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Mycelial Melanization of *Rhizoctonia solani* AG1 Affecting Pathogenicity in Rice

Heung Tae Kim*, Young Ryun Chung[1] and Kwang Yun Cho

Screening Division, Korea Research Institute of Chemical Technology, Daejon 305-606, Korea
[1]Department of Microbiology, Gyeongsang National University, Chinju 660-701, Korea

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The phenotype of *Rhizoctonia solani* KR-13 was randomly segregated to both melanin-producing (M+) and non-producing (M–) types through successive cultures on PDA. M+ type with dark melanin showed strong pathogenicity to rice and self-anastomosis. Meanwhile, M– type with white or less-melanized mycelia showed very weak pathogenicity and non-self-anastomosis. Melanin production of *R. solani* was affected by incubation temperature in both M+ and M– types, but not by light treatment. The application of tricyclazole, an inhibitor of fungal melanin biosynthesis, showed no controlling effect on *R. solani* causing rice sheath blight. Results of this study showed that melanization of mycelia of *R. solani* is an important pathogenicity factor in rice.

Keywords : melanin, pathogenicity, *Rhizoctonia solani*.

Rhizoctonia solani Kühn [teleomorph: *Thanatephorus cucumeris* (Frank) Donk] is a destructive and widespread fungal plant pathogen with wide host range including rice, turfgrass, vegetables, and many other field crops (Cu et al., 1996; Burpee and Martin, 1992; Doupnik, 1993; Keinath, 1995). Rice sheath blight caused by *R. solani* anastomosis group (AG) 1-IA has become increasingly important in most rice production regions because of the use of high yielding cultivars with large number of tillers and more frequent application of nitrogen fertilizer (Cu et al., 1996; Savary et al., 1995).

Most well known pathogenicity factors of *R. solani* include cellulolytic enzymes and infection structures. *R. solani* secretes plant cell wall lytic enzymes such as cellulase and pectinase to infect host cells (Baker and Walker, 1962). The formation of infection cushion and lobate appressorium are also known to lead to host infection (Matsuura, 1986; Murray, 1982). There have been few reports about the relationship between mycelial melanization and pathogenicity in *R. solani*. Hyakumachi et al. (1987) showed that, in some AGs of *R. solani*, melanin accumulated in hyphae by incubating fungal culture for 6 weeks. Particularly in AG 2-2 of *R. solani*, melanin biosynthesis was correlated with the anastomosing ability of hyphae and the ability to grow in soil (Hyakumachi and Ui, 1987). The loss of ability to grow in soil, which resulted from non-production of melanin, was correlated with the decrease of pathogenicity to seedlings and mature roots of sugarbeet. While mycelial melanization was related with the ability to grow in soil, it was not clear if mycelial melanization is essential to pathogenicity. Therefore, there is a need to determine whether mycelial melanization directly influences the pathogenicity of *R. solani*. However, in some plant pathogenic fungi such as *Magnaporthe grisea* and *Colletotrichum lagenarium*, melanization of appressorium was reported as an important factor to pathogenicity (Chida and Sisler, 1987; Kubo et al., 1984; Suzuki et al., 1982; Woloshuk et al., 1982; Yamaguchi et al., 1983). Studies with melanin-deficient mutants of *M. grisea* or *C. lagenarium* showed that those mutants were not able to cause typical disease by failure in penetration to host epidermis.

During the rice sheath blight inoculation experiments, pathogenicity variations were observed when mycelial agar disc was used for inoculum of *R. solani* AG 1-IA isolate KR-13. In this study, the relationship between mycelial melanization of *R. solani* KR-13 and its pathogenicity was presented through the evaluation of disease severity of rice sheath blight pathogens with different degree of melanization. Effects of temperature and light on melanin formation, and the inhibitory effect of tricyclazole, a fungicide known as inhibiting biosynthesis of melanin in some plant pathogens such as *M. grisea* and *C. lagenarium*, on the disease development of *R. solani* were also examined.

Materials and Methods

Fungal isolate. Isolates of *R. solani* were recovered from diseased sheaths of rice (cv. Nakdong) collected from Yusung area in Korea. Diseased rice sheaths were surface-sterilized using 1% sodium hypochlorite in distilled water and placed on potato dextrose agar (PDA, Difco) at 25 ± 1°C. Several isolates of *R. solani* were obtained by single hyphal tip isolation. The pathogenicity of

*Corresponding author.
Phone) +82-42-860-7432, FAX) +82-42-861-4913
E-mail) htkim@pado.krict.re.kr

these isolates was confirmed on 3- week-old rice seedlings in the greenhouse. Most isolates belonged to AG 1-IA, while one isolate, KR-13, was selected for further experiment since this specific isolate showed the strongest pathogenicity. During the 15-day incubation of this isolate on PDA at 25°C, KR-13 was segregated into two types based on its mycelial color: one type (M+, color scale=3) produced dark brown mycelia, and the other type (M–, color scale=0) produced white mycelia on PDA.

Culture conditions for mycelial growth and melanin production. Mycelial growth rate of two types of *R. solani* KR-13 was investigated on PDA. Mycelial agar discs (diameter: 5 mm) from 15-day-old cultures of M+ and M– types were inoculated on PDA and incubated at 20, 25, and 30°C until most actively growing mycelia reached the edge of the petri plate. Mycelial growth of subcultures was recorded as diameter of fungal colony. To investigate the effect of light on melanin production in the mycelia of *R. solani* KR-13, mycelial agar discs were also incubated on PDA for 15 days at 20, 25 and 30°C either under alternating 14-h light or under darkness. Melanin production in the fungal mycelia became evident after at least 15 days incubation at 25°C. Melanin accumulation in mycelia was determined based on rating scales of mycelial color as follows: 0=white; 1=brownish white; 2=light brown; 3=dark brown.

Extraction and characterization of melanin. Isolation of melanin from the mycelial mats grown on PDA was carried out by using the method of Ellis and Griffith (1974). Extraction and analysis of melanin were performed under reflux in an atmosphere of nitrogen. Identity of melanin from the extraction was confirmed as follows: 1) solubility and appearance in distilled water; 2) color; 3) solubility in 1 M of KOH; 4) precipitation in HCl; 5) solubility in organic solvents; 6) reaction to oxidizing agent (NaOCl); and 7) gradient of log absorbance in visible light (400-600 nm).

Pathogenicity test. Rice plant seedlings (cv. Nakdong) grown for 3 weeks in paddy soils contained in pots (5 cm in diameter, 7 cm in height) were used to test pathogenicity of *R. solani* isolates. Each pot contained three seedlings, and pots were placed on sub-irrigated benches in the greenhouse. Mycelial agar discs (5 mm in diameter) of *R. solani* grown on PDA at 25°C for 15 days were inserted into the sheath of fourth-leaf rice seedlings 3 cm above soil surface. The inoculated plants were immediately placed in a dew chamber at 28 ± 1°C and 100% relative humidity. After 10 days inoculation, the length of lesion from the inoculation site was measured. Variants of *R. solani* KR-13 in melanin production were also subjected to the pathogenicity test by the same method.

Effect of tricyclazole. Tricyclazole, an inhibitor of melanin biosynthesis, was used to test its effect on the development of rice sheath blight caused by *R. solani*. The controlling effect of tricyclazole was also compared with *M. grisea* and *C. lagenarium* causing rice blast and anthracnose of cucumber, respectively. Ten (10) ml of tricyclazole solution at the rate of 0.4, 2, 10, 50, 100 μg/ml in acetone with 500 μg/ml Tween 20 was sprayed on rice seedlings at the fourth-leaf stage grown in a pot (5 cm in diameter, 7 cm in height) 24 h before inoculation of *R. solani* and *M. grisea*. One-leaf stage cucumber plants were treated with tricyclazole as described above before *C. lagenarium* inoculation. After application of tricyclazole, conidial suspensions of *M. grisea* and *C. lagenarium* (10^6 conidia/ml) were sprayed onto the plants just before run-off, and inoculated plants were incubated in a dew chamber at 25°C for 24 h. The plants were then removed from the dew chamber, and were placed in a growth chamber (>85% relative humidity, 25°C) for one week for disease development. The disease development of rice sheath blight was evaluated as described above. The severity of rice blast and cucumber anthracnose disease was measured based on diseased leaf areas. The control values indicating the controlling activities of tricyclazole were calculated as follows:

$$\text{Control value (\%)} = \left(1 - \frac{\text{Disease severity of treated plant}}{\text{Disease severity of untreated plant}}\right) \times 100$$

Results

Cultural characteristics of M+ and M– types. After incubation for 15 days at 25°C, cultures of *R. solani* KR-13 were randomly classified into M+ and M– types (Fig. 1A). In the comparison between M+ and M– types, several different cultural characteristics were observed (Table 1). M+ type of *R. solani* KR-13 produced melanin in mycelia, which was attributed to self-anastomosis fusion, and showed strong pathogenicity to rice plant. However, whitened mycelia of M– type produced much more sclerotia than M+ type, and were not able to conduct self-anastomosis. The pathogenicity of M– type was very weak in the greenhouse test compared with the M+ type (Fig. 1B).

Effects of temperature and light on mycelial growth and melanization. The M+ type (color scale=3) grew much faster than M- type (color scale=0) at all tested temperatures (Fig. 2). Incubation temperatures affected the melanization in both M+ and M– types of *R. solani* KR-13, while light treatment had no effect on melanization (Table 2). Neither M+ nor M– type produced melanin at 20°C regardless of light/dark treatment. However, melanin production of M+ type was the most at 25°C after 15 days with an average color scale of 2.0-2.2. M– type also produced melanin at 25 and 30°C, but the amount of melanin was significantly less than that of M+ type at 25°C.

Characteristics of extracted melanin. The identity of melanin extracted from KR-13 strain was compared with melanin from other isolates of fungal pathogen (Table 3). The extracted pigments of KR-13 were insoluble in distilled water and formed dark brown flocculent precipitates. These were also insoluble in both HCl and organic solvents. However, pigments were completely soluble when placed in 1 M of KOH at 100°C for 2 h. In the reaction test to oxidizing agent (NaOCl), these pigments were decolorized. The absorption spectra from 400 nm to 600 nm of alkaline solution of melanin pigments did not show any absorption

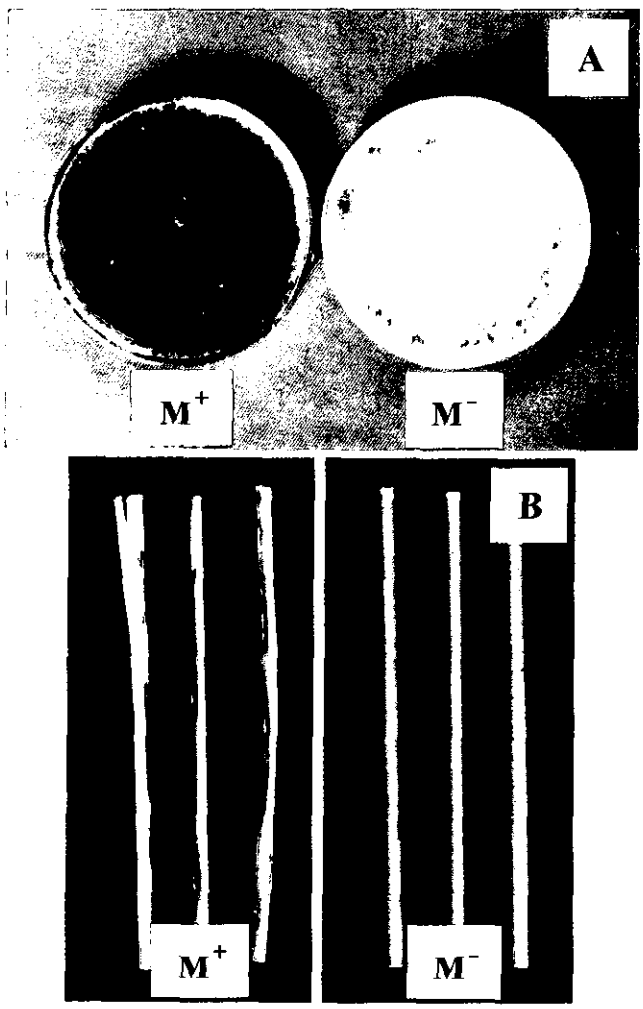

Fig. 1. Morphological culture type (**A**) and pathogenicity (**B**) of *Rhizoctonia solani* KR-13 incubated for 15 days.

peak, and the plot of log absorbance versus wavelength was linear with negative slope.

Pathogenicity and effect of tricyclazole. The pathogenicity of *R. solani* KR-13 on rice sheath varied depending upon the level of melanization in the mycelium (Fig. 3). Lesion length on rice sheath by *R. solani* KR-13 variants was correlated with the level of melanization of each variant. *R. solani* KR-13 variant with white or brownish white color produced lesions smaller than 6 cm in length, whereas, the other variant with dark brown color produced big necrotic lesions 8-10 cm in length. High positive correlation (r^2=0.9769, $p \leq 0.01$) between levels of melanization and lesion length was observed.

Tricyclazole treatment had little effect on rice sheath blight development even at 100 μg/ml (Fig. 4). The same fungicide, however, inhibited development of rice blast and cucumber anthracnose almost completely at the rate of 10 μg/ml.

Discussion

Rhizoctonia solani isolate KR-13 showed unusual variation of melanization in mycelia and pathogenicity among subcultures. Since the variation of melanin production in *R. solani* subculture is unusual and the relationship between melanin production and pathogenicity is not well understood, this study conducted experiments on the role of melanin during plant infection by *R. solani* KR-13.

Dark brown mycelia of *R. solani* KR-13 produced large brown necrotic lesions on rice sheath, while light brown or white mycelia produced very small lesions. This result suggests that melanin of mycelia might be associated with pathogenicity. Dark brown pigments extracted from the mycelia of *R. solani* KR-13 in this experiment were found to have typical melanin characteristics compared with those of *Rhizoctonia solani* AG 2-2 and *Verticillium dahliae* (Ellis and Griffiths, 1974; Hyakumachi et al., 1987). The production of melanin was shown to be affected by many factors such as temperature, light, and nutrients in *Aureobasidium pullulans* (Lingappa et al., 1963). In *R. solani* KR-13, temperature appeared to be an important factor in melanin biosynthesis. As shown in Table 1, melanin was formed most at 25°C, and less at 20 and 30°C, regardless of its type.

Previous studies have shown that melanin pigments play an important role in the pathogenicity and the survival

Table 1. Characteristics of melanin-producing (M+) and non-producing (M–) types of *Rhizoctonia solani* KR-13

Type	Mycelial color[a]	Anastomosis[b]	Pathogenicity[c] (cm)	Sclerotium
M+	Dark brown	Self-anastomosis	9.3 ± 0.8	Few
M–	White	Non-self-anastomosis	4.5 ± 0.7	Abundant

[a] Mycelial color of *R. solani* KR-13 was observed 15 days after incubation on potato-dextrose agar at 25°C.

[b] Mycelial discs of *R. solani* KR-13 were paired about 3 cm apart on a water agar plate. After the mycelia have grown and overlapped, mycelial fusion was observed under light microscope.

[c] Rice plants (cv. Nakdong) grown in paddy soils were used for inoculation of the pathogen. For inoculation, mycelial discs of different types of *R. solani* KR-13 were cut from the 15 day mycelial colonies, and inserted into sheaths of rice plants. Ten days after inoculation, the evaluation of disease severity was conducted by measuring the height of lesion from the inoculation site.

ability of some fungi in certain environments (Kubo et al., 1984; Kuo and Alexander, 1967; Old and Robertson, 1970; Woloshuk et al., 1981). In *M. grisea* and *C. lagenarium*, melanin is indispensable to pathogenicity. Woloshuk et al. (1980) reported that melanin-deficient mutants of *M. grisea* were non-pathogenic or rarely infected rice plants. In the pathogenicity test of *R. solani* AG 2-2 to sugarbeet, self-anastomosing isolates producing the melanin appeared to have stronger pathogenicity to both seedlings and mature roots of sugarbeet than non-self-anastomosing isolates that did not produce melanin in mycelia and sclerotia (Hyakumachi and Ui, 1987). Since melanin non-producing strain of *R. solani* AG 2-2 loses ability to grow in soil, Hyakumachi and Ui (1987) speculated that melanin might be correlated with fungal survival, and that lack of melanin results in loss of pathogenicity. In *R. solani*, however, there have been no reports about the effect of melanin on its pathogenicity except for the relationship between melanin and survival in soil.

Application of tricyclazole, a commercial melanin biosynthesis inhibitor, did not control rice sheath blight caused by *R. solani* KR-13, suggesting that the pathway of melanin production in *R. solani* may be different from that in *M. grisea* (Okuno et al., 1983; Suzuki et al., 1982). Hyakumachi et al. (1987) reported that Na_2-EDTA, tricyclazole, and chlobenthiazone did not affect melanin biosynthesis in mycelia of *R. solani* AG2-2. In this study, change in hyphal and agar media color from dark brown to red, which indicates the accumulation of shunt products

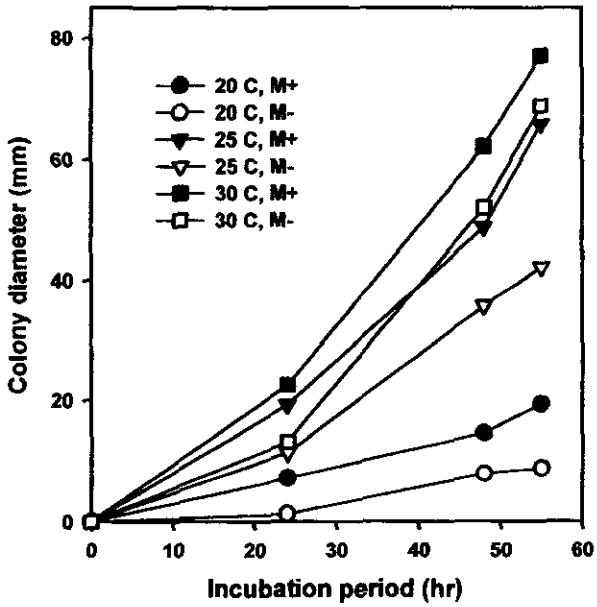

Fig. 2. Mycelial growth of melanin-producing (M+) and non-producing (M–) *Rhizoctonia solani* KR-13 at various temperature on PDA.

Table 2. Effects of temperature and light on biosynthesis of melanin in *Rhizoctonia solani*

Type of mycelia[a]	Temperature (°C)	Light[b]	Average of color scales[c]
M+	20	L/D	0
		D	0
	25	L/D	2.2 ± 0.2[d]
		D	2.0 ± 0.4
	30	L/D	0.8 ± 0.4
		D	1.0 ± 0.1
M–	20	L/D	0
		D	0
	25	L/D	1.2 ± 0.1
		D	1.4 ± 0.4
	30	L/D	0.6 ± 0.2
		D	1.0 ± 0.4

[a] Type of mycelia has been classified as melanized (M+) or not melanized (M–).
[b] Photoperiod was 14 h.
[c] Color of mycelia was determined based on four rating scales (0= white; 1=brownish white; 2=light brown; 3=dark brown) 15 days after incubation at each indicated temperature.
[d] Each value is the mean standard deviation of three replicates. One replicate was performed in 10 petri dishes.

Table 3. Diagnostic characteristics of melanin produced by *Rhizoctonia solani* KR-13

Characteristics	*Rhizoctonia solani* KR-13	*Rhizoctonia solani* A-14[a]	*Verticillium dahlia* 84[b]
Solubility in H_2O	Insoluble	Insoluble	Insoluble
Color	Brown black	Brown black	Brown black
Solubility in 1 M-KOH (100°C, 2 hrs)	Soluble	Soluble	Soluble
Pigment precipitation in HCl	Fast	Fast	Slow, 1 h
Solubility in solvents (acetone, methanol, ethanol)	Insoluble	Insoluble	Insoluble
Reaction to oxidizing agent (NaOCl)	Decolorization	Decolorization	Decolorization
Gradient of log absorbance vs. wavelength (400-600 nm) plots	-0.0041	-0.0025	-0.0015

[a] Hyakumachi et al., 1987.
[b] Ellis and Griffiths, 1974.

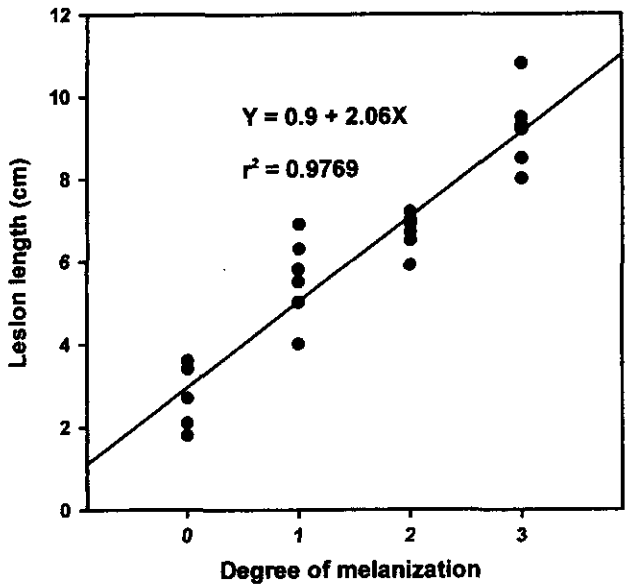

Fig. 3. Relationship between melanin content in the mycelium of *Rhizoctonia solani* KR-13 and its pathogenicity on rice plants. Melanin content was evaluated using four color scales: 0=white; 1 =brownish white; 2=light brown; and 3=dark brown.

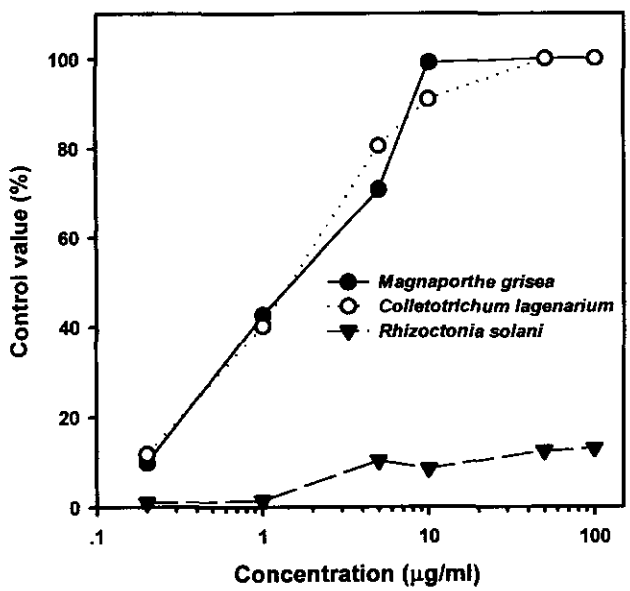

Fig. 4. Inhibitory effect of tricyclazole against *Rhizoctonia solani*, *Magnaporthe grisea*, and *Colletotrichum lagenarium* causing rice sheath blight, rice blast, and cucumber anthracnose, respectively.

such as 2-hydroxyjugulone and flaviolin, was not confirmed by the amendment of tricyclazole in PDA inoculated with *R. solani* (data not shown). Wheeler (1983) also reported that *R. solani* could not metabolize intermediate of melanin biosynthesis like 1,3,6,8-tetrahydroxynaphthalene, scytalone, and 1,3,8-trihydroxynaphthalene to melanin in *in vitro* assay with homogenate prepared from sclerotia. These results suggest that the melanin of *R. solani* may be different from dihydroxynaphthalene (DHN) melanin, which has been reported from other plant fungal pathogens. For that reason, it is likely that tricyclazole, an inhibitor of DHN-melanin biosynthesis, could not effectively control rice sheath blight in the greenhouse test.

Results of this study suggests that melanization of the mycelia of *R. solani* AG 1-IA might be related with the variation of pathogenicity to rice sheath and with self-anastomosis. The capability of melanin production in M+ and M– type isolates of *R. solani* KR-13 was, however, not genetically stable maybe due to the multinucleate characteristics. Even after the mycelial discs of M+ type have been transferred to the new PDA, both types of *R. solani* continued to grow. To determine the mechanism of pathogenicity variation in association with melanization, genetically stable melanin-deficient mutants must be selected.

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