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Pathogenicity and Host Range of a Potential Mycoherbicide, Isolate BWC98-105, Causing White Root Rot on *Trifoliorum repens*

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White root rot of wild white clover (*Trifoliorum repens*) caused by isolate BWC98-105 has been first reported in Korea. Typical symptoms on root include water-soaked and dark-brown rot, resulting in complete blight of the whole plant. The fungus grew well at 20-28°C and produced abundant sclerotia at 10-15 days after full mycelial growth on potato dextrose agar. Sclerotia were brown to dark-brown in color and 1-3 mm in length. When white clover plants were inoculated with mycelial suspension (10^5 cfu/ml) of isolate BWC98-105, the plant shoots were killed within 4-6 days and the roots were completely blighted. Sclerotia were also formed on the surface of the root covered with whitish mycelia within 10-15 days in the field. All nine isolates developed high incidences of white root rot disease on white clover seedlings, of which the symptoms were similar to those observed in the fields. Hence, their pathogenicity was confirmed on white clover. The infection rate of the fungal isolates varied from 78.5% to 95.2%, among which BWC98-105 was the most virulent isolate. The weeding efficacy of the fungus was maintained until the following year, leading to a significant reduction of reshooting. The fungus was specifically parasitic to white clover, but not to four lawn species including zoysiagrass (*Zoysia japonica*) under greenhouse test. The fungus also had no response to some *Gramineae* species including rice, but caused little damage to five species of *Leguminosae*.

Keywords : Pathogenicity, host range, mycoherbicide, BWC98-105, white root rot, *Trifoliorum repens*

White clover (*Trifoliorum repens*) is a problematic weed species worldwide. It is one of the major perennial sedges that propagates mainly by rhizome, and grows and flowers from spring to autumn. Formation of abundant underground rhizomes spreads vegetatively from the root systems and their irregular sprouting habits make it difficult to control them even by herbicides. White clover is controlled by mechanical means (mowing) and application of herbicides that must be reapplied to control new plants arising from regeneration of treated plants from the roots. Biological control is most effective when used as a component of an integrated pest management system, and in combination with chemical pesticide (Boyetchko, 1997; Rosskopf et al., 1999).

In the experimental fields of the National Yeongnam Agricultural Experiment Station at Milyang, Korea in 1998, white root rot symptoms were first observed on white clover plants in lawn ground in July, which rapidly developed in August (Hong et al., 1998, 1999). Infected roots of white clover were blighted, densely covered with mycelia including brown sclerotia, and eventually were completely killed (Hong et al., 2001, 2002). The causal fungus was isolated from the lesions of infected plants. So far, 25 isolates of the fungus (unidentified) have been isolated from several lawn ground infested with *Trifoliorum repens* in 1998-1999. The isolates somewhat varied in their sclerotia production in terms of size and colony color.

To use an organism as a biological control agent, selection of the promising isolate is most important (Danial et al., 1973; TeBeest, 1988; Walker, 1981; Weidemann, 1988). It should be highly pathogenic to target organism(s) but not pathogenic to other crop plants. The purpose of this research was to determine whether the pathogen could be used to control white clover. The fungus *Sclerotinia* sp. isolate BWC98-105 was selected because of its broad spectrum of host, and since it causes root rotting. Because *Sclerotinia* sp. has a wide host range, it should be determined whether such hosts are pathogenic on many crop plants and not only on grasses.

Materials and Methods

Isolation and culture condition. White clover (*T. repens*) plants showing typical symptoms of root rot lesions were collected from lawn ground contaminated with diseased white clover during the summer of 1998 through 1999. Diseased stems and roots with grown white mycelia were cut into 2-3 mm pieces and surface-sterilized by submerging them in 70% ethyl alcohol for 1 minute

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and 2% sodium hypochloride also for 1 minute, and then washing them with sterilized water. The sterilized pieces were transferred aseptically to water agar containing 200 μg chloramphenicol, 88 μg ampicillin, and 70 μg streptomycin.

Hyphal tips of the colonies formed were transferred to acidified potato dextrose agar (PDA, pH 5.5) and cultured for 5 days at 28°C. A mycelial disc (d-5-mm) of the culture was preserved in a -70°C deep freezer in Cryovial (Nunc, Inc., IL., USA) until use, containing 1:1 mixture of 1.5 ml of 40% glycerol and 10% skim milk (Aoshima et al., 1983). The nine isolates used in this study were selected based on their characteristics among 25 isolates collected from diseased white clover from 12 regions in Korea. Isolates were characterized by mycelial color, and by sclerotia production, size and color.

Inoculum production. For the production of mycelia to be used as inoculum, five pieces of agar disc with mycelia were cultured in a 250 ml Erlenmyer Flask with 150 ml of potato dextrose broth (Difco, MI., USA). The culture was then placed in a rotary shaker (Jeiotech, Korea) at 120 rpm at 28°C for 7 days. The fully grown mycelia were harvested and homogenized with a homogenizer (Nihonseiki Kaisha, Japan) at 10,000 rpm for 0.5 minute. The mycelial suspension containing mycelial fragments of 10^5 cfu/ml was adjusted with the use of a hemocytometer under 100x microscope.

Preparation of seedlings. Rhizomes of white clover were collected from lawn ground in March to April 1998. Whole plants with rhizomes were transplanted into plastic pots (21 × 17 cm) containing fertilized upland soil ($N\text{-}P_2O_5\text{-}K_2O$ = 15-15-15 g/m^2). The seedlings were grown to 10- to 15-cm tall for the pathogenicity and host range test.

Pathogenicity test to white clover. Mycelial suspension of isolates (3.2×10^5 mfu/ml containing 0.5% dextrose in distilled water) was sprayed on the whole plant and soil drench. The inoculated plants were incubated in a dew chamber controlled at 28°C for 16 hours, then the pots were placed on a greenhouse bench at 25-32°C for lesion development. Symptom progress was observed and recorded at 5 days after inoculation. In addition, a number of infected shoots, dead shoots, reshoots, and diseased underground rhizomes were recorded. Where applicable, the data were processed by SAS GLM procedure (International Rice Research Institute) and analyzed by Duncan's multiple range test.

Host range test. To study the host range of this mycoherbicide agent, 4 lawn grasses, 4 *Gramineae* spp., 4 *Leguminosae* spp., and 19 weed species were inoculated with mycelial suspension of the isolate BWC98-105 as described in the pathogenicity test. Plants used in the host range tests were grown in a plastic pot (21 × 17 cm) containing fertilized upland soil ($N\text{-}P_2O_5\text{-}K_2O$=15-15-15 g/m^2). Each pot contained 30 seedlings and grew to the third to fifth leaf stage. Development of the symptom on plants was observed 15 days after spraying with the mycelial suspension. Non-treated plants sprayed with water were used as controls.

Results and Discussion

Fungal characteristic. The fungus grew well at 20-28°C and abundant sclerotia were produced at 10-15 days after full mycelial growth on PDA. Sclerotia were brown to dark-brown, 1-3 mm in size, and were scattered on the medium (Fig. 1).

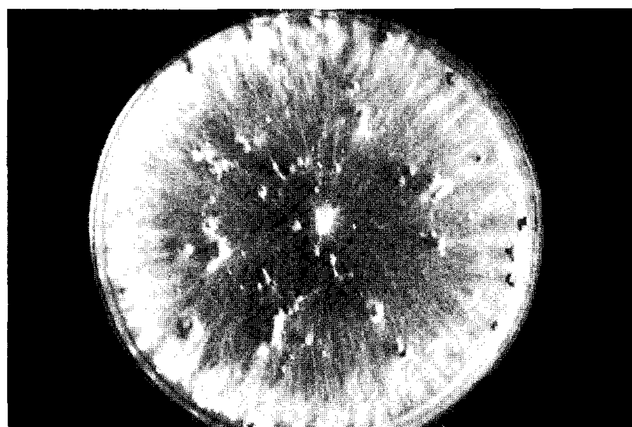

Fig. 1. Isolate BWC-105 with whitish mycelia grown on PDA medium for 5 days; sclerotia, 1-3 mm in size and brown to dark-brown in color, were formed 10 days later.

Pathogenicity. All nine isolates developed white root rot on white clover seedlings as observed in the fields, thus, were confirmed as the pathogen of *Trifoliorum repens* (Table 1). When inoculated with mycelial suspension, the rate of infection was 78.5% to 95.2% on shoots, among which BWC98-105 was the most virulent isolate. Other isolates, BWC99-124 and BWC99-220, were also virulent, showing weeding efficacy of 90.2% and 90.5%, respectively. Infected shoots were killed in 10-15 days. Symptoms first appeared on stems of the plants 1-2 days after inoculation in greenhouse conditions. Water-soaked lesions became larger with time, with the lesions rapidly expanding around the stem, blighting the whole plant completely within the next

Table 1. Weeding efficacy of host-specific isolates on *Trifoliorum repens*

Isolate	Sampling site	Weeding efficacy[a]
BWC98-105	Miryang	95.2[b]
BWC99-110	Seongju	83.8
BWC99-113	Yeongchon	82.5
BWC99-124	Jinju	90.2
BWC99-127	Daegu	78.5
BWC99-134	Daegu	81.7
BWC99-207	Cheongsong	83.3
BWC99-220	Miryang	90.5
BWC99-291	Miryang	79.6

[a] Weeding efficacy was examined 30 days after inoculation. 25-day-old seedlings of *Trifoliorum repens* were inoculated with 20 ml mycelial suspension containing 100 g fresh weight of mycelia in water per pot.
[b] Data are means of three replications; each replicate is the average of five values.

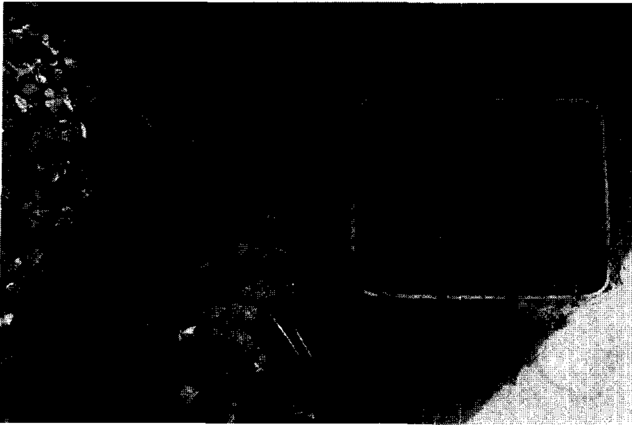

Fig. 2. Weeding efficacy of BWC-105 on *Trifoliorum repens* seedling when sprayed with mycelial suspension of 20 ml per pot (left: untreated control; right: mycelial suspension of BWC98-105 10 days later).

Fig. 3. Underground rhizomes with stems and leaves were killed by the fungus, from which whitish mycelia with abundant sclerotia densely emerged on the blighted root 10-15 days after inoculation with mycelia suspension (10^5 mfu/ml). As it did, the rhizomes can not sprout the new shoot of the wild white clover.

10-15 days (Fig. 2). Thereon, whitish mycelia densely emerged on the blighted root with abundant sclerotia, which can become the inoculum for the following year to suppress new plant emergence (Fig. 3). This means that the control efficacy of the fungus can be maintained year-round. Results of treatments with mycelial suspension of isolate BWC98-105 and flazasulfuron (10% ai.) were significantly different (Table 2). Higher weeding efficacy was observed with mycelial suspension treatment. Treatment with flazasulfuron as control showed 72.8% plant mortality at an average time of 15.6 days. This was lower than that of mycelial suspension which was 99.7% within 4 days. Time of plant mortality was shorter with mycelial suspension treatment than with flazasulfuron treatment. This result suggests that at least one time application in an appropriate environmental condition is needed for the effective control of white clover (Hong et al., 1999, 2001). For effective control, shoots, reshoots, as well as underground root formation must be considered. One of the difficulties encountered in attempting to kill weeds is their ability to regenerate, like white clover which mainly regrows by root and bindweed (Ormeno-Nunez et al., 1988; Reuveni et al., 1986). Therefore, shoots must be severely infected and must die before regeneration occurs. Ormeno-Nunez et al. (1988) observed regeneration in bindweed seedlings inoculated with *P. convolvulus*. Regeneration is particularly important in underground parts such as rhizomes because they are the last to collapse. High mortality will be achieved when all the underground rhizomes were killed at the time of fungal application.

Host range. All tested plants seemed to be non-host to isolate BWC98-105; white clover was the only test plant with white root rot lesions observed. No test plants produced typical symptom, but some plants in the genus *Leguminosae* sp. and some weed species produced slight disease symptom. There were no differences in overall plant size, leaf number, or plant vigor between any of the inoculated test plants and the corresponding control plants in 15 days (Tables 3, 4, 5, 6). In contrast, white clover became heavily infected; evidence of infection was apparent within 1-2 days on the whole plant. The result obtained in this study, as well as previous reports indicated that isolate BWC98-105 seemed highly pathogenic to white clover but either non-pathogenic or very weakly pathogenic to other plant species. In the studies of biological control of weeds with pathogens, most weed species are hosts to many pathogens (Boyette, 1991; Conway, 1976). TeBeest (1988)

Table 2. Weeding efficacy of isolate BWC98-105 on *Trifoliorum repens* when sprayed with the mycelial suspension

Treatment	Plant mortality (%)[a]	Time required to plant mortality (Day)	Percent of Plant Reshooted[b]
BWC98-101 (100 g F.Wt/m^2)	99.7	4	3.5 c[c]
Flazasulfuron (10%, ai.)	72.8	15.6	85.6 b
Untreated check	–	–	99.3 a

[a] Plant mortality was recorded as percent diseased area in the plot size of m^2.
[b] New sprouts were examined 2 months after inoculation.
[c] Numbers in each column followed by the same letter are not significantly different by Duncan's new multiple range test (p = 0.05).

Table 3. Response of lawn grasses to inoculation with mycelial suspension of host-specific isolate BWC98-105 from *Trifoliorum repens*

Plant[a]	Pathogenicity[b]
Creeping bentgrass (*Agrostis palustris* Huds.)	–
Kenterky bluegrass (*Poa pratensis* L.)	–
Perennial ryegrass (*Lolium perenne* L.)	–
Zoysiagrass (*Zoysia japonica* steud.)	–
Whiteclover (*Trifolium agrarium* L.)	+

[a] Host plants of 3-5 leaf stages were sprayed with mycelial suspension (containing 100 g fresh weight of mycelia in water) until run-off. The inoculated plants were incubated for 24 hours in a dew chamber at 28°C and then placed on a greenhouse bed. Data were collected 10 days after inoculation

[b] + = symptoms produced and heavily infected; – = no symptom developed.

Table 4. Response of some *Gramineae* sp. to inoculation with mycelial suspension of BWC98-105 from *Trifoliorum repens*

Plant[a]	Pathogenicity (lesion form)[b]
Rice (*Oryza sativa* L.)	–
Barley (*Hordeum vulgare* L.)	–
Maize (*Zea mays* L.)	–
Wheat (*Ttiticum aestivum* .L)	–
White clover (*Trifolium repens* L.)	+

[a] Host plants of 3-5 leaf stages were sprayed with mycelial suspension (containing 100 g fresh weight of mycelia in water) until run-off. The inoculated plants were incubated for 24 hours in a dew chamber at 28°C and then placed on a greenhouse bed. Data were collected 10 days after inoculation

[b] + = symptoms produced and heavily infected; – = no symptom developed.

Table 5. Response of *Leguminosae* sp. to inoculation with mycelial suspension of BWC98-105 from *Trifoliorum repens*

Plant[a]	Pathogenicity (lesion form)[b]
Soybean (*Glycine max* L. Merrill)	+
Peanut (*Arachis hypogaea* L.)	+
Green pea (*Pisum sativum* L.)	±
Cow pea (*Vigna sinensis* K.)	±
White clover (*Trifolium repens* L.)	++

[a] Host plants of 3-5 leaf stages were sprayed with mycelial suspension (containing 100 g fresh weight of mycelia in water) until run-off. The inoculated plants were incubated for 24 hours in a dew chamber at 28°C and then placed on a greenhouse bed. Data were collected 10 days after inoculation

[b] + = symptoms produced but was not heavily infected; ± = no symptom developed; ++ = symptoms produced and heavily infected.

Table 6. Host range of isolate BWC98-105 to some weed species

Family	Species[a]	Response[b]
Gramineae	*Echinochloa crusgalli* Beauv. var. *praticola* Ohwi	–
	Echinochloa crusgalli Beauv. var. *caudata* Kitagawa.	–
	Echinochloa crusgalli Beauv. var. *oryzicola* Ohwi	–
	Leersia japonica Makino	++
	Oryza sativa	±
Cyperaceae	*Cyperus difformis* L.	++
	Cyperus serotinus Rottb.	++
	Fimbristylis miliacea Vahl.	++
	Scirpus hotarui Ohwi	++
	Eleocharis kuroguwai Ohwi	+
Pontedriaceae	*Monochoria vaginalis* Presl	++
	Monochoria korsakowii Regel et Maack.	++
Leguminosae	*Aeschynomene indica* L.	++
Alismataceae	*Sagittaria trifolia* L.	++
	Sagittaria pygmaea Miq.	+
Commelinaceae	*Aneilema japonica* Kunth.	++
Polygonaceae	*Polygonum hydropiper* L.	+
Typhaceae	*Typha latipholia* L.	++
Onagraceae	*Ludwigia prostrata* Roxb.	–
Compositae	*Bidens tripartita* L.	–

[a] Host plants of 3-5 leaf stages were sprayed with mycelial suspension (containing 100 g fresh weight of mycelia in water) until run-off. The inoculated plants were incubated for 24 hours in a dew chamber at 28°C and then placed on a greenhouse bed. Data were collected 10 days after inoculation.

[b] – = no symptom developed; ± = symptom produced but not developed; + = symptoms produced but with little infection; ++ = symptoms produced and heavily infected.

has recently discovered that *Colletotrichum gloeosporioides* f. sp. *aeschynomene*, the incitant of anthracnose on northern jointvetch (*Aeschynomene virginica*) has a much broader host range than originally reported by Daniel et al. (1973). Daniel suggested that some of the highly desirable characters for biological weed control agents are genetic stability and specificity to the target weed.

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