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Aggressiveness of Three Snow Mold Fungi on Creeping Bentgrass Cultivars under Controlled Environment Conditions

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Snow molds are the most important winter diseases of turfgrass in the United States and Canada. Eight isolates of three snow mold fungal species (three isolates of *Typhula ishikariensis*, three of *T. incarnata*, and two of *Microdochium nivale*) were collected from infected turfgrasses on golf courses. The isolates were evaluated for their relative aggressiveness on three cultivars (L-93, Penncross, and Providence) of creeping bentgrass (*Agrostis palustris*) under the same controlled conditions. Four plant ages (15, 19, 23 and 27 week-old plants from germination to inoculation) were evaluated for their susceptibility to the three pathogens and for the recovery of the plants. Regardless of age or cultivar of the host plant, *M. nivale* was found to be more aggressive and faster to infect and colonize than *Typhula* species. After three weeks recovery, *M. nivale*-inoculated plants showed higher disease severity than plants inoculated with the two *Typhula* species. Plants infected by *Typhula* species displayed no significant difference in disease severity. As creeping bentgrass plants get older, the severity of disease caused by three snow molds gradually decreases. This effect was observed in all cultivars tested, suggesting expression of age-related resistance as the bentgrass plants matured.

Keywords : aggressiveness, creeping bentgrass, pink snow mold, recovery, typhula blight

Snow molds are economically and aesthetically important winter diseases of turfgrass in the northern hemisphere (Hsiang et al., 1999). Three common snow molds exist on golf courses in United States and Canada: speckled snow mold, gray snow mold, and pink snow mold caused by *Typhula ishikariensis* Imai, *T. incarnata* Lasch ex. Fr., and *Microdochium nivale* Samuels and Hallett (teleomorph: *Monographella nivalis* (Schaffnit) Mull, respectively (Chang et al., 2006b; Smith et al., 1989).

Typhula snow molds (also known as Typhula blight) caused by *T. ishikariensis* and *T. incarnata* on turfgrass usually appear as circular, straw-colored patches as the snow melts in early spring. At the spring thaw, these fungi produce black or reddish brown sclerotia, which remain resting structures during the summer, and become primary inoculum sources in the next fall through winter (Hsiang et al., 1999; Vargas, 1994). Meanwhile, the circular patches of *M. nivale* are covered with mycelium after the snow melts and often have pinkish margins due to asexual sporulation (Smith et al., 1989). Ecologically, *Typhula* snow molds predominantly occur after prolonged snow cover, whereas *M. nivale* can be active in cool, wet conditions during late fall and early spring even in the absence of snow cover (Chang et al., 2006b). However, in the presence of snow cover all three snow mold species typically occur as complex patches at the same time and location (Couch, 1995; Vargas, 1994).

In many cases, the patches caused by these snow molds are temporary and the turfgrass is able to recover four to six weeks after resuming growth. Some severe outbreaks of snow molds can kill the turfgrass and will require overseeding or re-sodding. Extensive disease development can delay recovery well into the growing season seriously affecting playability and revenue generation. In spring many snow mold-damaged areas on golf courses are colonized by annual bluegrass (*Poa annua* L.) before the infected turfgrass fully recovers. Snow molds can be a significant factor influencing the rate of annual bluegrass colonization into the preferred stand of turfgrass (Smith et al., 1989).

Unfortunately, differential effects of labeled fungicides at the fungal species level have led to tank-mixed applications of multiple fungicides on golf courses for many years (Hsiang et al., 1999). A consensus on the most effective tank-mixed combinations has not emerged. This disagreement is most likely due to geographic and chronic variability in the disease complexity (Smith et al., 1989). Furthermore, public concerns about the effects of fungicide use on local ecosystems and human health have resulted in restriction on the use of many pesticides (Vargas, 1994).

For these reasons, the optimal means for snow mold control could include the use of resistant turfgrass cultivars (Burpee et al., 1990; Worf, 1988). No report of cultivars

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highly resistant to snow molds has been made (Hsiang et al., 1999), although evaluation of resistance to snow molds has been extensively conducted in bentgrass species (*Agrostis* spp) (Chang et al., 2006a, 2007; Wang et al., 2005). Based on previous reports (Chang et al., 2006a, 2007; Litschko et al., 1987; Simpson et al., 2000; Wang et al., 2005), snow mold resistance of creeping bentgrass (*A. stolonifera* L.) to *Typhula* species or of perennial ryegrass (*Lolium perenne* L.) to *M. nivale* is known to be expressed quantitatively, not qualitatively.

Knowledge of the role of quantitative resistance in promoting recovery from snow mold damage would be valuable (Burpee, 1992). To our knowledge, there is no published study that includes an evaluation of creeping bentgrass cultivar recovery time from snow mold disease. The ability to recover quickly from a turfgrass disease outbreak has been reported to be associated with various factors such as cultivar character, soil moisture, and nutrient status (Burpee, 1992; Vincelli et al., 1997). Vincelli et al. (1997) reported that swift recovery from a disease outbreak could be a valuable varietal trait, particularly if the cultivar possesses inadequate levels of resistance to a particular pathogen.

Despite the occurrence of snow molds as mixed patches on many golf courses (Couch, 1995; Vargas, 1994), a comparison of their aggressiveness on different creeping bentgrass cultivars with regard to bentgrass' recovery from has not been studied. Knowing the recovery ability of creeping bentgrass from snow mold infection may be a key to effectively managing these diseases. In the present study, we evaluate the relative aggressiveness of the three snow mold fungi (*T. ishikariensis*, *T. incarnata*, and *M. nivale*) on three creeping bentgrass cultivars at various plant ages. We also evaluate the recovery ability of plants infected and colonized by the three fungi. Understanding the relative aggressiveness of the fungal species may help breeders to screen breeding materials and germplasms for the resistance of each pathogen with a specific breeding target.

Materials and Methods

Plant materials. Three creeping bentgrass cultivars (L-93, Penncross, and Providence) were used for this study. Plant preparation, maintaining, and hardening were conducted by following the procedures in Chang et al. (2006a). The three cultivars (0.01 g seeds) were evenly sown into a plastic pot (5.3×5.3×5.1 cm) containing commercial potting soil mixture (Metro Mix 366-P, Scott's company, Marysville, OH). 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 weekly with scissors to a height of 0.6 cm from the beginning two weeks after germination until the onset of hardening for acquirement of snow mold resistance of tested plants (Årsvoll, 1977; Gaudet and Chen, 1987). Fertilizer (SunGrow Company, Austin, Texas) was applied at 0.02-0.005-0.02 g (N-P-K) per pot biweekly for four weeks after germination until two weeks prior to acclimation.

Plants were transferred to a controlled environment chamber for acclimation 21 days prior to their scheduled inoculation. Hardening conditions consisted of 10°C and 10-h day length for seven days; 5°C and 8-h day length for seven days; and 2°C and 6-h day length for seven days. The pots were placed into plastic trays containing distilled water to ensure constant moisture.

Fungal isolates and inoculum. Three isolates of *T. ishikariensis* and *T. incarnata*, and two isolates of *M. nivale* were randomly selected from a number of isolates previously collected from golf courses in Wisconsin (Table 1) (Chang et al., 2006a). Inoculum preparation and inoculation were conducted by following the procedure in Chang et al. (2006a). Briefly, five 5-mm diameter plugs were taken from the edge of colonies growing on potato dextrose agar (PDA) and transferred to 20 ml potato dextrose broth (PDB) in plastic Petri dish (diameter 9 cm) and grown for 20 days at 10°C±1 with no light. For each isolate, four to

Table 1. List of snow mold isolates used in this study

No.	Code	Classification	Source
1	NE 10.9.2	*Typhula ishikariensis*	Big Stone Golf & Sports Bar, Three lakes, WI
2	NW 26.9.3	*T. ishikariensis*	Eastwin Valley Par 3 Golf Course, Two rivers, WI
3	NW 69.8.5	*T. ishikariensis*	NorthBrock Country Club, Luxemburg, WI
4	NE 108.8.3	*T. incarnata*	Vernon Hills golf course, Peshtigo, WI
5	SE 57.3.5	*T. incarnata*	Wander Springs golf course, Greenleaf, WI
6	SW 5.4.5	*T. incarnata*	Blackhawk golf course, Janesville, WI
7	SE 25	*Microdochium nivale*	Delbrook golf course, Delavan, WI
8	SW 47	*M. nivale*	Mascoutin golf course, Berlin, WI

six Petri dishes of mycelium were harvested, mixed, and then air-dried for 30 minutes under a laminar flow hood. Residual water in air-dried mycelia was finally removed by vacuum-filtration for 3 minutes under 21 lbs/inch^2 of pressure through cheesecloth. Mycelia were weighed and homogenized in a blender with sterile, distilled water for 30 seconds. The chopped mycelial suspensions were then adjusted to the desired concentration using sterile, distilled water.

Inoculation procedure. Four plant ages (15, 19, 23, and 27 weeks old) of each cultivar were inoculated with 0.1 and 0.3 g/mL mycelial suspensions of *M. nivale* and *Typhula* species, respectively. Sterile pipettes were used to deliver 1 ml of the mycelium suspension directly to the soil surface in the center of each pot. Immediately after inoculation, distilled water was applied to the foliage with a hand sprayer until runoff. Inoculated pots were then placed in a plastic box (70×40×15 cm, Rubbermaid, Wooster, OH) with approximately 30% of the total volume of the box filled with moist potting soil (1 soil : 1 distilled water in volume). The box was covered with a lid to maintain the high humidity required for disease development and then transferred to a controlled environment chamber maintained at 2°C and 0-h day length for 21 days, 5°C and 6-h day length for seven days, and 10°C and 8-h day length for ten days. Randomization of pots and boxes in each replicate were based on a randomized block plot design in ARM 6.1.12 (Gylling Data Management Inc., Brookings, SD).

Disease evaluation and statistical analysis. Progress of disease development was monitored by checking every seven days for five weeks after inoculation. At five weeks after inoculation, disease severity was assessed by a visual estimation of the percentage of diseased areas. After five weeks' post-inoculation, the infected plants were moved to greenhouse for progress of their recovery by assessing the disease severity up to eight weeks after inoculation. This experiment was conducted twice using a randomized block plot design with three replications. Data are analyzed using mean values of the two experiments since no significant difference between the runs (P<0.01) was detected. All statistical analyses were conducted using general linear models procedure (PROC GLM) in SAS 7.1 (SAS Institute Inc., 1999). Mean comparisons were done by using the least significant difference (LSD, P=0.05).

Results

Susceptibility of all three creeping bentgrass cultivars to three snow mold fungal species significantly increased during the three-week incubation period. During incubation,

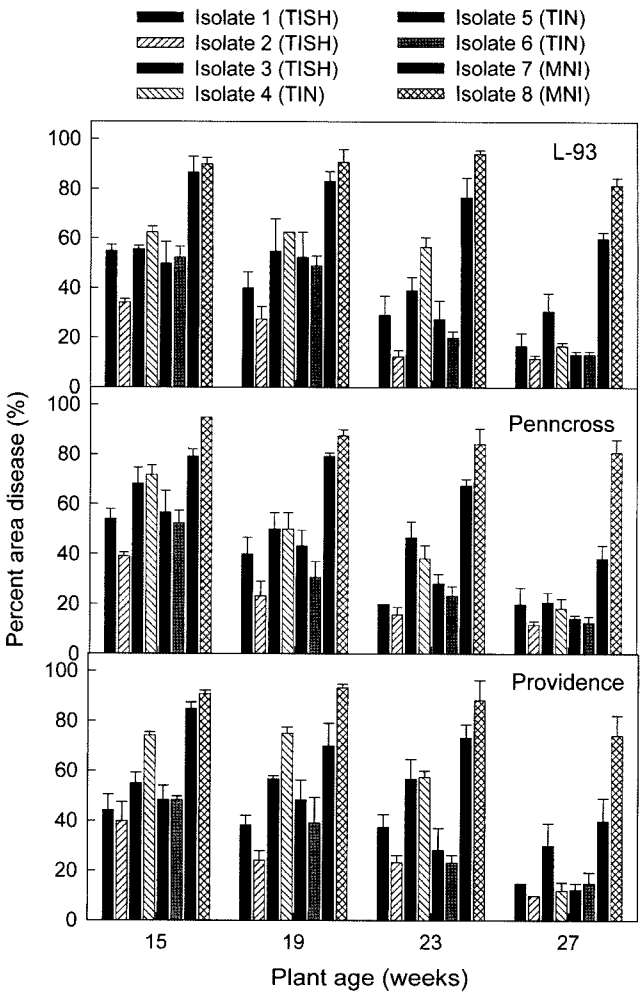

Fig. 1. Disease severity (percent diseased area) of three cultivars of creeping bentgrass (L-93, Penncross, and Providence) inoculated with eight isolates of three snow mold fungi (TISH; *Typhula ishikariensis*, TIN; *T. incarnata*, and MNI; *Microdochium nivale*) in the growth chamber. Plants with four ages (15, 19, 23 and 27 week-old plants from germination to inoculation) were inoculated. Bar represents standard error of the mean.

the mycelium of the fungi caused water-soaked symptoms on the leaves of infected plants. Infected and colonized plants were matted or appeared slimly with mycelium. Eventually, the infected plants were killed as the plants dried. *T. incarnata* and *T. ishikariensis* produced tiny brown and black sclerotia, respectively on the leaf sheaths of infected plants.

Varying levels of aggressiveness were observed among eight isolates of the snow mold fungi to the creeping bentgrass cultivars (Fig. 1 and Table 2). The ANOVA of the disease severity showed highly significant effects of fungal genus, and isolate within species on the disease progress of those cultivars. *M. nivale* isolates were found to be much more aggressive than ones of *Typhula* species regardless of plant ages. There was no significant difference in disease

Table 2. Analysis of variance for snow mold severity (percent diseased area[a]) on three cultivars (L-93, Penncross, and Providence) of creeping bentgrass inoculated with eight (three *Typhula ishikariensis*, three *T. incarnata*, and two *Microdochium nivale*) isolates in the growth chamber

Source of variation	df	Mean square	*F* value	*P*
Experiment	1	2.5	0.08	0.7757
Replication (Experiment)	2	3.4	0.11	0.8945
Genus	1	89,182.5	2,892.3	<0.0001
Species (genus)	1	0.19	0.01	0.9379
Isolate (species)	5	4,666.4	151.3	<0.0001
Cultivar	2	58.0	1.88	0.1533
Age (cultivar)	6	101.8	3.30	<0.01
Cultivar × isolate (species)	14	65.1	2.11	<0.05
Age × isolate (species)	21	262.3	8.51	<0.0001
Error	519	30.8	–	–

[a]Rated five weeks after inoculation.

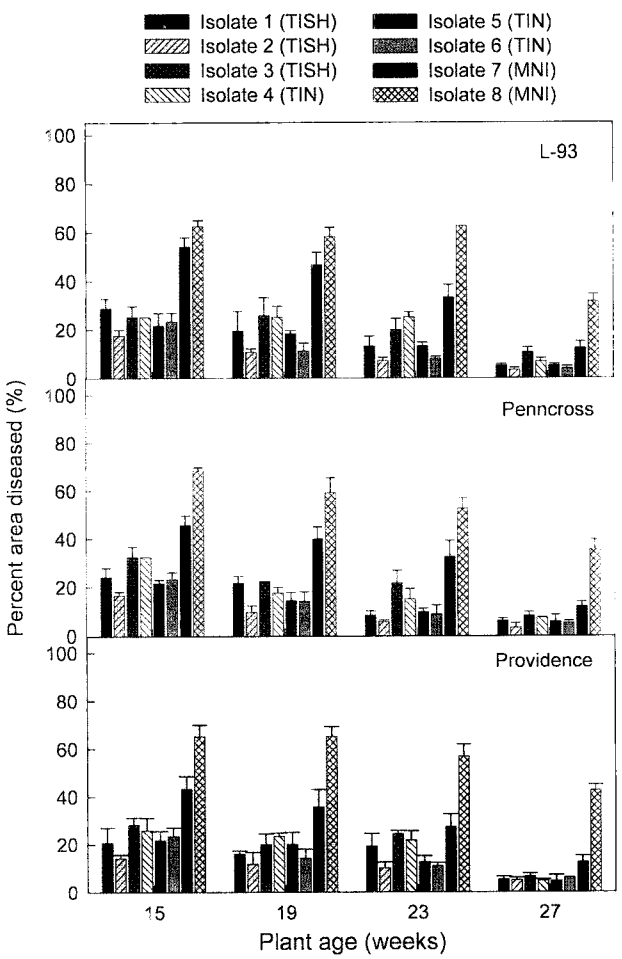

Fig. 2. Disease severity (percent diseased area) after recovery period of three cultivars of creeping bentgrass (L-93, Penncross, and Providence) inoculated with eight isolates of three snow mold fungi (TISH; *Typhula ishikariensis*, TIN; *T. incarnata*, and MNI; *Microdochium nivale*) in the growth chamber. Plants with four ages (15, 19, 23 and 27 week-old plants from germination to inoculation) were inoculated. Bar represents standard error of the mean.

severity between the both *Typhula* species. Within each species, a significant difference in the aggressiveness was observed among isolates. Isolates #3 of *T. ishikariensis*, #4 of *T. incarnata*, and #9 of *M. nivale* were more aggressive to creeping bentgrass cultivars than the other isolates of the same species tested.

The ANOVA of disease susceptibility showed significant effects of plant age (cultivar), and interactions of cultivar× isolate (species), and plant age×isolate (species) (Fig. 1 and Table 2). Cultivars were less susceptible to three snow mold fungi when older plants were challenged. Disease susceptibility was lowest in 27 week-old plants of the cultivars.

All tested cultivars gradually recovered during a recovery period of three weeks. After the three weeks' recovery, disease severity caused by *M. nivale* isolates was still significantly higher than those of two *Typhula* species (Fig. 2). However, we did not detect any difference between the *Typhula* spp. (Table 3). Among four different plant ages tested in our growth chamber experiements, 27 week-old plants showed the lowest susceptibility in all cultivars tested. Recovery progress of the infected plants had a similar trend in all ages and cultivars.

Discussion

This is the first study comparing relative aggressiveness among three snow mold fungal species on creeping bentgrass cultivars under the same controlled growth chamber conditions. The findings will help breeders design field evaluations of breeding materials and germplasms for resistance to specific pathogens. Among the three snow mold pathogens, isolates of *M. nivale* showed a higher level of aggressiveness than the two *Typhula* species, even at a lowest concentration (0.1 g/mL mycelial suspension) of an inoculum. In our preliminary study, plants inoculated with

Table 3. Analysis of variance for snow mold severity (percent diseased area[a]) after recovery period (three weeks) of three cultivars (L-93, Penncross, and Providence) of creeping bentgrass inoculated with eight isolates of three snow mold fungi (three isolates of *Typhula ishikariensis*, three of *T. incarnata*, and two of *Microdochium nivale*) in the growth chamber

Source of variation	df	Mean square	*F* value	P
Experiment	1	69.4	2.09	0.1493
Replication (Experiment)	2	12.6	0.38	0.6848
Genus	1	88924.1	2670.3	<0.0001
Species (genus)	1	85.6	2.51	0.1138
Isolate (species)	5	4556.9	136.8	<0.0001
Cultivar	2	117.5	3.53	<0.05
Age (cultivar)	6	178.2	5.35	<0.0001
Cultivar × isolate (species)	14	96.8	2.91	<0.001
Age × isolate (species)	21	281.8	8.46	<0.0001
Error	519	33.3	–	–

[a]Rated eight weeks after inoculation.

M. nivale showed too fast disease development at 0.2 and 0.3 g/mL mycelial suspension, thereby we couldn't use the same concentration as ones used for *Typhula* species (data not shown).

This might be due to the wide range of temperatures over which the *M. nivale* is active (Couch, 1962; Lebeau, 1964; Nakajima and Abe, 1990). The optimal temperature range for disease development of *M. nivale* is 0-7.2°C (Couch, 1962). Lebeau (1964) reported *M. nivale* as a dominant pathogen on turfgrass at 3°C. Meanwhile, both *Typhula* species favored lower temperatures than *M. nivale* (Smith, 1981). Another possible explanation is the association of high pathogenicity of *M. nivale* on creeping bentgrass without snow cover. In nature, severe damage by *Typhula* spp. can only occur in areas with a long duration of snow cover, since plants weaken due to carbohydrate depletion, where as *M. nivale* can be active regardless of snow cover (Smith et al., 1989). The findings may indicate that *M. nivale* is able to infect and colonize healthier plants than *Typhula* spp. The dissimilarity of aggressiveness and infection pattern of both groups may be caused by a different phylogenetic origin.

Despite a significant variation in aggressiveness among isolates within each *Typhula* species, no significant difference in disease severity between *T. incarnata* and *T. ishikariensis* isolates was observed. The phenomenon differed from reports by Matsumoto (1994) and Arsvoll (1977) stating that *T. incarnata* is considered less virulent than *T. ishikariensis* in winter wheat or timothy (*Phleum pretense* L) and fescue (*Festuca pratensis* L). Additional study of the aggressiveness over variety of isolates and environmental conditions is necessary to further our understanding of the fungi.

Within each species, the isolate effect was noted at $P<0.0001$. This result is supported by our previous study (Chang et al., 2006a, 2007) which concluded that variation in aggressiveness among isolates of *Typhula* snow mold fungi was found in creeping bentgrass cultivars. This suggests that the variation within *Typhula* species might be caused by high rates of outcrossing and sexual recombination (Bruehl and Machtmes, 1978; Vergara et al., 2004) and by spontaneous mutation of asexual structure such as mycelium (De Visser and Rozen, 2005). The extent and frequency of sexual reproduction of these species in nature is unknown, although sexual reproduction in *Typhula* species has been demonstrated under controlled conditions (Chang et al., 2008; Yang et al., 2006). The isolate effect of *M. nivale* may arise from different factors within *Typhula* species. Therefore, the variation of *M. nivale* might be due to the migration or dissemination of propagules such as vegetative mycelia. Mahuku et al. (1998) suggested that the sexual structures are likely playing important roles in maintaining high genetic variability within population of *M. nivale*.

Significant interactions between Cultivar×Isolate (Species) or Plant age×Isolate (Species) were observed in this experiment. This is likely due to an aggressive pattern of isolates within each *Typhula* species. For example, isolates #1 and #3 within *T. ishikariensis* and #5 and #6 within *T. incarnata* showed similar aggressiveness to three bentgrass cultivars tested.

A cultivar effect was not found in disease responses of three creeping bentgrass cultivars to the three snow mold fungi. This result was similar to our previous conclusion (Chang et al., 2006a) that disease severity among creeping bentgrass cultivars to *T. ishikariensis* was not significant. However, the three cultivars showed inconsistent ranking, as represented by Cultivar×Isolate (Species) in Fig. 1. We could not make the distinction of the differential interaction because snow mold symptoms were insufficient to cause

differential reactions between bentgrass cultivars and snow mold fungi.

As expected, the three bentgrass cultivars became more resistant to the three snow mold fungi as plants grew older (Fig. 1). According to the report of Chang et al. (2006a), older bentgrass plants expressed higher resistance to snow mold than young plants similarly acclimated under controlled conditions. In addition, the expression of age-related resistance has been found in the interaction of many snow mold pathogens and grasses including turfgrass (Arsvoll, 1977; Chang et al., 2006a; Gaudet and Chen, 1987; Nakajima and Abe, 1996). This expression is recognized to be associated with the rapid accumulation of high levels of carbohydrates (Bruehl, 1982; Gaudet et al., 1999) and slower metabolization of the carbohydrate, including fructan (Kiymoto and Bruehl, 1977; Yoshida et al., 1997) in old plants.

After three weeks' recovery, bentgrass plants infected and colonized by the snow molds showed quick recovery despite extensive damage. Wang et al. (2005) stated that diseased creeping bentgrass clones dramatically recovered from the damage. This appears to be due to the actively stoloniferous growth of creeping bentgrass, which is also promoted by cutting. After moving the diseased plants to greenhouse, we mowed the plants weekly under favorable growing conditions (18 to 28°C with light and dark cycle of 16 and 8 hours). After recovery, the ranking of disease severity between *M. nivale* and two *Typhula* species was not different from that of two fungi before recovery, although the difference decreased. This suggests that *M. nivale* induced more serious injury in the crowns and roots as well as leaves than *Typhula* snow molds.

No difference of disease severity in bentgrass cultivars between the *Typhula* species was observed after a recovery period of three weeks. We speculate that the two species might have similar infection sites on bentgrass plants.

The recovery progress from snow mold damage was generally faster in the older plants than the younger plants (data not shown). This may be due to the more advanced crown development of the older plants, which resulted in partial infection. Therefore, fall seeding on golf courses should be carried out in early fall. This will allow new seedlings to go into the winter months on a more mature state and allow for a more winter hardy plant. This phenomenon can be used to manage turf on golf courses, because overseeding or resodding is a required practice on many golf courses. More research including field evaluations is required to understand the interaction between snow mold fungi and creeping bentgrass species.

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