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Detecting Seed-Borne Fungi of Rice and Transmission of *Helminthosporium oryzae* in Germinating Seed

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벼種子隨伴眞菌의 檢定 및 種子發芽에 따른 깨씨무늬병균의 傳染經路

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SUMMARY

From the sample seeds of rice cultivar Palkoeng with the brown spot symptom, *Helminthosporium oryzae* was detected in 86~92% of hulls including empty glumes, lemma, palea, and rachilla in five to seven days of incubation. When the seeds were dehulled, the percentage detection decre ased to 74~83% of pericarps. The fungus sporulated on whole surface of the seed and was detected within about ten cell layers deep of endosperm but not detected in embryo. *Fusarium moniliforme* was found in embryo as well as pericarp and endosperm. The frequency of *F. roseum*, *Trichoconis padwickii*, *Curvularia* spp., *Alternaria tenuis*, *Cladosporium cladosporoides* and *Phoma* sp. was less than three percent of hulls and pericarps. In seven days of incubation, 60~87% of the germinating seeds showed the symptom of *H. oryzae* while 13~40%were apparently healthy. This fungus in hilum of infected rice transmitted through pericarp to plumule shoot and radicle of the germinating seed.

INTRODUCTION

The brown spot of rice caused by *Helminthosporium oryzae* Breada de Haan has been prevalent incurring severe loss in yield depending upon severity of infection in the fields(1, 6, 7, 13). The infected seeds with difuse symptom among healthy seeds were hardly detected to result in diseased seedlings when they were sown(4, 9, 7). Ganguly (3) thought that the seed-borne infection of this fungus was of little importance in the later stage of rice growth, acknowledging its importance for the early stage. Thomas(18) reported that the naturally infected rice seeds sown in sterilized soil gave rise at least to 20% diseased seedlings

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whereas the percentage increased to 60% when artificially inoculated seeds were sown. Ocfemia (12) observed that the diseased seedlings from the infected seeds with *H. oryzae* varied from 10 to 100% depending upon the ambient conditions including soil temperature. Although Fazli and Schroeder(2) confirmed the mycelial infection of the fungus in endosperm of rice seed, neither its infection in the other parts of the seed nor its route of transmission to seedling have been reported.

Since the transmission of brown spot after extrusion of the secondary leaf may have a high possibility due to seco ndary infection from the inoculum of same individual seedlings as well as of other sources, the present investigation was mainly concentrated on the early stage of the seed germination before extrusion of the secondary leaf or adventitious root. The purpose of the present experiment was to detect seed-borne fungi of rice, location nf *H. oryzae* in the seed, and then to clarify its route of transmission to plumule and radicle of germinating rice seed.

MATERIALS and METHODS

Infected seed sample used: The seeds of rice cultivar Palkoeng which were severely infected with *H. oryzae* at a farmer's field in Jinan were collected in the autumn of 1977 and used in the present investigation.

Detection of seed-borne fungi: More than two hundred unhulled or dehulled seeds were examined by water agar plate method(5, 11). Twenty five seeds were placed per petri dish of 9cm diameter containing 1.2% water agar. The seeds were treated with 1% NaOCl for five minutes before plating for surface disinfection. The plated seeds were placed under 12 hr alternative light and darkness in an incubator of about 20°C for seven days. The source of light was a standard cool white fluorescent lamp of 1,200 lux at approximately 35cm distance. The examination of sporulation on seeds was executed under a stereoscopic microscope with a substage illumination.

For detection of the location of *H. oryzae* infection in the seed components, the dehulled seeds that had been soaked in water of ambient temperature for 12 to 24 hr were separated into pericarp, embryo, and endosperm with an ethanol flamed dissecting knife under a dissecting microscope or a magnifier. Each of the separated components was dipped a few second into one percent chlorine before plating and incubation.

The embryo extraction technique by Shetty et. al.(14) was adopted for the examination of infection in the seeds. Three hundred seeds were soaked in five percent sodium hydroxide solution for about 20 hr at about 25°C. Embryos were collected in a vial and rinsed a few times in water, dehydrated in methyl alcohol for two minutes, and then stained with trypan bule in sodium hydroxide for five minutes. After rinsing the stained embryos in water again, they were boiled in lactophenol for a few minutes until they became relatively clear. The embryos in lactophenol then were poured in a petri dish to examine under a stereoscopic microscope.

Tansmission of *Helminthosporium oryzae* to germinating seed: After confirmation of each seed infected with *H. oryzae* by its sporulation on 1.2% water agar plate in a few days of incubation, the seedling tests were executed by the test tube agar method of Neergaard(11). Each seed was transferred into each test tube containing the water agar and kept until the symptoms appeared on coleoptile or radicle of germinating seed, or on primary leaf developing subsequently. The percentages of infected plumules or radicles were recorded by categories of the symptom. The infected parts of the young seedlings showing necrotic lesion or spot were cut into a cross section and adjacent next parts above and below apparently healthy were cut in succession into about two milimeter width. They were separately plated and incubated for confirmation of the causal fungus.

The emerging plumule shoot or radicle showing the symptom was cut into 16 to 32 μm thickness with a cryostat microtome. Distilled water or polyethylene glycole was used as supporting material. Except the replacement of cotton blue with

trypan blue, the staining procedure was followed by the method of Shipton and Brown(15). The ungerminated seeds during the incubation period were also cut with the microtome and prepared in the similar way for examination under a compound microscope. In order to remove starch grains in endosperm, the sections were treated with five percent potassium hydroxide solution for about an hour at room temperature.

RESULTS

Detection of *Helminthosporium oryzae* and other fungi: *H.oryzae* was observed in 92% hulls and 83% pericarps of the infected rice seeds of cultivar Palkoneg in six days of incubation. The brown spot fungus began to sporurate on unhulled seeds in one day whereas the sporulation on dehulled seeds began one day later(Table 1). Regardless of

Table 1. Percentage infection a of *Helminthosporium oryzae* on rice seeds of heavily infected cultivar Palkoeng on 1.2% water agar at about 20°C under alternative light and darkness.

Incubation period (day)	Unhulled seeds b		Dehulled seeds b	
	Non-treated (%)	Pretreated with NaCl (%)	Non-treated (%)	pretreated with NaOCl(%)
1	18v	13v	0v	0w
2	39w	36w	22w	3w
3	62x	64x	37x	24x
4	71y	75y	56y	49y
5	82z	86z	72z	74z
6	85z	90z	76z	79z
7	90z	92z	81z	83z

a. One hundred seeds per treatment were examined in three replications.

b. Numbers followed by same letters within each column are not significantly different at 5% level.

seeds unhulled or dehulled and chlorine pretreated or not, the daily increase in sporulation was prominent until five days. However, from five to seven days it was not significantly different. The treatment with one percent sodium hypochlorite the for five minutes before plating showed a low percentage sporulation for the initial two days in comparison with the nontreated seeds. With the dehulled seeds, the low percentage sporulation due to the chlorine pretreatment continued until four days. From the fifth day of incubation the percentage sporulation of pretreated seeds became higher than that of nontreated seeds.

The rice seeds infected heavily with *H. oryzae* carried various other fungi in the low frequency (Table 2). *Pyricularia oryzae, Fusarium roseum,*

Table 2. Frequency of seed-borne fungi a/ detected on unhulled and dehulled seeds of rice cultivar Palkoeng infected severely with *Helminthosporium oryzae.*

Fungi detected	Unhulled seeds	Dehulled seeds
Helminthosporium oryzae	273	250
Pyricularia oryzae	6	2
Fusarium roseum	7	5
Fusarium moniliforme	4	3
Trichoconis padwickii	3	2
Alternaria tenuis	8	6
Cladosporium cladosporoides	7	2
Curvularia lunata	6	2
Curvularia intermedia	4	1
Epicoccum purpurascens	5	0
Nigrospora oryzae	3	0
Unidentified spp.	6	2

a/Three hundred seeds were examined for dehluled or unhulled on 1.2% water agar in five to seven days of incubation at about 20°C under 12 hr alternative light and darkness.

F. moniliforme, Trichoconis padwickii, Alternaria tenuis, Cladosporium cladosporoides, and *Curvularia* spp. on pericarps were in decreased frequencies from those detected on hulls. *Epicocum purpurascens* and *Nigrospora oryzae* were not detected on dehulled seeds although they were detected on unhulled seeds.

Location of *Helminthosporium oryzae:* *H. oryzae* was observed in every part of the seed

including palea, lemma, rachilla, empty glumes and the awn end of hull as well as pericarp all around the seed. However, the most heavily infected part was the joint part of the two empty glumes between rachilla and pedicel with unhulled seed. A few to several cell layers of endosperm particularly in the dorsal part initiated from the point of hilum were discolored into dark brown in the heavily infected seeds. The mycelium of the brown spot fungus in such discolored part of endosperm was possible to observe after removing starch grains.

Testing of the seed components disclosed that no embryo was infected with *H. oryzae* whereas seven percent of endosperms were infected with this fungus(Table3). The location of the fungus into endosperm was shallow within about ten cell layers deep even with very heavily infected seeds when examined histologically. *Fusarium moniliforme* was detected in every component of its infected seeds including embryo. *F. roseum* and *Alternaria tenuis* were detected in pericarp but not in embryo and endosperm. Within the low percentages, *Pyricularia oryzae*, *Trichoconis padwickii*, *Cladosporium cladosporoides*, and *Curvularia* spp. were detected by their respective sporulations. Tests of embryo extraction, component plating, and histopathology disclosed that embryo of rice seeds was not infected with *H. oryzae*.

Table 3. Percentage recovery of fungi [a/] from different parts of rice seeds of cultivar Palkeong infected severely with *Helmiothosporium oryzae*.

Fungi dected	Pericarp (%)	Embryo (%)	Endosperm (%)
Helminthosporium oryzae	82	0	7
Pyricularia oryzae	1	0	0
Fusarium roseum	2	0	0
Fusarium moniliforme	1	1	1
Trichoconis padwickii	1	0	0
Alternaria tenuis	2	0	0
Cladosporium cladosporoides	1	0	0
Curvularia lunata	1	0	0
Curvularia intermedia	1	0	0
Unidentified spp.	2	1	1

a One hundred seed were dissected and plated on 1.2% water agar and incubated for six days at about 20°C under alternative light and darkness.

Transmission of *H. oryzae* to germinating seed: Symptom on young seedling: Though lower percentage of dehulled seeds was nongerminated, moreplumule shoots died soon after germination in comparison with unhulled seeds. The plumules died after germination from the dehulled seeds were three times higher those from the unhulled seeds (Table 4). With unhulled seeds, development of brown spots on coleoptile and primary leaf was higher than that of brown discoloration at the basal part of radicle. This trend, however, was reve-

Table 4. Results of rice seedling test of cultivar Palkoeng infected severely with *Helminthosporium oryzae* by test tube agar method[a].

Category	Unhulled seeds % observation	Unhulled seeds amount[b] sporulation	Dehulled seeds % observation	Dehulled seeds amount[b] sporulation
Nongermination	26	##	15	##
plumule shoct died soon after germination	10	+	32	+
Brown spot on coleoptile and primary leaf	8	—	11	—
Brown discoloration at basal part of radicle	7	—	12	—
Lesion on both shoot and radicle	9	—	17	—
No apparent symptom	40	—	13	—

a After confirmation of each seed infected with *H. oryzae* by its sporulation on 1.2% water agar plate in petri dish in a few days of incubation, each of 100 seeds in each test tube containing the water agar capped loosely with aluminium foil was kept at about 20°C under 12 hr alternative light and darkness for seven days.

b + Less than 500 conidia per kernel.
Between 500 and 1,500 conidia per kernel.
Above 1,500 conidia per kernel.

rsed with dehulled seeds so that the development of brown spots on coleoptile became lower than that of brown discoloration at the basal part of radicle.

Nongerminated unhulled seeds had the most abu ndant sporulation while nongerminated dehulled seeds showed the moderate sporulation. The approximate number of conidia per kernel was above 1,500 for the unhulled seeds and falled in between 500 and 1,500 for the dehulled seeds. The shoots-died soon after germination carried less than 500 conidia per plumule. The other type of symptoms such as brown spot on coleoptile or brown discoloration at the basal part of radicle did not show sporulation during the seven day period of the seed germination and seedling growth. When the seedlings with these symptoms were kept for longer period than one week, all of them resulted in seedling blight with the sporulation of *H. oryzae* within two weeks.

Transmission to plumule: The mycelium of *H. oryzae* in hilum and adjacent dorsal portion of rice seed developed towards ventral side to reach the pericarp covering embryo in a few days of incubation before emergence of plumule. When the developing plumule within embryo was about to emerge out of pericarp the scutellum and epiblast surrounding the growing plumule were heaving up to split the pericarp layers that had been invaded by the mycelium. The tip of upheaving scutellum or epiblast was naturally in contact with the mycelium in the split part of pericarp. The tip was subsequently infected with the mycelium from the split pericarp. The severity of symptom on the infected part of scutellum or epiblast depended upon degree of the mycelial growth and transfer from the pericarp (Fig. 1).

In light infection the hair of scutellum and epiblast shielded off the invading mycelium so that coleoptile emerging through the cleft between scutellum and epiblast was not directly exposed to the mycelium from pericarp. Brown discoloration on apical part of scutellum and epiblast was observed after most of the hairs were covered with the mycelium. The lighter the mycelial pro-

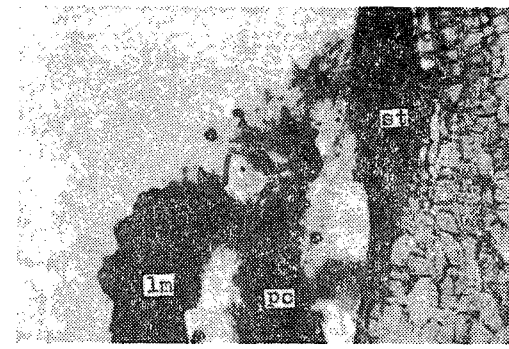

Fig. 1. Necrotic lesions at the edges of lemma(lm), pericarp(pc), and scutellum (sts) infected with *Helminthosporium oryzae* in five days at about 20°C under 12hr alternative light and darkness. X400.

liferation in the split part of pericarp the longer time required for *H. oryzae* to cause the discoloration. The similar relationship between pericarp and scutellum or epiblast existed also between scutellumor epiblast and coleoptile. The mycelial transmission onto the primary leaf from the brown spot on coleoptile was not accomplished within the seven day period of growth. In heavy infection the scutellum and epiblast did not show hair extrusion but showed the brown discoloration on the epical part that was projected out of split pericarp layers. In such case the emerging coleptile became in direct contact with the invading mycelium from scutellum or epiblast. The growth of coleoptile was retarded in comparison with light infection during the early period. The brown spot appeared in a few days after emergence wer e extended to merge one with another until whole coleoptile became dark brown during the subsequent period of incubation. Some primary leaves extruded out of the diseased coleoptile while others did not extrude depending upon the severity of infection.

Transmission to radicle: In the early period fo incubation when the developing radicle within embryo was about to emerge out of pericarp, coleorhiza was heaving up to split pericarp which had been infected with the mycelium developed from hilum. The mycelium in the split part of pericarp infected the tip of upheaving coleorhiza.

Along with further growth and emergence of radicle from inside, coleorhiza was also split resulting in its split tip projected out of the pericarp. The mycelium in the projected tip of the split coleorhiza infected the radicle. The symptom appeared as brown discoloration at the basal part of radicle. In heavy infection the growth of radicle was ceased due to the invading mycelium in a few days after its extrusion. The mycelium losing the area for further growth towards the tip of radicle began to develop towards the basal part(Fig. 2).

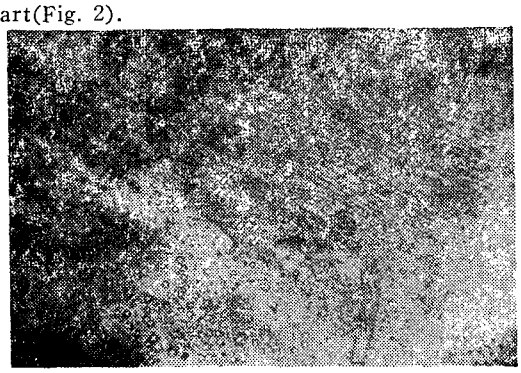

Fig. 2. Necrotic lesions at the basal part of radicle infected with *Helminthosporium oryzae* in five days of the seedling test. X150.

In light infection, however, radicle continued to grow even with the brown discoloration at the basal part of raidcle. Component plating and histological investigation revealed that the mycelium of *H. oryzae* developed further beyond the discolored portion into apparently healthy part of rad icle.

DISCUSSION

The location of *Helminthosporium oryzae* in rice seed and its transmission route to plumule and radicle of the germnating rice were clarified in the present investigation. Since the mycelium of the brown spot fungus could infect several to a little more than ten cell layers deep into endosperm beyond hilum and adjacent dorsal pericarp of rice seed, appropriate measure of control should be applied before sowing such seeds as might include the infected seeds.

Differently from *Pyricularia oryzae* of rice blast of which the location of infection was greatly influenced by the seed morphology(8), *H. oryzae* was little affected by the seed morphology showing its sporulation all around the seed pericarp including the ventral portion of embryo. These results might be ascribed to the fast growing thick mycelium with large conidia of *H. oryzae* as compared with *P. oryzae*.

The pericarp infection with *H. oryzae* was as high as 83% while that with *P. oryzae* was only 25% with the rice seeds of their respective heavy infections on hull layers. In spite of the wide difference between the two fungi, the depth of mycelial infection into endosperm was not so markedly different. Though Fazli and Schroeder(2) ob served the mycelium of *H. oryzae* in endosperm of the rice seeds, they did not report the depth of infection. The mycelium in endosperm did not reach plumule or radicle within a week period of incubation. Therefore, it must be unable to cause the development of symptom on plumule or radicle of germinating seed during the initial stage of rice seedling development. However, it may become the important source of inoculum to incurr the brown spot epidemics during the subsequent period of growth.

The transmission of pericarp-borne *H. oryzae* to germinating seeds was so rapid and severe that coleoptiles and radicles with the brown spot symptom accounted to the high percentage in one week of the infected seed plating. Since most of the pericarp infection was accompanied by the hull infection, the mycelial transmission of this fungus from pericarp to emerging plumule and radicle may usually be augmented with that from lemma. The abundant sporulation on the high percentage nongerminated seeds plus plumule shoots are considered to be the primary source of inoculum for the secondary infection (3, 12, 13).

Although the myeclium of *H. oryzae* was not observed in the embryo of the infected seeds, the fungal invasion proceeded more easily or faster into embryo rather than into endosperm. This is probably related to the route of water supply into the rice seeds associated with the split of pericarp

covering embryo during the initial period of the seed germination. Accordingly, further investigation on the vascular system of rice seeds may possibly lead to full clarification of the infection process of this fungus in germinating rice.

In spite of the fact that *F. roseum* had the ability of higher percentage infection in pericarp than in hull(8), such a phenomenon was not observed in the present investigation. This might have been related with the time of infection, for the percentage infection of some other fungi varied according to the artificial inoculations before, during, and after the flowering period(2, 17). Further investigation is required with such seeds as harvested from the rice infected at different stages of growth. Investigation on the competitive ability of *F. roseum* with *H. oryzae* and *P. oryzae* may provide another way of solution to the question.

H. oryzae was not detected in embryo of rice seeds infected heavily with this fungus in the present study. The mycelium in hilum of infected seed transmitted through pericarp to plumule and radicle of germinating rice. The percentage seedlings with typical symptom of *H. oryzae* were very high in comparison with the case of the blast fungus. Symptomless carrier seedlings in addition to thos ewith the mycelium in endosperm may have a significant role for the epidemics of the disease during the later period of rice growth in the field.

摘　　要

깨씨무늬病에 甚하게 罹病된 벼八紘 供試種子는 培養 5~7日에 護穎, 內外穎 및 珠柄의 86~92% 그리고 果皮의 74~83%가 *Helminthosporium oryzae*에 感染되어 있음을 보였다. 同菌은 種粒全面에 分生胞子를 形成하였으며 胚乳의 約10餘細胞層까지 感染되어 있었으나 胚組織에서는 檢出되지 않았다.

*Fusarium moniliforme*는 果皮, 胚乳 및 胚組織에서 檢出되었으며 *F. roseum*, *Trichoconis padwickü*, *Curvularia* spp., *Alternaria tenuis*, *Cladosporium cladosporoides* 및 *Phoma* sp.는 3% 以下의 穎 및 果皮에서 檢出되었다.

培養一週日에 60~87%가 깨씨무늬 病徵을 나타냈으며 13~40%는 外觀上 健全하였다. 깨씨무늬病菌은 感染된 種子의 臍點으로부터 果皮를 거쳐 幼芽및 幼根에 傳染 發病케 하였다.

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