **47**: 191-192.

NELSON, R. 1964. The perfect stage of ***Helminthosporium cynodontis***. Mycologia **56**: 64-69.

NISHIKADO, Y. 1928. Studies on the Helminthosporiose of Japanese graminaceous plants. Spec. Rep. Ohara Inst. Agr. R. IV. (in Japanese with English summary).

TALBOT, P. H. B. 1971. Principles of fungal taxonomy. 274 pp. Macmillan, London.

TINLINE, R. D. 1959. Studies on the perfect stage of ***Helminthosporium sativum***. Canadian J. Botany **29**: 467-498.

YAMAMOTO, W., M. MAEDA, and T. IDONO. 1956. Studies on the brown spot of ***Zizania latifolia*** Tuca. with special reference to comparison with the brown spot of rice plants caused by ***Helminthosporium oryzae*** Breda de Haan. Sci. reptr. Hyago University of Agr. Vol. 2, No. 2 series: Agr. Biol.

三種 *Helminthosporium* spp. 的種屬關係

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本研究是在探討水稻 (*Oryza sativa*)，茭白 (*Zizania latifolia*) 和 *Zizania aquatica* 上引起葉褐點病 (brown leaf spot) 病原 *Helminthosporium* 的種屬問題。過去認爲在水稻上的是 *H. oryzae*，在茭白上的是 *H. zizaniae*，在野生稻上的是未鑑定的 *H.* sp. 利用三者的單胞菌株相互交配，根據它們是否形成有性世代的子實體，判別三者是否同屬於一種，也就是 *Helminthosporium oryzae*。實驗的結果證明三者相互交配能形成子囊殼，子囊和子囊胞子，所以三者應該是同屬 *H. oryzae*。

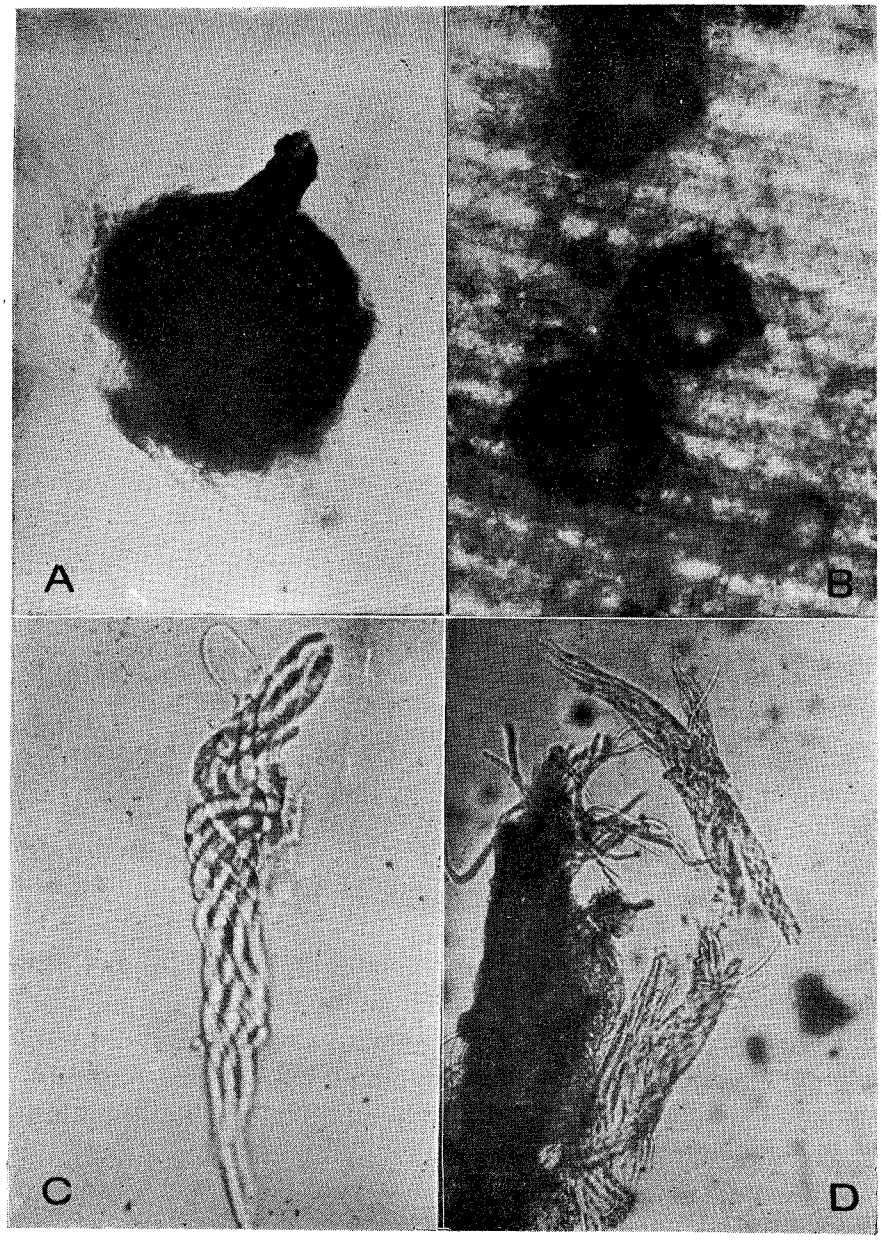

Fig. 1. Fruiting body of ***Cochliobolus miyabeanus***, the perfect state of *H. oryzae*. A and B: perithecia; C and D, ruptured asci and helicoid ascospores.