THE KOREAN JOURNAL OF MYCOLOGY
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Vol. 26, No 3, p387-395 September 1998
Printed in S. KOREA

Relationship between Virulence and Cultural Characteristics in *Calonectria ilicicola*

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*Calonectria ilicicola*의 병원성과 배양적 특성간의 상호관계

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ABSTRACT: Mycelial growth, production of microsclerotia and perithecia, and presence of double-stranded RNA were examined in *Calonectria ilicicola* isolates from several hosts to detect morphological and/or genetic markers for comparison with levels of virulence. Variability in disease severity, production of microsclerotia and perithecia, and mycelial growth was observed across all isolates. None of 35 isolates of *C. ilicicola* examined contained detectable levels of double-stranded RNA. Disease severity on soybean cultivars correlated positively with production of both microsclerotia and perithecia. Mycelial growth correlated negatively with production of perithecia. Virulence on the cultivars and production of microsclerotia and perithecia were greater in isolates of *C. ilicicola* from soybean than those from peanut. These results suggest that the ability of isolates to produce microsclerotia and perithecia is a component of inoculum potential. Perithecia production may serve as a useful marker for characterizing virulence or host specialization in this homothallic fungus.

KEYWORDS: ***Calonectria crotalariae*, Cultural characteristics, *Cylindrocladium parasiticum*, Inoculum potential, Red crown rot**

The soilborne fungus *Calonectria ilicicola* Boediun and Reitsma [anamorph: *Cylindro cladium parasiticum* Crous, Wingf. and Alfenas (Crous *et al.*, 1993), syn. *C. crotalariae* (Loos) Bell and Sobers (Bell and Sobers, 1966)] is the causal agent of red crown rot of soybean [*Glycine max* (L.) Merr.] (Berggren *et al.*, 1985; Berner *et al.*, 1986). This disease was first reported from the United States in 1973 (Rowe *et al.*, 1973) and from Louisiana in 1976 (Berggren *et al.*, 1985). Since then, red crown rot has become one of the major yield-reducing diseases in Louisiana (Berggren *et al.*, 1985; Berner *et al.*, 1986).

C. ilicicola produces abundant microsclerotia, which are the primary inocula; these can survive several years in soil or in host debris (Rowe *et al.*, 1974). However, the roles of conidia and ascospores in the disease cycle are not known. Crous *et al.* (1992) reported that sporulation, growth, and vesicle formation in *Cylindrocladium* species decreased in media of low osmotic potential and suggested that this may explain the fact that distribution of these fungi in South Africa was limited to areas with high humidity. Support-

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ing evidence was presented by Nishijima and Aragaki (1973) who recognized that occurrences of papaya collar rot caused by *C. ilicicola* were related to high rainfall.

The existence of races in *C. ilicicola* has been proposed by several authors (Black and Beute, 1984; Hadley *et al.*, 1979; Rowe and Beute, 1975) because of variability in disease severity within a field (Rowe *et al.*, 1973), prevalence of the sexual stage (Bell and Sobers, 1966), a multinucleate hypha (Jones, 1981), and hyphal anastomosis (Black and Beute, 1984). Although no firm evidence for races has been reported, variability in virulence among isolates of *C. ilicicola* was demonstrated on cultivars both peanut (*Arachis hypogaea* L.) (Rowe and Beute, 1975) and soybean (Kim *et al.*, 1992). In addition, isolates from soybean produced more disease on soybean than did isolates from peanut (Kim *et al.*, 1992), which suggests that host specialization may exist in *C. ilicicola* on soybean.

Investigators have studied virulence in fungi using both morphological phenotypes (Pearson *et al.*, 1986; 1987) and molecular markers, such as isozymes, restriction fragment length polymorphisms (RFLP), mitochondrial DNA and plasmids, and double-stranded RNA (dsRNA) (Michelmore and Hulbert, 1987). Such an approach may be useful to understand variability in virulence reported for *C. ilicicola* (Kim *et al.*, 1992). The objective of this study was to compare mycelial growth, production of microsclerotia and perithecia, and dsRNA in isolates of *C. ilicicola* from several hosts to identify morphological and/or genetic markers that correlate with levels of virulence in these isolates.

Materials and Methods

Fungal isolates and virulence

Thirty-five isolates of *C. ilicicola* from various hosts and geographic origins were used in this study (Table 1). Isolates from soybean and peanut were examined for production of microsclerotia and perithecia, mycelial growth rate, and dsRNA content whereas isolates from Hawaii were included only in dsRNA analysis. Previous laboratory inoculation tests (Kim, 1994) determined virulence of 25 isolates of *C. ilicicola* from peanut and soybean on soybean cultivars Asgrow 7986, Braxton, Cajun, Centennial, Forrest, and Hartz 7126. Soybean plants were grown in a greenhouse in 72-cell (5×5×7 cm) plastic trays (67×35×7 cm) filled with a commercial potting mixture (Peat-lite mix®, Conrad Fafard Inc., Springfield, Massachusetts) for 10 days. Seedlings were removed from the plastic trays, washed with tap water to remove soil, then placed on wet paper towels. Seedlings were inoculated in the crown region with mycelium of *C. ilicicola* in PDA discs (5 mm in diameter) cut from margins of actively growing cultures (10 days old). Inoculated seedlings were kept in a dark chamber (90×70×190 cm) at 25°C. Control plants were inoculated with PDA discs (5 mm in diameter) without mycelium and incubated as described. Disease severity was evaluated 9 days after inoculation based on a 0~5 scale as follows: 0=no visible symptoms; 1=reddish necrotic stem lesions <10 mm in length; 2=stem lesions 10~40 mm; 3=stem lesions extending up to cotyledon (40~50 mm), and slight leaf yellowing; 4=stem lesions ≥40 mm with water-soaking of stem above cotyledons, loss of cotyledons, severe leaf yellowing; and 5=seedlings dead. These tests were conducted twice in a completely random design with five and six replicates, and pooled data were used for comparisons with cultural characteristics of the fungus.

Cultural characteristics

Mycelial growth was measured after trans-

Table 1. Geographic origin, host, date collected, and provider/collector of *Calonectria ilicicola* isolates included in this study

Isolate	Geographic origin	Original host	Date collected	Provider/collector
BH1	Louisiana	*Glycine max*	1991	1[a]
BH2	Louisiana	*G. max*	1991	1
BSD	Louisiana	*G. max*	. . .[b]	2
SG911	Louisiana	*G. max*	1991	1
SG912	Louisiana	*G. max*	1991	1
SG913	Louisiana	*G. max*	1991	1
SG914	Louisiana	*G. max*	1991	1
SG915	Louisiana	*G. max*	1991	1
SG916	Louisiana	*G. max*	1991	1
SG917	Louisiana	*G. max*	1991	1
SGD	Louisiana	*G. max*	. . .	2
323	North Carolina	*Arachis hypogaea*	. . .	3
417	North Carolina	*A. hypogaea*	. . .	3
447	North Carolina	*A. hypogaea*	. . .	3
48	North Carolina	*A. hypogaea*	. . .	3
1PN	North Carolina	*A. hypogaea*	1986	3
2PN	North Carolina	*A. hypogaea*	1986	3
3PN	North Carolina	*A. hypogaea*	1986	3
4PN	North Carolina	*A. hypogaea*	1986	3
C31	North Carolina	*A. hypogaea*	1973	3
J1	North Carolina	*A. hypogaea*	1985	3
J2	North Carolina	*A. hypogaea*	1985	3
J3	North Carolina	*A. hypogaea*	1985	3
S38	North Carolina	*A. hypogaea*	. . .	3
S44	North Carolina	*A. hypogaea*	. . .	3
148(24023)[c]	Hawaii	*Acacia koa*	. . .	4
156(24024)	Hawaii	*Carica papaya*	. . .	4
725	Hawaii	*Leea coccinea*	. . .	4
729	Hawaii	*L. coccinea*	. . .	4
846	Hawaii	*Medicago sativa*	. . .	4
872	Hawaii	*L. coccinea*	. . .	4
894	Hawaii	*M. sativa*	. . .	4
895	Hawaii	*M. sativa*	. . .	4
1102	Hawaii	*Anthurium andraeanum*	. . .	4
1809(76649)	Hawaii	*Howeia forsterana*	. . .	4

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[b] Unknown.
[c] Numbers in parenthesis are ATCC numbers.

ferring a plug (5 mm in diameter) of actively growing mycelium in PDA to the center of a Petri dish (90 mm in diameter) containing 20 m*l* of PDA. These were incubated in darkness at 25°C. Colony diameters were measured 5 days after inoculation. This test was conducted twice with five replicates each.

Production of microsclerotia was quantified on similar cultures that were incubated in darkness at 25°C for 4 weeks. Cultures were comminuted in water (200 m*l*) for 2 min using a Waring Commercial Laboratory Blender

Table 2. Disease severity[a], microsclerotia production[b], mycelial growth[c], and perithecia production[d] by isolates of *Calonectria ilicicola*

Isolate	Disease severity	Microsclerotia/cm^2		Mycelial growth(mm)		Perithecia/cm^2	
		Test 1	Test 2	Test 1	Test 2	Test 1	Test 2
SG915	3.5	. . .[e]	. . .	28.2	39.4	297	. . .
SG917	3.4	174	252	49.0	46.4	391	. . .
SG915	3.4	198	223	38.0	39.4	267	88
BH2	3.3	160	213	24.2	22.8	355	79
SG916	3.2	174	193	37.8	36.6	317	113
SG913	3.0	163	255	39.4	30.0	364	48
2PN	2.8	127	216	27.0	25.2	413	141
SG911	2.8	. . .	. . .	35.6	34.4	244	. . .
SG912	2.6	188	243	38.6	42.0	268	. . .
1PN	2.5	62	75	29.0	24.3	309	16
J3	2.0	182	210	44.0	43.4	0	0
48	1.9	235	293	40.4	42.6	0	0
4PN	1.8	142	182	29.4	32.2	249	76
447	1.4	186	230	47.6	43.4	0	0
323	1.3	90	143	54.4	48.8	0	0
SGD	1.3	175	196	29.6	39.0	12	0
BH1	1.0	171	233	44.2	45.6	201	. . .
C31	0.6	169	245	66.2	45.3	65	24
3PN	0.6	146	142	33.4	17.0	59	. . .
BSD	0.5	208	233	35.6	37.4	. . .	59
S38	0.3	201	230	43.8	45.6	0	0
J1	0.1	27	60	42.4	49.4	0	0
J2	0.1	48	47	45.8	46.0	0	0
417	0.1	108	127	33.6	33.6	0	0
S44	0.1	205	197	27.0	20.8	0	0
$LSD_{0.05}$	0.5	25	37	4.3	2.8	83	14

[a] Disease severity was rated using a scale of 0 (no visible symptoms) to 5 (dead plants) 9 days after inoculation of 10-day-old plants.
[b] Microsclerotia were counted 4 weeks after inoculation of potato dextrose agar (PDA).
[c] Mycelial growth was measured 5 days after inoculation of PDA.
[d] Perithecia were counted on PDA 3 weeks after inocubation under continuous fluorescent light.
[e] Not tested.

(Dynamics Corporation of America, New Hartford, Connecticut). The slurry of mycelium and microsclerotia was passed through nested sieves of 250 μm and 45 μm. Microsclerotia on the 45 μm sieve were collected and volume was adjusted to 100 m*l* with tap water. After sufficient dilution, 10 m*l* of the diluted suspension was applied to a counting dish and numbers of microsclerotia were determined as means of three counts per plate. Microsclerotia production was calculated using the following equation: total number of microsclerotia=counted number of microsclerotia × original volume (100 m*l*) × dilution factor. This test was conducted twice with four and five replicates, respectively.

Production of perithecia was determined from cultures grown on PDA in darkness at 25°C for 10 days. At that time, aerial mycelia were removed with a sterile spatura and cultures were incubated under continuous fluorescent lights for 3 additional weeks. Perithecia production was quantified as the mean of numbers from 10 random 1-cm^2

areas on each plate. This test was conducted twice with three and five replicates, respectively.

DsRNA anlysis

Isolates were grown on potato dextrose broth for 2 weeks and maintained under ambient laboratory condition (ca. 25°C). Mycelia were harvested by vacuum filtration through filter paper (Whatman no. 1), lyophilized for 24 hr, then stored at −20°C. The extraction procedure for dsRNA was that of Valverde *et al.* (1990) which was modified from Morris and Dodds (1979). The extracted dsRNA was electrophoresed in 6% polyacrylamide gels at 100 V for 2 hr. Gels were stained in ethidium bromide and observed UV light. The dsRNA nature of samples was verified following treatment with DNase. Isolates (FL40C and FL173) of *Diaporthe phaseolorum* var. *caulivora* (Lee, 1991) known to contain dsRNA were used as controls in dsRNA extraction.

Analysis of data

Statistical analyses were conducted using SAS (1988). Analysis of variance was determined using PROC GLM procedure. Relationships among dependent variables were examined using PROC CORR.

Results

Variability in disease severity, production of microsclerotia and perithecia, and mycelial growth was observed among isolates of *C. ilicicola* (Table 2). Isolates that induced more severe disease generally produced more microsclerotia and perithecia than did isolates with lower virulence (Table 2). However, mycelial growth among isolates was inconsistent. Nine isolates of *C. ilicicola* did not produce perithecia in either test, whereas a single isolate (SGD) produced few perithecia in test 1 only (Table 2). Isolates that failed to produce perithecia exhibited moderate or low virulence (Table 2).

Disease severity correlated positively with production of microsclerotia and perithecia in tests 1 and 2 (Table 3). However, correlation coefficients were markedly higher for perithecia than for microsclerotia. Disease severity correlated negatively with mycelial growth in test 1 only (Table 3). In both tests, mycelial growth correlated negatively with production of perithecia but did not correlate

Table 3. Correlation coefficients among disease severity[a], microsclerotia[b], mycelial growth[c], and perithecia[d] produced by isolates of *Calonectria ilicicola*

	Disease severity		Microsclerotia/cm^2		Mycelial growth(mm)	
	Test 1	Test 2	Test 1	Test 2	Test 1	Test 2
Microsclerotia	0.1934[e]	0.3404				
	0.0648[f]	0.0002				
Mycelial growth	−0.2555	−0.1212	−0.0184	0.0697		
	0.0040	0.1834	0.8616	0.4650		
Perithecia	0.7628	0.6633	0.0681	0.2038	−0.3283	−0.4907
	≥0.0001	≥0.0001	0.5287	0.1320	0.0003	0.0002

[a] Disease severity was rated using a scale of 0 (no visible symptoms) to 5 (dead plants) 9 days after inoculation of 10-day-old plants.
[b] Microsclerotia were counted 4 weeks after inoculation of potato dextrose agar (PDA).
[c] Mycelial growth was measured 5 days after inoculation of PDA.
[d] Perithecia/cm^2 were counted on PDA 3 weeks after inocubation under continuous fluorescent light.
[e] Correlation coefficients.
[f] *P* values.

with disease severity (Table 3).

Production of microsclerotia and perithecia was greater in isolates of *C. ilicicola* from soybean than those from peanut. Mycelial growth by peanut isolates was greater but only in test 1 (Fig. 1). Soybean isolates were more virulent on soybean than were peanut isolates (Tables 1 and 2).

None of the *C. ilicicola* isolates tested contained detectable levels of dsRNA, but dsRNA was detected in isolates of *D. phaseolorum* var. *caulivora* which served as controls.

Discussion

Morphological phenotypes have been used as markers to characterize pathogenicity or virulence among isolates of plant pathogens. Molecular markers such as isozymes, RFLP, mitochondrial DNA and plasmids, and dsRNA also have been applied extensively for this purpose (Michelmore and Hulbert, 1987). Because variability in virulence among *C. ilicicola* isolates was observed on legume plants such as soybean (Kim *et al.*, 1992) and peanut (Rowe and Beute, 1975), this research focused on identifying cultural and/or molecular characteristics in this fungus that may be related to virulence.

Rowe and Beute (1975) reported a wide range of virulence in *C. ilicicola* on peanut but no relationship between virulence and growth rate in culture or geographic origin of the isolates. Results from this study also showed no consistent association between virulence and diameter growth *in vitro* for isolates of this fungus from soybean and peanut. Pearson *et al.* (1986, 1987) found that colony morphology of *Macrophomina phaseolina* isolates from soybean or corn differed on chlorate-amended media, suggesting that host-specific isolates may be identified using this marker. We did not test whether growth of *C. ilicicola* on such media was related to virulence or other cultural characteristics. Positive correlations between virulence and production of microsclerotia or perithecia were observed among isolates of *C. ilicicola*, but the correlation was much stronger for perithecia than for microsclerotia. Elliston (1985) found that strains of *Cryponectria parasitica* that were free of detectable dsRNA produced abundant perithecia and stromata in culture, whereas dsRNA-containing strains with low virulence did so inconsistently. Colony morphology and growth rate in *C. parasitica* were diverse regardless of the presence of dsRNA. These observations suggested that cultural characteristics such as perithecia and stromata might be useful indicators for selecting dsRNA-containing strains. Similarly, production of perithecia and possibly microsclerotia may be useful indicators of virulence in *C. ilicicola*.

The finding that isolates demonstrating higher levels of virulence produced more perithecia than did isolates with moderate or low virulence suggests that perithecia in this fungus may be involved in generation of virulent genotypes. This may imply that sexual reproduction plays an important role in morphological and pathogenic variation, as suggested by Klittich *et al.* (1988). Hyphal anastomosis in *C. ilicicola* (Black and Beute, 1984) also has been observed to produce genetic variation. The latter may be the cause of frequent spontaneous production of morphological variants, i.e., sectors, when mycelia of *C. ilicicola* are grown on artificial media (our unpublished data). Such sectoring may be critical for ensuring variability in organisms that lack sexual recombination, such as *Fusarium moniliforme* (Klittich *et al.*, 1988). These results suggest that the high level of variability in isolates of *C. ilicicola* may result from sexual and/or nonsexual mechanisms for genetic recombination.

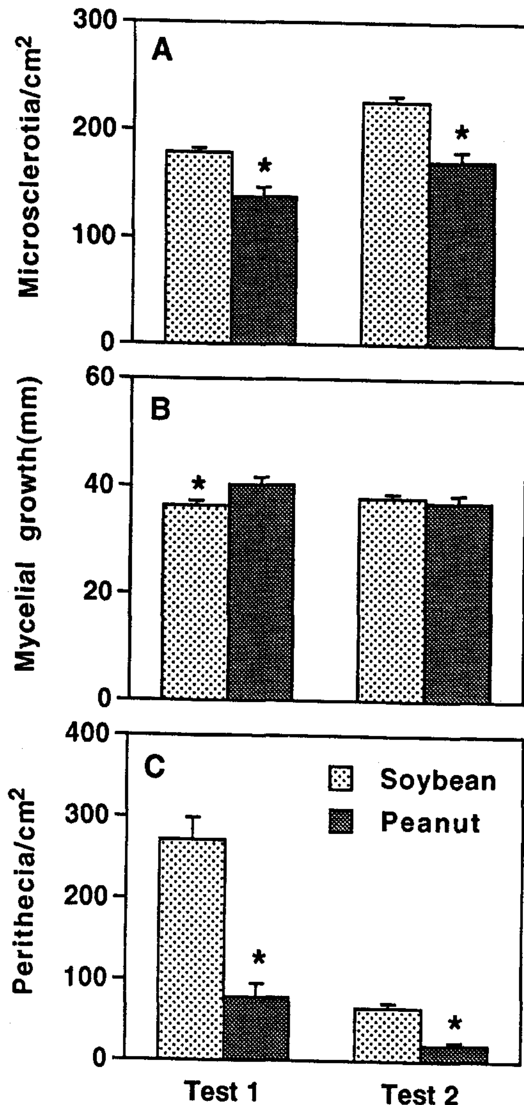

Fig. 1. (A) Production of microsclerotia 4 weeks after inoculation on potato dextrose agar (PDA), (B) mycelial growth 5 days after inoculation on PDA and (C) production of perithecia on PDA by soybean and peanut isolates of *Calonectria ilicicola* 3 weeks after incubation under continuous fluorescent light. Bars indicate standard errors of the means; within tests, an asterisk indicates difference ($P \leq 0.05$) based on analysis of variance.

Soybean isolates exhibited greater production of perithecia and microsclerotia than did peanut isolates, which agrees with previous findings that show greater virulence in isolates from soybean (Kim, 1992). This supports the hypothesis of host specialization in *C. ilicicola* (Kim, 1994). Isolates of *M. phaseolina* from soybean colonized soybean roots more extensively than did isolates from sorghum, which suggests that host specialization may exist in this soybean pathogen as well (Cloud and Rupe, 1991).

Microsclerotia are the primary inocula of *C. ilicicola* and, as such, have a critical role in producing red crown rot on soybean. They are produced in fibrous roots infected by the fungus and are released into soil upon deterioration of the host roots (Johnston and Beute, 1975). Therefore, the ability of isolates to produce microsclerotia is an important component of inoculum potential in *C. ilicicola*. The role of perithecia in the disease cycle is not understood, but they may be important for generating virulent genotypes through genetic recombination. In addition, perithecia production may serve as a useful marker for characterizing virulence or host specialization in this homothallic fungus.

적 요

여러가지 기주에서 유래한 *Calonectria ilicicola* 균주의 병원성에 대한 형태적, 유전적 표지를 검출하기 위하여 균사생장, 균핵, 자낭각 그리고 dsRNA를 조사하였다. 시험된 모든 균주에서 병원성, 균핵수, 자낭각수 와 균사생장 등의 다양한 변이가 관찰되었으나 dsRNA는 검출되지 않았다. 균의 병원성은 균핵과 자낭각 생산과 각각 정의 상관관계가 있었으나, 균사 생장은 자낭각의 생산과 부의 상관관계를 보여 주었다. 땅콩에서 유래한 균주보다 콩에서 유래한 균주가 기주인 콩에 더 강한 병원성을 보였으며 균핵과 자낭각의 생산도 더 많았다. 이와 같은 결과로 볼 때 *C. ilicicola*의 균핵과 자낭각 생산력은 inoculum potential의 구성요소로써 작용하며, 이중 균의 자낭각 생산력은 병원성과 기주분화를 나타내는데 유용한 표지로써 이용될 수 있을 것이다.

Acknowledgments

We thank M. M. Blackwell, G. E. Holcomb, and K. G. Jones for critical review of this manuscript. We thank D. K. Berner, M. K. Beute, and J. Y. Uchida for providing the isolates of *Calonectria ilicicola.*

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