

Difference of Susceptibility on Bentgrass Cultivars to Pink Snow Mold Caused by *Microdochium nivale*

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ABSTRACT. The susceptibility of cultivars of three bentgrass species (creeping, colonial, and velvet bentgrass) was evaluated on detached leaves assays with pink snow mold 9 isolates caused by *Microdochium nivale* in Petri dishes and whole plants under controlled conditions. The pink snow mold isolates obtained from infected turfgrasses on golf courses in Wisconsin were tested on response of fungicides and temperature. Detached leaf assay and susceptibility of bentgrass cultivars were evaluated with potted adult seeding during 80 days. Nine isolates were susceptible to two fungicides and were significantly different among isolates. Mycelial growth was varied in response of temperatures among isolates. There were significant differences in development and colonization of the fungus on detached leaf assay among bentgrass species include cultivars. There were significant differences on whole plants in disease severities among the three bentgrass species, particularly between tetraploids (creeping and colonial) and diploid (velvet) species, and among cultivars within each species, indicating that there are varying levels of susceptibility in species and cultivars to *M. nivale*. This study could be applied to evaluate the susceptibility of bentgrass to pink snow mold and also to predict a prospective evaluation of bentgrass cultivars to pink snow mold in fields in a breeding program.

Key words: Detached leaf assay, Diploid, *Microdochium nivale*, Tetraploids

Introduction

Pink snow mold (Fusarium patch), caused by *Microdochium nivale*, (Fries) Samuel & Hallet (synonym *Fusarium nivale* (Fr.) Sorauer) is one of the important diseases causing winter damage on turfgrasses in Wisconsin and other regions with similar climatic conditions (Smith et al., 1980, Ulrich and Fredey, 1994). Pink snow mold describes the symptom of *M. nivale* on the leaves of grass after the disappearance of the permanent snow cover. The mycelium and the spores of the fungus take on, acquire, become a better than assume a pink color after exposure to the light (Smith et al., 1980). The fungus affects a number of turfgrass cultivars in late fall, winter and early spring. Outbreaks occur under cold and wet weather conditions, with circular and discolored patches becoming apparent in the turfgrass. The leaves of affected plants tend to coalesce. Also, a faint white or dull pink mycelium may develop at the margins of patches. *M. nivale* survives unfavorable periods in infected grass plants and dead debris (Richard et al., 2000). Susceptible grasses include: bentgrass, bermudagrass, annual bluegrass, Kentucky bluegrass, annual ryegrass, perennial ryegrass and tall fescue

(Cough, 2000). Cough (2000), describes 85 grass hosts, many of which are forage grasses (Cough, 1973).

Pink snow mold severity can be reduced by several cultural practices such as moisture control and maintenance of turf vigor, and fertility control such as the application of late fall nitrogen fertilizer (Madison, 1960; Smith et al., 1989). Currently, pink snow mold management, is highly dependent on chemical fungicide application (Smith et al., 1989). Affordable fungicides such as PCNB are no longer registered for use in the United States due to environmental concerns (Smith et al., 1989). Therefore, there is a need for alternative disease management strategies such as host resistance to *M. nivale* in bentgrass cultivars. Resistant cultivars would greatly reduce the costs and environmental impacts of fungicide application. However, little research on current cultivar resistance has been done. A good understanding of both the pathogen and the host biology is required to implement host resistance by selecting resistant cultivars (Chang et al., 2011). The breeder needs information regarding different isolates of the pathogen and their virulence variability to understand potential host resistance to the pathogen (Chang et al., 2011). Field evaluation for cultivars resistant to pink snow mold is often inefficient due to several many factors as a environmental, space (Smith et al., 1989; Browne and Cooke, 2004). For screening of bentgrass cultivars resistance to *M. nivale* isolates, good methods, has not been developed for in vitro using artificial inoculum with several isolates. A new method for controlled growth

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chamber experiments is needed.

The objectives of this study, I evaluated the susceptibility of commercial bentgrass cultivars and breeding lines to pink snow mold, and compared the virulence of selected isolates as well as their sensitivity fungicide.

Materials and Methods

Identification of isolates

Isolates of *M. nivale* were collected from 60 golf courses in Wisconsin during 2000 and 2001. Isolations were acquired or isolated from pink snow mold symptomatic leaves, and isolates were purified by Single-spore (Chang et al., 2011). Isolates of ten *M. nivale* strains used in this study were selected from 160 isolates, which were previously collected from more than one hundred golf courses in Wisconsin during 2001. The isolates were chosen from different regions based on their genetic diversity. Ten isolates were analyzed to detect their varieties using specific primers with a theoretical melting temperature around 62°C. Two primer pairs were generated. The first primer pair as the forward and the reverse, Y13MF / Y13MR (CTTGAGGCGGAAGATCGC / ATCCCTTTCCGGGGTTG) for the detection of *M. nivale* var *majus* and the second primer pair as the forward and the reverse, Y13NF / Y13NR (ACCAGCCGATTTGTGGTTATG / GGTCACGAGGCAGAGTT CG) for the detection of *M. nivale* var *nivale* (Chang et al., 2011).

Response of Fungicide and temperature

The sensitivity of 10 isolates, contact (PCNB) and systemic fungicide (Heritage) labeled for pink snow mold control was tested with 1 µg a.i / ml of Heritage and 5 µg a.i / ml of PCNB. Investigated temperature was 5°C for 10 days and 20°C for 4 days.

Plant materials and managements

Ten bentgrass (*Agrostis* spp.) cultivars (6 creeping (*A. stolonifera*), 3 colonial (*A. stolonifera*) and 1 velvet (*A. canina*)) were used for in both detached leaf assay and whole plants experiments (Table 1). Cultivars of creeping and colonial bentgrass or velvet bentgrass cultivars were sown evenly, respectively (Chang et al., 2011). The plants were grown in the greenhouse at 18 to 28°C with light and dark cycle of 16 and 8 hours, respectively. The plants were mowed with scissors at 0.5 cm to 0.7 cm ranges until 80 days old, at which time they were adult plants. Water soluble fertilizer (21-5-20: SunGrow company, Texas) at 2 g per m² was applied weekly after was the beginning of mowing at 21 days (Chang et al., 2011). These plants were used for all in vitro experiments. For growth chamber tests, adult plants were grown for 80 days old before being moved to growth chambers maintained at 5°C for 1 week. *M. nivale* was

Table 1. Bentgrass cultivars and sources used.

Bentgrass Species	Cultivars	Lot number
Creeping	Penncross	L36-0-109
	Penn A4	M52-9-A4-1
	Penn G2	M33-0-57
	PST-9BNC	C8-1-9BNC-BS
	Penneagle II	C8-1-OPN-BS
	Penlinks II	C8-1-OVN-BS
Colonial	Alister	M65-1-9F7-F
	Glory	M65-1-9HG-F
	Bardot	M65-1-9HB-F
Velvet	Greenwich	M146-1-81

grown PDB at 20°C for 7 days to produce mycelium. On the inoculation day, the cultured mycelium was homogenized and adjusted to a concentration of 0.15 g mycelium at 1 ml. The plants were inoculated using ground mycelium 1 ml of each isolate, and the inoculated plants were kept for 1 week at 10°C in the dark. Inoculated plants were placed at 15°C under 8 hours lights for 1 week. Disease infected area was visually rated twice 3 days interval after inoculations (Chang et al., 2011).

Detached leaf assay

For detached leaf assay, individual leaves were collected 2 weeks after mowing and cut into 5 cm long pieces. The pieces were then surface disinfected in 0.3% sodium hypochlorite for 1 min and washed four times with tap water and twice with sterile distilled water. Detached leaf blades pieces were placed in a trifoliate pattern in a plastic Petri dish on top of a filter paper, which was moistened with 2.0 ml of sterile water containing 30 µg/ml of benzimidazole. For inoculations, inoculation was placed onto the transected end of the leaflets with mycelium plug (PDA agar plugs) from 4 day old *M. nivale* isolates (Ting and Greg, 1997). The Petri dishes were incubated at 20°C under 8 hours light for 3 days. Lesion length of mycelium infection on each leaf pieces was measured 3 days after inoculation.

Determination of amount of inoculums

The creeping bentgrass cultivar ‘Penncross’ and velvet bentgrass cultivar ‘Greenwich’, were used to determine ideal inoculums concentration for growth chamber experiment. Eighty day old plants were cold hardened in a growth chamber for 15 days. Three *M. nivale* isolates (SE25, SW47 and NW70) were used in the experiment. Mycelium suspension inoculums were prepared at final concentration, of 0.05 g, 0.1 g, 0.2 g, and 0.4 g fresh mycelium weight per mL with sterile distilled water. The inoculation was made by

injecting 1 ml of the inoculums suspension directly onto the plants in the center of the pots using a micropipette (Chang et al., 2011).

Evaluation of susceptibility of bentgrass cultivars/line

Mycelium suspensions were produced by taking twenty culture plugs of 5 mm in diameter from the colony edge and transferred to 100 ml Difco potato dextrose broth (PDB) and grown at 20°C±1 for 10 days in the dark. Mycelia were harvested from PDB culture by filtration on cheesecloth. After drying for 15 min under a clean hood, the weights of fresh mycelia were measured. The mycelia were homogenized for 30 seconds in a blender with sterile distilled water. These suspensions were adjusted with sterile distilled water to a final concentration of 0.2 g fresh mycelium weight per ml for growth chamber inoculations (Chang et al., 2011).

Inoculations of bentgrass one cultivar seeding were used 4 isolates (SW5, SE90, SW8, SW65). Inoculations of bentgrass cultivars seeding were used to develop the localized inoculation directly on the plants as a final concentration of 0.2 g fresh mycelium weight per ml per pot using a micropipette for all growth chamber assays. Incubation was used to develop the plastic containers method. The plastic incubation containers were filled by mixing 4 L of potting soil (Metro Mix 366-P, Scott's company) and 4 L of distilled water for maintaining high humidity before inoculated plant was placed (Chang et al., 2011).

The inoculated pots were arranged in a randomized complete block design with four replications in a plastic container (70×40×15 cm, Rubbermaid, Wooster, OH). To keep moisture in the plastic containers for 10 days, the inoculated pots were applied by evenly spraying on the plants with distilled water using a hand sprayer until saturation, were covered with containers cover to maintain the high humidity to disease infection and development. Incubation containers were maintained at 10°C in the dark for 10 days in growth chamber. Disease infection and colonization were assessed at 7 days and 10 days after inoculation (Chang et al., 2011).

Disease assessment and statistical analysis

Disease severity in growth chamber experiments was assessed by measuring diameter (millimeter) of infection areas using a digital caliper (Mitutoyo Co.) 3 days (detached leaf assay) 7 days (whole plant evaluation) after inoculation. Incubation period was determined by examining the plant in each pot for disease infection and colonization.

All statistical analyses were conducted using general linear models procedure (PROC GLM) in SAS (SAS institute Inc., Cary, NC). Means were compared with Fisher's protected least significant differences test at $P < 0.05$. For all primary and derived parameters, analysis of variance (ANOVA) was used to test the significance of main effects (cultivars within

Table 2. Means response to temperature and inhibition of mycelium growth for *M. nivale* isolates effects.

Isolates	Mycelial growth (mm)		Inhibition of mycelial growth (%)	
	5°C	20°C	Heritage (1 ppm)	PCNB (5 ppm)
NW48	55.7 ^{ab} ^y	60.5 ^b	50.53 ^a	79.17 ^a
NW48	53.2 ^c	57.8 ^c	43.90 ^{ab}	73.30 ^{ab}
SW65	48.0 ^d	56.2 ^d	42.87 ^{ab}	73.87 ^{ab}
SW5	52.5 ^c	58.5 ^c	39.70 ^{abc}	67.70 ^{ab}
SW8	46.9 ^d	48.4 ^f	33.77 ^{abc}	61.90 ^{abc}
NW14	16.7 ^f	19.0 ^g	25.63 ^{bc}	77.57 ^a
NE117	54.1 ^{bc}	57.1 ^{cd}	22.27 ^{cd}	54.77 ^{bc}
NE90	59.2 ^a	64.4 ^a	4.67 ^{de}	46.00 ^c
SE90	39.9 ^e	52.3 ^e	2.80 ^e	73.70 ^{ab}

^z Values are means for five replications for 9 isolates.

^y Means in a column followed by the same letter are not significantly different according to Fisher's LSD ($P = 0.05$).

species and isolate) and the first order interaction (cultivar x isolate). The source of variation for bentgrass species was partitioned into two orthogonal contrasts: creeping bentgrass and colonial (tetraploidy) vs. velvet bentgrass (diploid), and creeping bentgrass vs. colonial (different genomes within tetraploidy). The experiments were repeated twice, and the results of each experiment were analyzed separately.

Results

Response of isolates of fungicide and temperature

The response of temperature on the mycelial growth of 9 isolates was a significant difference ($P < .0001$) among isolates (Table 2). However, the same isolates were not different between 5°C and 20°C. Fungicides sensitivities of the isolates were shown for significant differences in low concentration among isolates.

Identification of the isolates and amount of inoculums

Nine isolates used in this study were identified for *M. nivale*. For evaluation of susceptibility of bentgrass, the creeping bentgrass cultivar 'Penncross' and velvet bentgrass cultivar 'Greenwich' were used to determine ideal inoculums concentration for growth chamber experiment. ANOVA detected that there were effects of concentration of inoculums and isolates on disease infection and colonization in two bentgrass cultivars (Fig. 1). There was a significant difference with increased concentration of inoculums to three *M. nivale* isolates. Mycelium suspension inoculums were the most high at 0.4 g fresh mycelium weight per mL

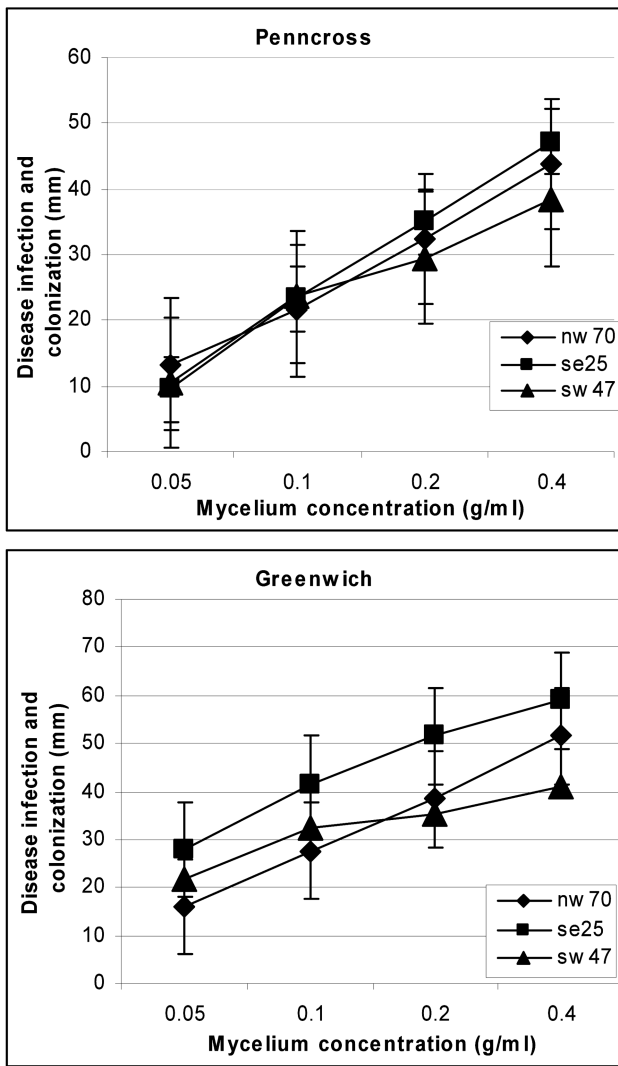


Fig. 1. Effect of disease infection and colonization two bentgrass cultivars/line inoculated with four concentrations of three isolates of *M. nivale* at 10°C in the dark after cold hardening at 5°C for 15 days.

with sterile distilled water.

Detached leaf assays and virulence of isolates

Detached leaf assays in Petri dishes (Fig. 2, 3), there were significant differences ($P < 0.05$) of disease infection and colonization of *M. nivale* 9 isolates to bentgrass cultivars within species and isolates had a significant effect. There were significant differences among the 10 commercial bentgrass cultivars for 9 isolates (Fig. 2). The millimeter of disease infection and colonization lesions size based on measuring results by caliper were lower for cultivar “Pennlinks II” and cultivar “Penncross”, creeping bentgrass species, than for cultivar “Glory”, colonial bentgrass species and cultivar “Greenwich”, velvet bentgrass species. The creeping

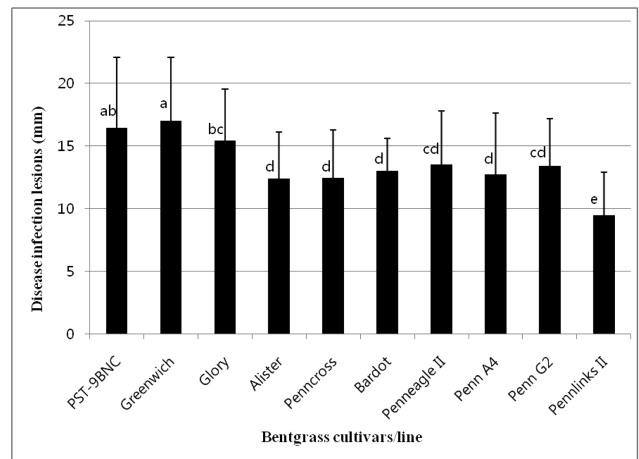


Fig. 2. Susceptibility of detached leaf assay of ten bentgrass cultivars/line inoculated with 9 isolates of *M. nivale*. Means were compared with Fisher’s protected least significant differences test at $P < 0.05$.

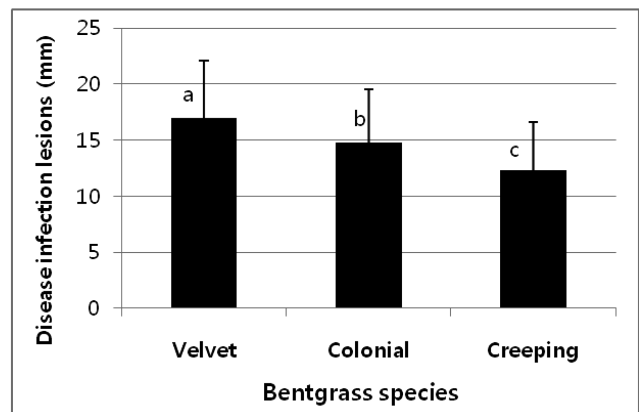


Fig. 3. Susceptibility of detached leaves of ten bentgrass cultivars/line inoculated with 9 isolates of *M. nivale*. Inoculation was placed onto the transected end of the leaflets with mycelium plug from 4 day old *M. nivale* isolates. The Petri dishes were incubated at 20°C under 8 hours light for 3 days. Lesion length of mycelium infection on each leaf pieces was measured 3 days after inoculation.

bentgrass species showed the lowest on disease infection and colonization lesions among bentgrass species (Fig. 3).

The pink snow mold isolates were also significant difference ($P < 0.05$) in virulence based on results for disease infection and colonization (Fig. 4). In the detached leaf assay only isolate SW65 showed the most an virulence among isolate on ten bentgrass cultivars/line ($P < .0001$).

Evaluation susceptibility on bentgrass cultivars/line

Based on whole plants (Fig. 5), there were significant

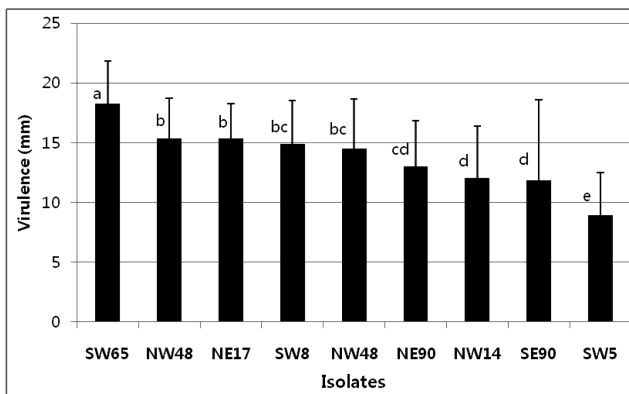


Fig. 4. Virulence of *M. nivale* isolates of ten bentgrass cultivars/line of collected from coulf course. Infection lesions sizes were measures on 3 days after inoculation. Means were compared with Fisher's protected least significant differences test at $P < 0.05$.

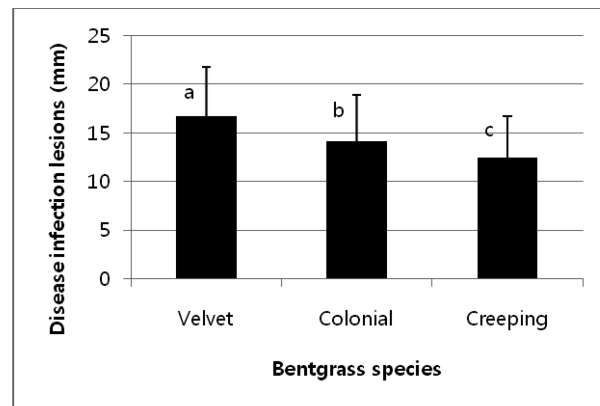


Fig. 6. Susceptibility of ten bentgrass cultivars/line inoculated with four isolates (SW5, SE90, SW8, SW65) of *M. nivale* at 10°C in the dark after cold hardening at 5°C for 15 days.

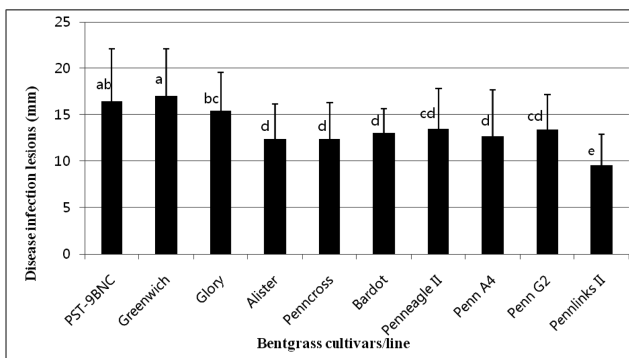


Fig. 5. Susceptibility of ten bentgrass cultivars/line inoculated with four isolates of *M. nivale* at 10 in the dark after cold hardening at 5 for 15 days. Inoculations of bentgrass one cultivar seeding were used 4 isolates (SW5, SE90, SW8, SW65). Means were compared with Fisher's protected least significant differences test at $P < 0.05$.

differences ($P < 0.05$) of disease infection and colonization of *M. nivale* isolates to 3 bentgrass species. Cultivars within species and isolates had a significant effect. There were significant differences ($P < 0.05$) among the 10 commercial bentgrass cultivars for 9 isolates. The millimeter of disease infection and colonization size based on measuring results by caliper were lower for cultivar "PennlinksII". ANOVA showed significantly differences among bentgarss species (Fig. 6). The creeping bentgrass were lower on disease infection lesions than colonial and velvet bentgrass.

Discussion

A range of growth differences of mycelial was detected

after 4 days at 20°C, with lesion sizes varying between 18.9 mm and 64.3 mm. The sensitivity of mycelial relative growth in heritage amended agar varied more than PCNB (Table 1). Although some isolates are less sensitive to heritage than others, on whole plants assays, virulence among isolates was not significantly different ($P < .0001$).

Infecting Detached leaves of colonial 3 cultivars, velvet 1 cultivars and creeping 6 cultivars with isolate SW65 showed the largest lesion lengths (range : 17.1 mm to 22.6 mm), and was the lowest lesion lengths (range: 7.8 mm to 10.4 mm) with SW5 isolate as compared with other isolates. However, on whole plant evaluation, these isolates were not significantly different as compared with others. On whole plant, differences in the virulence among the colonial cultivars/lines were associated with resistance in cultivars of "Glory" and creeping cultivars/lines were the most resistance cultivars of "Pennlinks II". In the previous studies, the varieties of selections from Northern climates had the greatest resistance to *M. nivale* and that the stolonized bentgrasses were more resistance to this pathogen than the seeded sorts. Colonial (*A. tenuis*) lines were less resistant than the velvet lines or creeping lines (Could, 1978). On creeping bentgrass lines, "Penncross" is one of the most resistant cultivars (Smith et al., 1980).

Pink snow mold symptoms caused by *M. nivale* were successfully reproduced by direct inoculation onto plants using a mycelium suspension. A high level of disease infection and colonization was obtained by incubating inoculated plants at 10°C with saturated moisture on the plants using plastic container containing saturated culture media for 10 days after inoculation. Disease development was observed by three days after inoculation with peak symptom expression occurring at 10 days in susceptible cultivars (Chang et al., 2011).

The growth chamber method developed in this study showed consistent disease development by *M. nivale* isolates throughout detached leaf assay and in the growth chamber. According to this newly modified method (Chang et al., 2011), pink snow mold symptoms caused by *M. nivale* were successfully reproduced by direct inoculation onto plants using a mycelium suspension. A high level of disease infection and colonization was obtained by incubating inoculated plants at 10°C with saturated moisture on the plants using plastic container containing saturated culture media for 10 days after inoculation. Disease development of whole plant evaluation in the growth chamber was observed by three days after inoculation with peak symptom expression occurring at 10 days in susceptible cultivars.

Virulence varied significantly among *M. nivale* isolates in this experiment (Fig. 4). I noted an interesting isolate effect in that the isolates caused different amount of disease in the cultivars. Isolates SW5 caused less disease infection and colonization in all the cultivars compared to isolates SW65, NW48 and NE17. This is very important information for turfgrass breeders since it suggests that cultivars breed for resistance against one or a few isolates can hold up their resistance may have risks (Diamond and Cooke, 1999). In field condition for a longer period of time where due to genetic shift in the pathogen population, the host resistance tends to break down faster (Chang et al., 2011).

Comparison of the susceptibility test for commercial bentgrass cultivars to *M. nivale* in growth chamber experiments that the growth chamber method is a useful technique to evaluate commercial bentgrass cultivars for susceptibility and resistance to *M. nivale* isolates (Chang et al., 2011).

In this study, these inoculation methods in *M. nivale* have the potential application as easy approach for evaluating resistance in all cultivars/line of pink snow mold on turfgrass.

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벤트그라스 품종이 *Microdochium nivale* 에 의한 자주색설부병에 대한 감수성 차이

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요 약: 벤트그라스 3종류의 품종에 대한 감수성을 잎 조각 검정과 전체식물에 대하여 9개의 *Microdochium nivale* 균주를 이용하여 온도가 조절된 공간에서 평가 하였다. 위스콘신 골프장에서 감염된 병반에서 얻은 자주색 설부병 균주에 대하여 온도와 살균제에 대한 반응시험을 하였다. 잎 조각 검정과와 전체식물을 이용한 시험은 80일된 잔디로 평가하였다. 9균주는 2종류의 살균제에 감수성이 었으며, 온도에 대하여 균주간에 차이를 보였다. 잎조각을 이용한 검정에서도 벤트그라스 품종과 종간에도 뚜렷한 차이를 보였다. 전체식물을 이용한 시험에서도 품종과 종간에 병 심각성에 차이를 보였는데, 특별히 4배수체인 크리핑과 콜로니얼 벤트그라스와 2배체인 벨벳벤트그라스 종과 품종간에 *M. nivale* 균주에 대한 감수성 차이를 보였다. 이 연구는 자주색 설부병에 대한 벤트그라스 평가에 응용될 수 있고, 육종을 하는데도 품종간에 전망적인 예측 평가를 하는데 필요한 자료로 쓰일 수 있을 것이다.

주요어: 잎조각 검정, 2배체, *Microdochium nivale*, 4배체