SCREENING ECOTYPES OF *POA ANNUA* VAR. *REPTANS* FOR SUSCEPTIBILITY TO PINK SNOW MOULD CAUSED BY *MICRODOCHIUM NIVALE*

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ABSTRACT

Screening ecotypes of *Poa annua* var. *reptans* for susceptibility to pink snow mould caused by *Microdochium nivale*

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Annual bluegrass (*Poa annua)* ecotypes collected from golf course putting greens were evaluated for resistance to pink snow mould (*Microdochium nivale)* under natural and controlled conditions. Field plots consisted of thirty ecotypes transplanted into existing creeping bentgrass (*Agrostis stolonifera*) putting greens in the fall, which allowed the turf to cold acclimate. Inoculation occurred in early winter. Weekly visual evaluation conducted immediately after snowmelt over six weeks revealed significant differences in the ability of the ecotypes to resist pink snow mould attack and to recover after snowmelt. Cold chamber plots consisted of the same ecotypes used in the field study plus two bentgrasses transplanted into tubes. The ecotypes were acclimated in a cold chamber before they were inoculated with infested wheat bran. Visual evaluation during the incubation period revealed significant differences in the ability of the ecotypes and the bentgrasses to resist pink snow mould attack.

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Environmental Concerns and the Golf Industry

Over the last several years, the golf industry has been enjoying incredible growth. According to a survey by the Royal Canadian Golf Association in 2002, there are more than 2100 golf courses in Canada and more than 4.95 million golfers. Approximately 20 new golf courses are constructed in Ontario annually (Ted MacIntyre, Editor, Ontario Golf Magazine, 2003, personal communication). Increasing interest in the game of golf, coupled with the increase in competition between golf courses, has created a demand for improved aesthetics and playability of putting greens. Golfers demand superior quality turf that provides a uniform playing surface and they do not accept declining turf quality due to environmental stresses, including pest problems. This is reflected in the frequency and intensity of management practices performed by golf course turf managers to maintain putting green turf, including regular pesticide applications.

There are public concerns about pesticide use and its effects on surface and ground water, human health, and wildlife. With the first ban on aesthetic pesticide use implemented in Hudson, Quebec in 2001, municipal pesticide bans are becoming a factor that golf course turf managers across Canada must consider. Hudson By-law 270 was challenged by a Lawn Care company, but the By-law was upheld by the Supreme Court of Canada. According to the By-law, pesticide use is "prohibited for purely aesthetic use … to minimize the use of allegedly harmful pesticides in order to promote the health of its inhabitants" (Anonymous, 2001). Other municipalities across Canada, including Toronto, have implemented similar pesticide bans.

In Quebec, the new Pesticides Management Code Regulations, which came into effect on April 3, 2003, obliges all golf courses to present a pesticide reduction plan to the Quebec Ministry of the Environment. This plan must be signed by an

agronomist member of OAQ (Ordre des Agronomes du Quebec) and must be sent to the Ministry every three years starting April 2, 2006. The Pesticide reduction plan must contain recommendations formulated by an agronomist to obtain the established percentage pesticide reductions over a three-year period (Anonymous, 2005)

Increasing concerns about pesticide levels in the environment and the threat of municipal pesticide bans has made the use of stress-resistant turf species of critical importance. Since 1982, the USGA has sponsored turfgrass breeding programs directed toward significantly reducing pesticide use (Kenna and Snow, 2000). Fungicide applications are expensive, have limited effectiveness, and may adversely affect the environment (Stier et al., 2003). Disease-resistant turf cultivars reduce the need for pesticide use. Turfgrass improvement efforts are an integral part of Integrated Pest Management (IPM) strategies, thus, new varieties with better abiotic and biotic stress resistance will have a positive role in IPM practices (Kenna and Snow, 2000).

Annual Bluegrass (*Poa annua* L.)

I) Establishment on Golf Greens

Currently, the turf species of choice for golf course greens establishment in Canada is creeping bentgrass (*Agrostis stolonifera* Huds.). Creeping bentgrass withstands traffic and tolerates extremely low mowing heights to provide a suitable putting surface (Eggens, 1998). Coexistence of creeping bentgrass and annual bluegrass (*P. annua*) on a putting green is governed by cyclic changes, such as the annual reproductive cycle of annual bluegrass (Cline et al., 1993). Annual bluegrass is favoured by cool weather during the spring and fall. Creeping bentgrass is favoured during mid-summer following seed production in annual bluegrass (Cline et al., 1993). Intensively managed greens are more susceptible to damage caused by turf equipment and golfers than other less

intensively managed areas of the golf course (Lush, 1988a). Disturbed areas of the green resulting from biotic (e.g. disease) and abiotic (e.g. traffic) stresses are quickly colonized by the opportunistic annual bluegrass. This turfgrass flowers and germinates all year around and regenerates readily from self-sown seed (Lush, 1988b). Annual bluegrass also seeds profusely, which is characteristic of a plant adapted to disturbed habitats (Lush, 1988a). Annual bluegrass is an undesirable invader because it has a lighter green colour and an upright growth habit (Lush, 1988a) which blends poorly with the bluish-green colour and prostrate growth habit of creeping bentgrass. It also produces an abundance of seed heads in flushes throughout the season (Lush, 1988a), and these seedheads can be produced below the height of mowing. The presence of annual bluegrass seedheads disrupts the uniformity of the putting surface and the aesthetic quality of the green. Susceptibility to injury by environmental stresses causes death or dormancy of annual bluegrass leaving greens patchy, which further affects the uniformity and the aesthetic quality of the green.

Annual bluegrass is generally susceptible to environmental stresses (Beard, 1970). Abiotic stresses include temperature extremes, ice damage, drought, and winter desiccation. Biotic stresses include several diseases (and their causal agents) such as anthracnose (*Colletotrichum graminicola*), brown patch (*Rhizoctonia solani*), dollar spot (*Sclerotinia homoeocarpa*), pink snow mould/Fusarium patch (*Microdochium nivale*), Pythium blight (*Pythium* spp.), red thread (*Laetisaria fuciformis*), and gray snow mould/Typhula blight (*Typhula* spp.); and many insect pests such as *Aetaenius spretulus* grubs, chinch bugs (*Blissus* spp.), grubs of the beetles belonging to the family *Scarabaeide*, sod webworm (*Crambus* spp.), turfgrass weevil (*Hyperodes* spp.), and cutworms (belonging to the family *Noctuidae*) (Beard et al., 1978).

Annual bluegrass thrives under shady, moist, cool conditions and continues to gain a competitive edge against creeping bentgrass until it ultimately dominates many golf green putting surfaces (Huff, 1996). When the performance of a pure

stand of annual bluegrass is observed, it is seen to have many qualities that make it a good turf choice. It forms a thick stand because of its dense growth and fine leaf texture (Piper and Oakley, 1927, Juska and Hanson, 1969). Leaf texture becomes finer and density increases as mowing heights are decreased (Beard et al., 1978). The resulting putting surface is smooth and uniform, which is the goal of the turf manager. However, golf courses managers who have annual bluegrass greens do not have an adequate seed source for the types of annual bluegrass that are adapted to their golf green environments for use in routine overseeding, repair work, or new green establishment (Huff, 1996).

III) **Origin and Diversity**

The Mediterranean is the hypothesized center of origin for annual bluegrass (Mitich, 1998). From there, it spread throughout temperate and alpine regions of the world (Beard, 1970; Mitich, 1998). Annual bluegrass was introduced to North America from Europe (Mitich, 1998). It is ubiquitously distributed and widely adapted to diverse habitats (Mitich, 1998), which explains its success as an invader. The high genotypic variability allows the potential to be a successful weed (Mitich, 1998). Life strategy of annual bluegrass varies from annual to perennial with all degrees of variation between these two forms (Beard, 1970).

Annual bluegrass originated as a result of a cross between *Poa supina* Schrad. x *Poa infirma* HBK (Tutin, 1957). Both parents are diploid species with 14 chromosomes (2n=2x=14) (Huff, 1996). Due to the differences in chromosomes between the two species, the resulting hybrid was sterile (1n=2x=14) (Huff, 1996). Fertility was restored by doubling of chromosomes to produce the polyploid annual bluegrass (2n =4x=28) (Huff, 1996). Hybridization and chromosome doubling events generated high levels of genetic variability (Huff, 1996).

Evidence for genetic variability of annual bluegrass can be established when comparing neighboring populations in the environment. Morphological and physiological variation exists between annual bluegrass populations found on golf greens and those found in less intensely maintained areas of the golf course (Lush, 1989). Differential selection pressures caused divergence of annual bluegrass populations into ecotypes exhibiting differences in reproductive strategies (Johnson et al., 1993; Lush, 1989).

Populations found in lower maintenance areas (rough) around the green exhibit a short vegetative stage, after which the plants produce numerous inflorescences (Johnson et al., 1993). Inflorescences have a spreading morphology making them more open to airborne pollen, which encourages outcrossing (Johnson et al., 1993). Populations found on putting greens have a longer vegetative growth stage and a shorter, more determinate flowering habit (Lush, 1989). Flowers are smaller, more compact, and tightly arranged (Johnson et al., 1993). Pollination occurs earlier on the green than in the rough, which limits pollen exchange (Johnson et al., 1993). The morphology of inflorescences on putting greens and early pollination favour self-pollination (Johnson et al., 1993). Fertilization can also occur before florets ever open (cleistogamy) in greens populations. As a result, certain plant habits have become fixed in many biotypes (Johnson et al., 1993).

Seed production is prolific in populations found in the rough (Johnson et al., 1993) and germination occurs in flushes in response to temperature (Lush, 1989,). Populations found on greens produce fewer and smaller seeds, which are capable of germinating at any time (Lush, 1989). Populations in rough areas exhibit an annual life cycle, while populations found on greens tend to be more perennial (Johnson et al., 1993, Lush, 1989).
Differences in vernalization requirements also highlight genetic variation within this species. Vernalization refers to low temperature requirements for vegetative meristems to become reproductive (Mahfoozi et al. 2001). It acts as an

environmental trigger to synchronize flowering to the spring season and promote vegetative growth the rest of the growing season. A study by Johnson and White (1997) revealed significant variation in responses to vernalization treatments among perennial-type populations. The length of the vernalization period varied among ecotypes, as well as resulting reproductive development. Populations requiring a shorter vernalization period sometimes produce multiple flowering culms. The number of inflorescences influences the ultimate seed yield of the population. Breeders need to know vernalization requirements to determine the flowering pattern of a population in order to synchronize flowering for cross pollination (Johnson and White, 1997).

Genetic diversity is also found when comparing freezing tolerance among populations of annual bluegrass. Studies by Dionne et al. (2001a, b) examined freezing tolerance of annual bluegrass ecotypes collected from golf greens in regions with varying winter climates. Results showed significant differences in the ability of these ecotypes to tolerate freezing stress (Dionne et al, 2001a). Further analyses indicated that variation in cold tolerance was related to the accumulation of specific proteins, which also varied between ecotypes (Dionne et al., 2001b). These differences in freezing tolerance and in biochemical traits observed between ecotypes may be the result of genetic adaptation to their specific growing conditions (Dionne et al., 2001b).

Thus, annual bluegrass is a highly variable species which can adapt to widely differing environmental conditions. Adaptation of annual bluegrass to its microenvironment occurs by natural selection for existing ecotypes, or by the development of new ecotypes through cross-pollination between genetically distinct plants (Beard et al., 1978).

IV) Evolution of *Poa annua* on Golf Greens

The populations found on putting greens may have originated from seed dispersed from populations found in lower maintenance areas of the golf course or from outside sources such as contaminated soil mixture or seed (Sweeney and Danneberger, 1995). Selection forces of turf management act through both physiological and developmental constraints and guide evolution to localized biotypes (Wu and Harivandi, 1993). Evolution occurs in response to management selection pressures, such that genotypes that tolerate intense management are favoured. Populations on putting greens are primarily self-pollinating, which further restricts gene flow. Over time, the ecotypes found on putting greens become more perennial and can survive multiple growing seasons (Beard et al., 1978). These ecotypes harbor desirable aesthetic qualities for golf greens and are uniquely adapted to the environmental conditions and management methods to which they are exposed (Huff, 2004).

A large genetic variability for various vegetative and reproductive traits was shown to exist between adjacent populations of annual bluegrass found on greens and roughs (Lush, 1989). This is caused by the strong selection pressure found in the microenvironment of the green that leads to the divergence of adjacent populations (Sweeney and Danneberger, 1995). Such divergence occurs when the frequency of adaptive alleles increases in only one of the populations. Over time, only a few individual genotypes may be able to survive the harsh management conditions of a golf green and the original population may experience genetic drift (Sweeney and Danneberger, 1995). For example, the ability of an ecotype of annual bluegrass found on a golf green to produce seed heads below the height of mowing is an important trait to ensure the persistence of the population. Mowing also limits the introduction of genes from outside sources by removing seed heads that could come into contact with airborne pollen. The population becomes reliant on self-pollination, so the genes linked to the production of short seed heads is conserved in subsequent generations.

Ecotypes found in less intensely maintained areas of the golf course are mown at higher heights, so the height of seed head production is less constrained. As strong selection continues, gene flow from one population to the other may be restricted and the populations may diverge resulting in genotypically diverse populations (Barrett and Husband, 1990). Turf management practices and physical environmental conditions act as selection forces to maintain genetic differences among populations (Wu and Harivandi, 1993). Management alters the environment to favour the genotype best adapted to that management (Hanson and Juska, 1969). Mowing provides a gradient of intensity of management (Wu and Harivandi, 1993), which drives evolution within the turf population.

Annual bluegrass populations found on golf greens are isolated from the original population, since most of the pollination occurs as selfing. Sweeny and Danneberger (1995) used Random Amplified Polymorphic DNA (RAPD) markers to estimate phenotypic diversity between green and fairway populations. They found that 4 of 5 markers varied between the populations. Their results suggest that populations have diverged for neutral loci, and that gene flow between populations may be limited. (Sweeney and Danneberger, 1995).

After every generation of selfing, the number of heterozygotes in the population is reduced by 50% (Huff, 1996). Populations found on older golf greens have already evolved into a turf type with a more perennial life cycle as the population persists over subsequent growing seasons (Beard, 1970). There is some disagreement whether the new population is a true perennial or a succession of overlapping populations originating from the seed bank (Lush, 1989). Either way, the new population persists on the green. This evolved type has been classified by some as *P. annua* var. *reptans* (Hauskn) Timm, and the annual biotype has been referred to as *P. annua* var. *annua* (L) Timm.

Annual bluegrass is a successful invader because it has evolved very effective survival strategies (Cline et al., 1993). Evolutionary change has been very rapid which is why annual bluegrass is such a widespread weed in various landscapes; this also suggests that selection for better turfgrass characters is possible (Wu and Harivandi, 1993). Ecotypes are the product of one or more genotypes interacting with the environment and altered by management (genotype x management x environment) (Hanson and Juska, 1969). Diversity in environments and management methods result in ecotypes that are unique to the golf green on which they are found. These ecotypes contain germplasm that may contribute desirable traits to a turf breeding program.

Fluctuations in allele frequencies can occur over time due to environmental effects (Sweeny and Danneberger, 1995). Infection by disease exerts strong selection pressure on the turf during evolution resulting in ecotypes that may become more resistant. Thus, it should be theoretically possible to identify ecotypes that have evolved on golf greens that are less susceptible to pink snow mould (David Huff, personal communication, 2003).

Pink Snow Mould

Pink snow mould is one of the most common and damaging diseases affecting overwintering turfgrass in temperate and alpine climates (Smith et al., 1989). The causal agent, *Microdochium nivale* (Fries) Samuels and Hallett, grows under snow cover and the disease is not apparent until snow recedes in spring (Hsiang, 1996). Turf is weakened or killed by snow mold which allows weed invasion or secondary infections by other pathogens (Smith et al., 1989). More details on this pathogen are provided below.

I) Symptoms

The development of *Microdochium nivale* in the field is favoured by high humidity and ambient temperatures between 0-7^{o}C (Tani and Beard, 1997, Couch, 1973). This pathogen may also develop in temperatures up to 18^{o}C under persistent drizzle or foggy conditions in the absence of snow cover (Tani and Beard, 1997). At low temperatures, turf grows more slowly and is more vulnerable to attack by the fungus (Vargas, 1994). There is also less competition from antagonistic fungi at low temperatures (Vargas, 1994; Hsiang et al., 1999). The disease symptoms caused by *M. nivale* occur in two forms: in the presence of snow cover, large bleached circular patches appear and may be covered with white mycelium (Hsiang, 1996; Smith et al., 1989); in the absence of snow cover, patches appear as smaller circular, water-soaked patches with a yellow to orange-brown colour which turns tan (Tani and Beard, 1997). Pink snow mould is the name given to the disease that becomes apparent after snowmelt. The symptoms that are observed in the absence of snow cover such as cool, wet weather in the fall and spring are referred to as Fusarium patch (Tani and Beard, 1997; Vargas, 1994; Smith et al., 1989; Couch, 1973). Although the American Phytopathological Society recommends the using the disease name pink snow mould for all the symptoms elicited by *M. nivale* on turgrass, pink snow mould and Fusarium patch do refer to different types of symptoms under different environmental conditions, and these names are in general use by turf managers to refer to the different symptoms.

Patches of pink snow mould range in size from 10-20 cm and often overlap to form large irregular patches (Couch, 1973; Hsiang, 1996; Smith et al., 1989). Patches will turn pink, especially at the margins, when exposed to sunlight (Smith et al., 1989; Tani and Beard, 1997). The hyphae assume a pink colour and pink sporodochia of the fungus develop after exposure to light (Dahl, 1933). Margins of patches may turn an orange-brown tone under moist conditions due to continued activity of *M. nivale* (Smith et al., 1989). Surviving turf plants in the

center of patches resume their growth when temperatures warm up and optimal conditions for pathogen development no longer exist (Smith et al., 1989).

II) Disease Cycle

Microdochium nivale survives warm, dry periods as dormant mycelium in diseased leaf tissue and thatch (Vargas, 1994; Couch, 1973; Hsiang, 1996; Smith et al., 1989; Tani and Beard, 1997). In autumn, with the occurrence of proper environmental conditions, spores may germinate or mycelium may grow and infect leaves (Sears et al., 1996). The resulting infection is characteristic of Fusarium patch with reddish-brown spots in the turf (Vargas, 1994; Tani and Beard, 1997). Conditions are particularly conducive to the development of pink snow mould in the presence of snow over unfrozen ground (Couch, 1973). After snow recedes, infection may continue during the spring and cause more symptoms of Fusarium patch (Vargas, 1994; Tani and Beard, 1997). Once temperatures rise and conditions become drier, the fungus becomes dormant. Disease is usually present in the same areas during subsequent years (Sears et al., 1996). Conidial production occurs soon after the development of optimum environmental conditions at the margins of diseased patches (Couch, 1973; Smith et al., 1989). Ascospores, produced by sexual reproduction, are rarely reported in research (Lees et al., 1995). However, high levels of genetic variability found among isolates of *M. nivale* indicate that sexual reproduction and dissemination of propagules does occur (Mahuku et al., 1998).

III) Microdochium nivale (Fries) Samuels and Hallett

The fungus *Microdochium nivale* was formerly known as *Fusarium nivale* (Smith et al., 1989). However, it has been established that it is not a true *Fusarium* species since it has annellated conidiogenous cells rather than phialides and has a different teleomorph than true *Fusarium* species (Seifert, 1996). *M. nivale* is the anamorph while the teleomorph is *Monographella nivalis* (Schaffnit) E. Muller

(Smith et al., 1989; Smiley et al., 1992). Several other names have been used to describe the anamorph, but *Microdochum nivale* is the most recent (Smith et al., 1989).

Isolates vary in morphology in culture (Smith et al., 1989). Isolates of *M. nivale* from cereals have been divided into two varieties (var. *majus* and var. *nivale)* based on conidial morphology (Gams and Muller, 1980 *in* Litschko and Burpee, 1987; Mahuku et al., 1998). Litschko and Burpee (1987) could not differentiate between turf and wheat isolates based on conidial morphology, conidiogenesis, response to fungicide applied *in vitro*, or asexual compatibility. Lees et al. (1995) used molecular, biological, and physiological markers to assess genetic diversity among isolates of *M. nivale* from winter wheat. Random amplified polymorphic DNA (RAPD) analysis distinguished two distinct groups which supported groupings based on width of conidia. RAPD profiles showed a high level of uniformity in the var. *majus* group and a greater degree of variation in the var. *nivale* group. Mahuku et al. (1998) assessed genetic diversity of turfgrass isolates using RAPD profiling and restriction fragment length polymorphisms (RFLP) analysis of the internal transcribed spacer (ITS) region and the intergenic spacer (IGS) region of rDNA. Restriction digestion of the amplified ITS region revealed that all isolates collected from turfgrass belonged to var. *nivale*. RFLP analysis demonstrated variation within and between populations and distinguished 60 distinct genotypes from 100 isolates. Genetic distance analysis provided evidence for the occurrence of host specialization (Mahuku et al., 1998). Isolates of *M. nivale* found on turfgrass had very high levels of genetic diversity (Mahuku et al., 1998). Sexual reproduction has been demonstrated under controlled conditions (Lees et al., 1995), but the extent and frequency in nature is unknown. The high levels of genetic variability found within populations imply that recombination or sexual reproduction and migration or dissemination of propagules occurs among turf isolates of *M. nivale* (Mahuku et al. (1998).

Mycelium of *M. nivale* is white, mats leaf blades together, and gradually turns a pink colour (Smith et al., 1989). In contrast to pink snow mould, aerial mycelium of Fusarium patch is more cobwebby and sparse; stretching from leaf to leaf, especially at the borders of patches (Smith et al., 1989). *In vitro,* cultures grow in temperatures between 0^{o} to 30^{o}C, with an optimum temperature of 22^{o}C (Dahl, 1933). The infection process was described by Dahl (1933) as follows: "mycelium develops along the outside of the leaf; when a hypha reaches a stoma, it enlarges at the terminal end and one or more infecting hyphae develop down through the stomatal opening into the intercellular spaces; progress through the tissue is intercellular until the cells begin to collapse, then it becomes intracellular as cells collapse and the fungus almost fills the space; the fungus then penetrates into the vessels". Conidia are borne on salmon-pink sporodochia which can be found on leaves and suspended on mycelium under moist conditions (Smith et al., 1989). Sporodochia develop through the stomata in rows (Dahl, 1933). Conidia are curved and may be 1- to 5-septate, depending on the strain, and lack a foot cell typical of *Fusarium* species (Smith et al., 1989; Smiley et al., 1992). Brief exposure to light in culture initiates sporulation (Smith et al., 1989). Turf isolates produce abundant loose or dense white or pink-coloured mycelium (Smith et al., 1989) that lack clamp connections (Smiley et al., 1992). Perithecia of the teleomorph are oval, papillate, and appear as black dots, but could not be induced in turfgrass isolates in culture (Litschko and Burpee, 1987; Smith et al., 1989). Asci are parallel-walled or spindle-shaped, and ascospores are hyaline, 2-4 celled, usually bearing 8 ascospores (Smith et al., 1989).

IV) Epidemiology

Although *M. nivale* is rarely isolated directly from soil, it is regarded as a soil-borne pathogen (Smith et al., 1989). The fungus is also seed-borne in cereals and grasses (Smith et al., 1989; Tani and Beard, 1997) and is capable of surviving on infected cereal straw for up to one year (Smith et al., 1989). Most

infection of grass seedlings and mature turf occurs from soil-borne or debris-borne mycelium (Smith et al., 1989). Sticky conidia or mycelium in leaf or soil fragments are dispersed by wind, water (rain splash, water films), mechanical operations (brushing, aerating, mowing), or pedestrian traffic (Smith et al., 1989). Wind dispersal of ascospores is not likely because perithecia are not yet shown to occur naturally (Couch, 1973; Smith et al., 1989), although Mahuku et al. (1998) found high levels of genotypic diversity within populations and low genetic differentiation among populations suggestive of sexual reproduction in Ontario populations of *M. nivale*.

Isolates are active over a wide range of temperatures which explains the wide geographical range of *M. nivale* as a pathogen on turf (Smith et al., 1989). Conditions that favour pink snow mould are poor drainage, high N fertility, matted leaf blades, thick thatch, soil pH above 6.5, persistent precipitation, temporary snow cover, heavy dews during clear weather when temperatures are low, alternate thaw and snow cover, and conditions under snow on unfrozen ground (Smith et al., 1989; Smiley et al., 1992; Vargas, 1994; Tani and Beard, 1997). Intermittent short periods of low temperatures during wet weather are more likely to predispose turf to attacks by *M. nivale* than continuous cold spells (Smith et al., 1989). Rate of spread is very slow when humidity or surface moisture is low (Smiley et al., 1992). *Poa annua* and *Agrostis* spp. suffer the most severe infections, but *P. pratensis* L., *Festuca*, and *Lolium* spp. also suffer damage (Braverman, 1986; Smiley et al., 1992; Vargas, 1994).

V) Management

Cultural management includes moisture control, fertility control, and use of resistant species or cultivars. Surface drainage and dew removal help prevent pink snow mould development (Smith et al., 1989; Tani and Beard, 1997). Improved air drainage helps turf surfaces to dry and autumn removal of organic material such as leaves also helps keep turf surfaces dry (Smith et al., 1989;

Tani and Beard, 1997). Management procedures that help control pink snow mould are the maintenance of low soil pH and of balanced soil fertility (N-P-K) (Couch, 1973; Smiley et al., 1992; Vargas, 1994). Fast-acting fertilizers should be avoided in autumn, while heavy applications of slow-release N fertilizers should be avoided at any time (Smith et al., 1989). Late fertilizer applications should be avoided as they promote lush shoot growth prior to the onset of winter temperatures (Couch, 1973; Vargas, 1994; Tani and Beard, 1997). If unavoidable, late applications should be coupled with a fungicide application (Smith et al., 1989). Heavy thatch or mulch favour disease development (Couch, 1973; Tani and Beard, 1997). Insulating winter protective covers provide temperature and moisture conditions which favour disease development and require preventive fungicide applications in the fall (Dionne et al., 1999). Use of resistant cultivars or less susceptible species would help reduce the need for fungicides and ensure healthier turf in the spring (Smith et al., 1989; Tani and Beard, 1997).

Chemical control of snow mould should be applied in late fall or early winter before the establishment of a permanent snow cover (Smith et al., 1989; Anonymous, 2002; 2003). Multiple fall applications, as well as extra sprays during midwinter thaws, may be required for adequate control (Tani and Beard, 1997). Fungicides should be applied to surrounding turf areas as well as greens and fairways to limit inoculum production from these sources (Smith et al., 1989). Fungicides listed by OMAF (Ontario Ministry of Agriculture and Food) as registered for pink snow mould control on turfgrass contain quintozene, propiconazole, chlorothalonil, thiophanate-methyl, iprodione, azoxystrobin, or a mixture of carbathiin, oxycarboxin, or thiram (Anonymous, 2002; 2003).

Breeding Disease Resistant Turfgrasses

I) History of Turfgrass Breeding

Breeding of turfgrasses originally occurred through natural and unconscious selection. Genetic interaction with the environment, including biological organisms, promoted genetic shifts toward improved survival strategies in cool-season grasses (Casler et al., 1996). Genotypes better adapted to specific environmental constraints and intensive management are better able to persist (Casler et al., 1996). Both adaptive and reproductive strategies contributed to persistence and adaptation to stresses. The presence of pathogenic organisms in turfgrass populations led to genetic changes towards more disease-resistant plants (Casler et al., 1996).

Domestication of turfgrass helped refine disease resistance. Individuals were selected within natural populations according to their phenotype. Transplanting or seeding wild species into environments different from those where they evolved exposed them to organisms that they had not encountered previously. The individuals genetically able to resist or tolerate these organisms contributed to the next generation of individuals. Early breeding concentrated on selection of biotypes exhibiting desirable traits resulting from natural variation in a species and collection from environments that contributed to that variation. These varieties were combined to maximize the desirable traits, and today, breeding procedures range from simple selection to molecular modification (Casler et al., 1996).

II) Breeding Strategy

Improvement of disease resistance requires the identification and the integration of the desired resistance traits into an established turfgrass species without compromising its performance. However, disease resistance is rarely the only objective. The cultivar should be adapted to the environment and cultural

conditions existing where it is intended for use (Vargas et al., 1979). *Poa annua* ecotypes that have been established for long periods of time on intensively managed putting greens have already evolved into a more perennial-type that tolerates low mowing and high traffic stress. These two traits are very important for putting green turf (Huff, 1996).

Collecting annual bluegrass ecotypes from existing and older putting greens could help accelerate the breeding process. Effective screening techniques are needed to identify those ecotypes that have naturally developed superior resistance to snow mould. In many cases, further breeding may not be required if these ecotypes already harbor the aesthetic, agronomic and environmental stress tolerance qualities that are desired. Since 1995, Dr. David Huff has been collecting natural ecotypes at The Pennsylvania State University with the objective of identifying ecotypes that could be registered as commercial seed sources (Dr. D. Huff, personal communication, 2003). This genetic material will be subsequently integrated in a turfgrass breeding program to develop cultivars with improved resistance to snow mould.

Cold Tolerance and Pink Snow Mould Resistance

I) Cold Hardening

Cold acclimation of turf occurs in the fall at the time when photoperiod and temperatures start to decline (Levitt, 1980). During that period, growth slows and photosynthates are being converted into storage carbohydrates and other organic reserves (Levitt, 1980; Pontis, 1989). The accumulation of storage carbohydrates contributes to the cold hardening process of plants and their ability to tolerate winter conditions (Levitt, 1980; Pontis, 1989). Cool-season grasses from temperate areas accumulate carbohydrate mainly in the form of fructans (Pollock and Cairns, 1991; Livingston, 1991)

Cold hardening occurs in two phases: first-phase cold hardening (1PH) occurs at temperatures above 0^{o}C and involves the accumulation of fructans in cell vacuoles; second-phase cold hardening (2PH) occurs at temperatures below 0^{o}C and involves hydrolysis of fructans into soluble sugars (Livingston, 1996). These sugars may act as cryoprotectants lowering the osmotic potential (Livingston, 1993) or by providing a protective shell around hydrophilic cell components to prevent adhesion with ice (Olien and Lester, 1985; Olien and Clark, 1993, 1995). Studies on winter cereals have indicated that more cold tolerant cultivars tend to accumulate higher levels of fructan in the fall and to utilize them more slowly in winter (Suzuki and Nass, 1988; Olien and Clark, 1993, 1995; Livingston, 1991, 1996; Livingston and Henson, 1998). The maintenance of higher carbohydrate reserves in the spring may contribute to a more vigorous spring regrowth. The level of cold hardening fluctuates from year to year (Tompkins et al., 2000) and is dependent on fall climate conditions (Levitt, 1980) and other factors including management and pathogen infection.

A study by Tompkins et al. (2000) noted that dehardening of *P. annua* and *A. stolonifera* in spring was accompanied by an increase in percent crown moisture when snow cover disappeared and soil temperatures rose. A higher level of percent crown moisture was found in *P. annua* than in *A. stolonifera*, which was associated with lower cold hardening levels in *P. annua.* The mechanism of cold hardening in the fall could be due to water loss or dry matter accumulation.

A study by Dionne et al., (2001a) found that fructan accumulation and utilization did occur in *Poa annua* in response to cold hardening temperatures. However, variation in cold tolerance among ecotypes was not associated with fructan levels, but rather with the accumulation of unidentified proteins during cold hardening (Dionne et al., 2001b).

II) Snow Mould Resistance and Cold Tolerance

There is generally a high variability in the susceptibility to a pathogen within a given plant species and, as well, in the observed virulence of various strains of a given pathogen. The expression of resistance to a pathogen can be the result of lower infection rates, slow colonization, reduced reproduction, etc. and is referred to as rate-reducing resistance. Plant resistance to a disease can be horizontal or vertical (Agrios, 1997). This relates to the fact that the plant must exhibit specific susceptibility towards the pathogen in order to have an infection (Agrios, 1997). The pathogen also requires genes that determine its pathogenicity towards the plant. Horizontal resistance is a general plant response to infection by any strain of a pathogen. It is controlled by multiple genes that produce small, additive effects (Agrios, 1997). Although all plants within a species are likely to have some level of horizontal resistance, it is possible to identify individuals that may be more resistant than others. Vertical resistance is specific to certain races or strains of the pathogen and is usually expressed as a defense reaction which prevents the pathogen from becoming established (Agrios, 1997). This resistance is gene specific (controlled by one or a few genes which each have a major effect) and is seen when the plant and the pathogen have co-evolved (Agrios, 1997). The centre of co-evolution will have the greatest diversity of resistance genes and compatibility factors of the plant and pathogen (Agrios, 1997).

Studies have been conducted on winter cereals to select snow mould resistant cultivars and to determine the mechanisms underlying resistance (Bengtsson, 1989; Hommo, 1994; Yoshida et al., 1998; Gaudet et al., 2001). Enhanced snow mould resistance in winter wheat has been attributed to higher fructan content in crowns in fall and elevated fructan content remaining in spring (Yoshida et al., 1998; Gaudet et al., 1999). By maintaining high fructan levels during winter, snow mould resistant wheat cultivars possess lower levels of mono- and disaccharides in crowns than susceptible cultivars (Yoshida et al., 1998). Snow

mould fungi might have decreased ability to metabolize fructan polymers compared with simple sugars, so resistant cultivars that maintain a higher proportion of fructans should be less susceptible (Gaudet et al., 1999) Hommo (1994), and Gaudet et al. (2001) have also examined crown water content and the accumulation of dry matter in relation to resistance levels of winter wheat and winter rye. A decrease in water potential caused by carbohydrate accumulation may be a mechanism to reduce or prevent growth of snow mould fungi (Gaudet et al., 1999). Water potentials in grasses decrease during hardening, which results in less extensive growth of snow mould fungi on hardened plants (Tronsmo, 1986 *in* Gaudet et al., 1999). The ability of wheat and rye to lose water at hardening temperatures was found to be related to resistance (Gaudet et al., 2001), but Hommo (1994) suggested that other resistance mechanisms may be acting in response to infection.

Several mechanisms have been associated with resistance to pink snow mould in winter cereals. These mechanisms have small additive effects and are likely under the control of multiple genes that are expressed to a greater degree by some cultivars. Therefore, pink snow mould resistance in winter cereals is likely horizontal.

III) **Pathogenesis-related Proteins**

Ergon et al. (1998) studied the effect of cold hardening and *M. nivale* infection on the expression of pathogenesis-related (PR) proteins. PR-proteins are synthesized in plants in response to pathogen attack. They may have antifungal activity, be deleterious to oomycetes, or induce plant defense reactions. Expression of PR-proteins are also induced by cold hardening and it has been reported that cold hardened plants have increased resistance to snow mould (Ergon et al., 1998). A rapid increase in simple sugars in response to low temperatures may be a stimulus for the accumulation of antifungal substances (Gaudet et al., 1999). Increases in levels of PR-proteins may be affected by

carbohydrate accumulation (Gaudet et al., 1999) which would explain why expression is greater in cold hardened plants. Some PR-proteins were shown to have characteristics typical of antifreeze proteins (AFPs) (Hon et al., 1995; Antikainen and Griffith, 1997; Griffith et al., 1997) and their production has been detected during cold hardening (Hon et al., 1995).

PR-proteins either cause a defense reaction or induce a defense reaction within the plant against the pathogen. The production of these proteins as a defense reaction is an example of vertical resistance. However, PR-protein accumulation might also be a horizontal (unspecific) response.

Analysis of the biochemical changes and the molecular differences between resistant and susceptible ecotypes will provide more clues about the mechanisms of resistance to pink snow mould in *P. annua*.

IV) Research Objectives

There are currently no clear hypotheses to explain the bases of snow mould resistance in grasses (Gaudet et al., 1999). Hommo (1994) suggested that differences in snow mould resistance among winter rye cultivars might actually reflect differences in winter hardening ability rather than a real resistance reaction. The identification of annual bluegrass ecotypes with contrasting levels of resistance to snow mould is a necessary first step towards genetic improvement program and future unraveling of the molecular genetic bases of resistance.

In this study, we hypothesize that genetic variability can be used to develop seed sources more resistant to *M. nivale*. More specifically, the objectives were to: 1) assess the extent of variability among ecotypes of turf-adapted *Poa annua* for resistance to pink snow mould; 2) determine the source of resistance (e.g. true resistance vs. avoidance mechanisms such as rapid regrowth); 3) characterize

the relationship between the provenance of the ecotypes and their level of resistance and; 4) evaluate the link between cold hardiness and disease resistance.

Chapter 1

Genetic Variability for Resistance to Pink Snow Mould Among Ecotypes of *Poa annua* var. *reptans* Assessed Under Field Conditions

1.1 Introduction

Annual bluegrass (*Poa annua* L.) is generally considered a weed because it tends to invade sports turf and home lawns (Beard, 1970). It is commonly an unsown component of golf course putting greens in temperate zones throughout North America (Beard, 1970; Mitich, 1998). Most of the past literature on annual bluegrass has emphasized controlling invasion on golf course greens (Beard, 1970). Some of the more recent literature has highlighted cultural requirements and characteristics of *P. annua* to provide guidelines for its management as a turfgrass species (Beard, 1970), including use of annual bluegrass as a major component of golf greens (Huff, 1996; Dionne et al., 2001a, b).

Poa annua var. *reptans* (Hauskins) Timm is a subspecies of annual bluegrass found on golf course greens (Beard et al., 1978). It is adaptable to conditions within the micro-environment in which it exists (Cline et al., 1993; Mitich, 1998). In response to management practices, this subspecies of annual bluegrass has evolved over time into a more perennial ecotype (Beard et al., 1978). Management practices have imposed physiological and developmental constraints on the turf and genotypes that tolerate intense management have been favoured (Wu and Harivandi, 1993).

Poa annua is considered unreliable as a turf choice for greens because of its susceptibility to environmental stresses (Eggens, 1979). It is commonly attacked by the pink snow mould pathogen, *Microdochium nivale* (Fries) Samuels and Hallett (Smith et al., 1989). Damage occurs under snow cover and is not

apparent until the spring when snow recedes (Hsiang, 1996). Circular patches with a bleached appearance are exposed and leaf blades are matted together with white mycelium which turns pink when exposed to light (Smith et al., 1989; Hsiang, 1996). These patches disrupt the aesthetic quality and playability of the golf green. Preventative fungicides are applied to turf in late autumn to early winter to control pink snow mould (Anonymous, 2002).

In order to reduce the need for pesticide use on the golf course, turf managers would like to use disease resistant turf cultivars as part of an integrated pest management (IPM) program (Fermanian et al., 2003). At present, there is no commercial seed source for *P. annua* adapted to the golf green environment available to turf managers for greens repair or overseeding (Huff, 1996). However, due to the adaptability and genetic variability of *P. annua,* it may be possible to identify ecotypes with superior levels of resistance to pink snow mould.

There is support in the literature that cold acclimation not only increases tolerance to freezing, but also promotes non-specific resistance to low-temperature pathogens (Griffith and Yaish, 2004). Although low temperature exposure was required for the expression of *M. nivale* resistance, the pattern of development of snow mould resistance and cold tolerance was shown to differ substantially (Nakajima and Abe, 1996). This could be indicative of distinct or only partially overlapping processes leading to snow mould and cold hardiness resistance. The accumulations of storage carbohydrates and of unidentified proteins during cold acclimation have been linked to the acquisition of cold tolerance in *P. annua* ecotypes (Dionne et al., 2001a, b). Accumulation of similar storage carbohydrates has been associated with pink snow mould resistance in winter cereals (Bruehl, 1982; Bengtsson, 1989; Wu and Harivandi, 1993; Hommo, 1994). It is, therefore, possible that biochemical changes that occur during cold acclimation of turfgrasses in the fall are associated with resistance to pink snow mould.

Cold hardiness levels for turfgrasses fluctuate from year to year (Tompkins et al., 2000). The degree of cold hardiness expressed by the plant depends on climatic conditions during the acclimation process (Levitt, 1980). Temperatures between 0^{o} to 5^{o}C will induce cold acclimation, but additional cold hardening, or second-phase cold hardening, can occur with further exposure to temperatures below 0^{o}C (Levitt, 1980).

Nakajima and Abe (1990, 1994, 1996) conducted a series of studies on snow mould resistance in winter wheat cultivars and environmental effects on disease development and cultivar resistance. The first study (Nakajima and Abe, 1990) screened cultivars for resistance to pink snow mould and examined optimum temperatures and incubation periods conducive to pathogen growth. The objective of the study was to reduce the time required to assess the level of resistance of winter wheat cultivars. The second study (Nakajima and Abe, 1994) looked at the effects of autumn climate on the development of resistance to pink snow mould in winter wheat and changes in resistance over time under snow cover. This second study found that differences in resistance were more evident after cold hardening had taken place and that the duration of snow cover influenced the degree of resistance expressed. The authors attributed this change in the level of resistance over winter to the accumulation of carbohydrates during cold hardening and the depletion of these reserves during the winter.

In their third study, Nakajima and Abe (1996) examined how different environmental conditions during cold hardening affected the level of resistance expressed in winter wheat. Conditioning at low temperatures was essential for expression of resistance to pink snow mould. Cultivars resistant to snow mould, but not to cold temperatures, required lower temperatures during cold hardening for full expression of resistance than cultivars moderately resistant to both snow mould and low temperatures. The researchers inferred a difference in the

pattern of development of snow mould resistance and the pattern of development of cold tolerance.

The objective of the current study was to screen *P. annua* var. *reptans* ecotypes collected from golf greens 15+ years old for resistance to pink snow mould under field conditions. To increase the chances of finding a naturally occurring population of *P. annua* with resistance to pink snow mould, it is important to start with a population that has adapted to golf course maintenance practices in a cold temperate climate, and hence the focus on older established golf greens $\geq$ 15 years old. Samples collected from populations assumed to have existed for long periods of time on old golf greens were screened for snow mould resistance in this study.

1.2 Materials and Methods

I) Plant Material

Naturally occurring ecotypes of *Poa annua* var. *reptans* were collected from golf course putting greens in Ontario and Quebec during the summer of 2002 (May – July) by Louis Simard. To ensure that the ecotypes collected were of the subspecies *reptans* (greens-type *P. annua*), samples were selected from greens that had been established more than 25 years ago without extensive renovation since that time. This time period would allow evolution of ecotypes in response to golf course management practices and traffic stress, so that breeding for these traits would be unnecessary. A list of collected specimens and the origin of each collection are found in Table 1.1. A cup cutter of 10.5 cm diameter was used to remove a 10 cm deep plug of turf and soil from one or two greens at each golf course. Individual turf plants were separated from the collected sample and then transplanted into 3.8 cm diameter forestry tubes containing 1 L of an 80:20 v/v sand:soil medium. Ecotypes of *P. annua* collected by Dr. David Huff at The Pennsylvania State University (Penn State) were grown from seed in the same type of forestry tubes using the same medium. The turf samples were grown in a

greenhouse where they were watered daily. Average daily temperature in the greenhouse was 21±3°C between May and September, 2002. Humidity readings were unavailable due to sensor malfunction. No supplementary light was supplied. Average daily (6 am to 6 pm) irradiance level between May and September, 2002 was 1581±128 KJ m^{-2} according to weather data collected by the University of Guelph weather station. Tubes were fertilized with 20-8-20 NPK fertilizer via irrigation at a concentration of 200-250 mg L^{-1} (approximate rate 475 L m^{-2}, 4-5 g N m^{-2} $week^{-1}$) and grass plants were cut back to a 1 cm height twice per week.

II) Inoculum Preparation

Three strains of *Microdochium nivale* were collected from Kentucky bluegrass (*Poa pratensis* L.) turf at the Guelph Turfgrass Institute in 1999 and from Aylmer, Quebec in April, 2002, courtesy of Dr. T. Hsiang. Leaf blades were placed in a 0.1% Tween-20 solution for 10 s, then surface-sterilized in a 1% $NaClO_3$ solution for 60 s. The tissue was then rinsed twice in autoclaved distilled water and plated on potato dextrose agar (PDA) amended with streptomycin at 100 ppm to inhibit growth of bacteria. The plates were incubated for 1 to 2 weeks at room temperature (20 to 25°C). *M. nivale* was isolated from the leaf tissue by removing a sample of hyphae from the colony margin with a surface-sterilized scalpel and transferring it to a fresh PDA plate. Plates were incubated at room temperature (20 to 25°C) in indirect sunlight until sporodochia were formed, after approximately 2 weeks. With exposure to light, the pink pigmentation of the hyphae and sporodochia intensified. Sporodochia were removed from each colony with a sterilized needle and placed in a 1.5 mL tube with 1 mL of autoclaved water. The tube was agitated with a vortex mixer for 10 s and the spore suspension was poured into a plate containing PDA. The solution was spread evenly over the surface of the PDA with a surface-sterilized glass rod. After incubation at room temperature in indirect sunlight for 2-3 days, a single-

spore colony was selected from each sample and replated on PDA for incubation at 4^{o}C for approximately 2 weeks.

Mason jars (500 mL) containing 50 g of wheat bran with 45 mL of water were autoclaved at 120^{o}C for 20 minutes 3 times at 24 hour intervals. The wheat bran was stirred in a flow hood after the first autoclaving. After the third autoclaving, the sterilized wheat bran was inoculated with six 5-mmdiameter plugs of PDA containing *M. nivale*. The liner lid of each Mason jar was inverted so the rubber gasket did not contact the glass to allow for some gas exchange. Many attempts at growing inoculum were unsuccessful until this modification in protocol. The inocula were allowed to grow for two weeks at room temperature (25^{o}C). Wheat bran cultures were opened occasionally and stirred in a flow hood during incubation to break up any clumps and increase the growth of the fungi through the medium. Each isolate was dried separately in a fumehood for 24 hours and then ground into a powder using a blender. The three strains were then combined in equal portions for application. Mixing strains possibly minimizes the effects of strain-specific differences in the host response. The ground wheat bran was easier to apply to turf and provided more uniform coverage than wheat bran that had not been ground.

III) Experimental Design

This study was conducted during the winter of 2002 – 2003 at two locations: the Guelph Turfgrass Institute (GTI) and the Victoria Park Golf Club West (Vic West) in Guelph, Ontario. The GTI plot was established on the USGA research green and the Vic West plot was established on an unused sand-based green. A soil probe of 2 cm diameter was used to remove a 15 cm deep plug of soil. The hole was enlarged using a forestry dibble to 3.8 cm in diameter, which is the same diameter as the forestry tubes containing the turf samples. Turf plugs (grass and media) were then removed from the forestry tubes and inserted into the enlarged hole. Thirty ecotypes were tested with 10 replications of inoculated ecotypes in a

randomized complete block design with rows spaced 20 cm apart. To facilitate fungicide application, another set of plots with the same thirty ecotypes but with only three replicate blocks were established adjacent to the test plots. Plots were established on September 27, 2002 (GTI) and on October 11, 2002 (Vic West) to allow turf to acclimate to fall conditions. Inoculum was applied to turf at Victoria West Golf Course on December 5, 2002 using infected wheat bran at a rate of 0.1 g cm^{-2}. For uninoculated control plots adjacent to the test plots, the fungicide Quintozene 75WP (Plant Products, Brampton, Ontario) was applied on December 6, 2002 at a rate of 250 g 100 m^{-2}. A datalogger was situated near each plot to record air and soil temperature throughout the winter season. Probes were inserted at 7 cm to measure soil temperature.

IV) Evaluation

Evaluation of field plots commenced when snow cover had receded completely on March 22, 2003. Ratings for winter injury were performed weekly using an adjusted Horsfall - Barrett scale (Green et al., 1998) to assess disease severity and subsequent re-growth over the next six weeks. The scale was applied as follows: 0 = 0% injury, 1 = 1 to 4% injury, 2 = 5 to 9%, 3 = 10 to 16%, 4 = 17 to 25%, 5 = 26 to 36%, 6 = 37 to 50%, 7 = 51 to 63%, 8 = 64 to 74%, 9 = 75 to 83%, 10 = 84 to 90%, 11 = 91 to 95%, 12 = 96 to 99%, and 13 = 100% injury. Since it was often difficult, if not impossible, to visually distinguish abiotic winter injury from biotic injury, both types of injury were evaluated together in this rating system.

V) Statistical Analysis

Ratings from field plots were transformed into a percent injury value except in the case of the recovery index and were analyzed using the General Linear Model procedure of SAS ver. 8.0 (SAS Institute, Cary, NC) to assess the statistical significance of the data. Tests for normality were conducted using the Proc

Univariate procedure of SAS ver. 8.0. Duncan's multiple range test was calculated to determine which ecotypes differed significantly with regard to their resistance to pink snow mould and to winter stresses. Recovery Index value was calculated by subtracting the mean Horsfall - Barrett rating score of each ecotype on April 18, 2003 from the mean rating score of each ecotype on March 22, 2003. This index value provided a comparative basis to ascertain the recovery potential of each ecotype. Index values were analyzed with Duncan's multiple range test. Regional differences in resistance were assessed using Duncan's multiple range test to analyze injury observed on field plots after snowmelt and after the recovery period. Regional differences in rate of recovery were determined using Duncan's multiple range test to analyze recovery index values. Correlation analysis was conducted between inoculated and uninoculated ecotypes to detect any relationship between the two groups.

1.3 Results

I) Climatic Conditions

The duration of the winter of 2002-2003 was long with permanent snow cover commencing on November 2, 2002 and not completely receding until March 22, 2003 in many areas. The field plot at GTI was located in an unprotected area exposed to desiccating winds, which resulted in shallow snow cover. Figure 1.1 illustrates the air and soil temperature fluctuations at the GTI field plot. A brief rise in ambient temperature to 5.5^{o}C in November caused soil temperatures to rise to 6.7^{o}C and then fall to 1.8^{o}C a week later when ambient temperature fell to 0.5^{o}C (Figure1.1). Soil temperatures dropped to below 0^{o}C in late November and remained below freezing in the following weeks with absolute minima of -5^{o} and -10^{o}C in mid-December and mid-January respectively (Figure 1.1). Annual bluegrass exposed to such low soil temperatures may have succumbed to root death. Freezing temperatures at crown level result in recurrent loss of annual bluegrass on golf greens (Dionne et a l., 1999). Soil temperatures at the Vic

West plot dropped to just below 0^{o}C in January and did not go below -1^{o}C throughout the winter (Figure 1.2). This indicated that they were covered by snow throughout the winter since air temperatures were well below 0^{o}C (Figure 1.2). As the snow melted, it became apparent that ecotypes had sustained high levels of injury ranging between 91.6% to 97.7% injury at the GTI plot as measured on March 22, 2003. Recovery was slow and, after six weeks, injury ratings ranged from 76.9% to 100%. The field plot at Victoria West Golf Club was located in a low area of the golf course that was surrounded by trees. This protected area was less exposed to winter winds, so snow cover was deeper than the GTI site and lasted 2-5 days longer than that of the GTI site. This resulted in milder soil temperatures that could explain why ecotypes that were transplanted at the Vic West site experienced much less injury. As evidence of lack of snow cover or shallow snow cover at the GTI site, soil temperatures were seen to dip down to –5 ^{o}C (27 Dec 02) or even –10 ^{o}C (24 Jan 03) while they remained near 0 ^{o}C at the Vic West site. Furthermore, air and soil temperatures fluctuated in the spring at the GTI and at the Vic West field plot, and, an unseasonable cold period occurred between April 3, 2003 and April 9, 2003 (Figures 1.1, 1.2). This climatic event may have contributed to the high degree of injury during the recovery period observed among the ecotypes at both sites. During the cold period in April, air temperature readings at the GTI plot (Figure1.1) dropped from 3^{o}C to -5^{o}C during the day and to -11^{o}C at night. The cold spell was accompanied by precipitation in the form of ice and snow which persisted as temporary snow cover for several days.

II) Ecotype Variability

A test for normal distribution using the Shapiro-Wilk (W) statistic revealed that the data from the GTI field plot was not normally distributed both after snowmelt (inoculated, $W = 0.76$, $p < 0.0001$; uninoculated, $W = 0.80$, $p < 0.0001$) and after the recovery period (inoculated, $W = 0.76$, $p < 0.0001$; uninoculated, $W = 0.75$, $p < 0.0001$). The high injury levels at the GTI were probably confounded

confounding winter stresses and not just due to disease. Therefore, we decided that the results should be excluded from this study, so these results are not presented. However, the data collected from Victoria West was normally distributed and showed statistically significant differences, so the analyses are presented here.

Ecotypes inoculated with *M. nivale* differed significantly for injury levels after snow had receded ($p<0.0001$). Figure 1.3 and Table 1.2 present the mean injury observed on the ecotypes after snowmelt on March 22, 2003. Ecotypes with the lowest levels of injury were assumed to be the least susceptible to pink snow mould and the ones with the highest levels of injury were assumed to be the most susceptible. The most susceptible ecotypes were significantly different from the least susceptible ecotypes, but the ecotypes that are in the mid- range did not differ significantly. Lack of statistical discrimination among ecotypes may be attributable to large variations in winter injury between replications.

The four least susceptible ecotypes were WM2 (ON), Q97-1-10 (US), WM9 (ON), and LO16 (QC) with mean scores ranging from 48% to 60% injury (Figure 1.3). Even though these showed the least amount of disease, the level of injury observed would not be acceptable for a putting green. However, these injury levels were significantly lower than those of the four most susceptible ecotypes; Q98-3-30 (US), RC17 (QC), MCC18 (QC), and CH12 (QC) ranging from 85% to 90% injury.

III) Recovery Period

Injury ratings of the inoculated ecotypes after the recovery period were dependent on their ability to initiate and sustain spring growth. Additional factors may have also affected the injury ratings including exposure to cold temperatures or the initiation of *M. nivale* growth due to the onset of the appropriate conditions for Fusarium patch disease. Figure 1.4 presents the mean injury observed on

the ecotypes after the recovery period on April 18, 2003. Ecotypes that had been inoculated with *M. nivale* differed significantly for the level of injury present ($p<0.0001$). There was a significant difference between ecotypes with the highest and lowest levels of injury, however most of the other ecotypes did not differ significantly from each other (Table 1.3).

The ecotypes with the least amount of injury on April 18, 2003 were FB6H (ON), Q97-1-10 (US), LO16 (Quebec), and SY2 (QC) with mean scores ranging between 21% and 30% injury (Figure 1.4). Two of these ecotypes, Q97-1-10 and LO16, were among the group of ecotypes least susceptible to pink snow mould according to ratings taken on March 22, 2003. The ecotypes with the most injury were the same ecotypes that were found to be most susceptible to snow mould injury after snowmelt: RC17 (QC), CH12 (QC), Q98-3-30 (US), and MCC18 (QC) with injury ratings ranging from 72% to 86%.

Correlation analysis revealed that injury ratings taken on March 22, 2003 were weakly but significantly correlated with injury ratings taken on April 18, 2003 ($r^2=0.47$, $p<0.0001$). The ability of the ecotypes to resist snow mould attack seemed to influence the amount of injury observed after six weeks of recovery.

IV) Rate of Recovery

The rate of recovery of the ecotypes was assessed by subtracting the mean injury ratings taken on April 18, 2003 from the mean injury ratings taken on March 22, 2003 for each ecotype and calculating the difference to specify a recovery index value (Figure 1.5). Statistical analysis of the rate of recovery among ecotypes indicated that there was a significant difference ($p=0.0074$) between the highest and lowest levels of regrowth, but most of the ecotypes were not significantly different from each other (Figure 1.5).

Although injury levels were still moderately high after the recovery period, two of the ecotypes with the lowest observed injury after the recovery period exhibited the highest levels of regrowth: FB6H with a recovery index of 3.9 and SY2 with a recovery index of 3.5 (Figure 1.5). Both of these ecotypes exhibited average injury levels after snow melt, so their high levels of regrowth resulted in lower levels of injury after the recovery period.

V) Regional Differences

Ecotypes collected from golf courses in Ontario were statistically less susceptible to pink snow mould than ecotypes from Quebec or the US ($p=0.0001$) with average injury values of 62.7%, 76.8%, and 72.4% injury respectively (Table 1.4). After the recovery period, Ontario ecotypes had significantly less injury (32.9%) than the other ecotypes (49.3% for both QC and US, $p=0.0074$) (Table 1.4). The Recovery Index of the ecotypes (difference between first and last ratings) was not significantly different between regions ($p=0.3$), so the performance of the Ontario ecotypes may be attributed to their capacity to resist pink snow mould.

VI) Effects of Winter Hardening

Replicated control plots that were uninoculated and treated with fungicide were used to assess the level of winter injury that occurred in response to stresses other than snow mould (cold temperatures, desiccation, ice injury, or other stresses that occur during winter). Ecotypes were significantly different from each other after snowmelt ($p=0.0037$) (Table 1.5) and after the recovery period ($p=0.0024$) (Table 1.6) in the absence of inoculum. Figure 1.3 compares observed injury levels of the ecotypes between inoculated and control plots after snowmelt (March 22, 2003) and Figure 1.4 compares observed injury levels between inoculated and control plots after the recovery period (April 18, 2003). The injury levels of inoculated plots were generally higher than the injury levels of

control plots, implying that injury was cumulative with abiotic and biotic components. The level of injury observed between inoculated and control ecotypes was significantly, but weakly, correlated after snowmelt (r^2=0.28, p=0.0029) and after the recovery period (r^2=0.14, p=0.0403). Although there was a statistically significant relationship between winter injury of inoculated plots and control plots both at snowmelt and at re-growth, the correlations imply a weak relationship.

1.4 Discussion

The major objective of this study was to assess the extent of genetic variability among *Poa annua* var. *reptans* ecotypes in order to select more resistant types. Some ecotypes showed significantly lower levels of susceptibility to pink snow mould attack than others providing evidence for genetic variability among ecotypes. The levels of injury that we observed in the field were too excessive to be acceptable on putting greens. However, this study represents only one year of data and may not provide sufficient data to make recommendations. It is thus important to repeat similar field studies over several years and locations to account for climatic differences and to confirm ecotype resistance over a range of conditions. Although the ecotypes tested did not perform at the level necessary for use on putting greens, the existence of genetic variability in susceptibility among ecotypes suggests the possibility of discovering more resistant types with continued screening.

Environmental conditions that prevail during fall acclimation will affect winter survival (Levitt, 1980). Exposure to low temperature in the fall has been linked to the acquisition of cold tolerance of turf species (Tompkins et al., 2000, Dionne et al., 2001a, b), as well as winter cereals (Olien and Lester, 1985; Suzuki et al., 1988; Livingston, 1991, 1996; Olien and Clark, 1993, 1995; Yoshida et al., 1998). Winter hardening has also been linked to pink snow mould resistance in winter cereals (Bengtsson, 1989; Hommo, 1994; Gaudet, 1994; Nakajima and Abe,

1994, 1996; Ergon et al., 1998; Gaudet et al., 1999, 2001). Climatic events that occur during cold acclimation in the fall and during spring recovery can influence the level of winter hardiness expressed by turf plants (Levitt, 1980; Tompkins et al., 2000). Insufficient exposure to low temperatures in the fall may result in low levels of resistance and could result in higher levels of injury. The climatic events that occurred during this study may have affected ecotype performance. Dehardening temperatures in early winter and spring may have influenced the level of resistance to pink snow mould achieved by the ecotypes and their subsequent recovery. Replication of the field screening would help confirm ecotype performance under varying environmental conditions that are bound to influence snow mould resistance in *P. annua*.

Ecotypes exhibiting the least amount of injury after the recovery period demonstrated either less susceptibility to the disease and winter stresses or high rates of regrowth. Rapid recovery of *P. annua* ecotypes may be dependent on the level of storage carbohydrates remaining in the spring. The level of remaining reserves depends on the levels accumulated during winter hardening and/or the rate of consumption during the winter (Gaudet et al., 1999). Enhanced snow mould resistance among winter wheat cultivars has been attributed, in part, to higher fructan contents in crowns during autumn and elevated fructan content remaining in the spring (Yoshida et al., 1998; Gaudet et al., 1999). Increases in the level of storage carbohydrates in the form of fructan have been observed during cold acclimation of *P. annua* var. *reptans* ecotypes and their levels were shown to vary among ecotypes (Dionne et al., 2001a).

Injury levels after the recovery period may have also been attributed to further infection by *M. nivale* under conditions conducive to pathogen growth. Prolonged cool temperatures accompanied by wet conditions in the spring may have initiated growth of *M. nivale* in the absence of snow cover to incite Fusarium patch disease (Couch, 1973; Vargas, 1994; Tani and Beard, 1997). Susceptibility of the ecotypes to Fusarium patch disease may be related to the

level of winter hardiness remaining after the commencement of dehardening. Increases in soil temperature and the disappearance of snow cover in the spring trigger dehardening in turf plants (Tompkins et al., 2000). The unseasonable drop in air and soil temperature in April, 2003 may have caused some additional injury to less winter hardy ecotypes that had already partially dehardened.

The injury ratings of ecotypes collected from Ontario were significantly lower than the ecotypes from the other regions both after snowmelt and after the recovery period. The regional differences in ecotype susceptibility illustrate genetic variability resulting from varying evolutionary pressure based on climatic and environmental differences between the regions. Since there was no difference in the rate of recovery between regions, the success of the Ontario ecotypes after the recovery period was probably influenced by higher levels of resistance to *M. nivale*. This is also reflected in the correlation data ($r^2 = 0.47$, $p<0.0001$), which indicated that less susceptible ecotypes had significantly lower injury ratings after the recovery period.

The ecotypes differed in their ability to tolerate winter conditions, which indicates genetic variability among ecotypes. Correlation data indicates that the ability of ecotypes to withstand winter conditions influenced how well they resisted pink snow mould, although the level of influence is only weakly correlated. The effects of cold hardening have been shown to influence both cold tolerance and pink snow mould resistance in winter wheat (Gaudet, 1994; Gaudet et al., 1999). Cold hardening has also been shown to influence the level of cold tolerance expressed in *P. annua* var. *reptans* ecotypes (Dionne et al., 2001b). Inoculation resulted in higher injury ratings than the controls, supporting the suggestion by Nakajima and Abe (1996) that the mechanisms that influence resistance to pink snow mould are distinct from those conferring cold tolerance. It is, however, likely that winter hardening predisposed the ecotypes to reduced susceptibility to pink snow mould by influencing winter survival mechanisms.

In future studies, the ecotypes should be evaluated over a period of many years at multiple locations so that the level of pink snow mould resistance may be characterized under different winter hardening regimes. Biochemical analysis of the most and least susceptible ecotypes should be conducted during cold acclimation in the fall to assess how winter hardening influences the expression of pink snow mould resistance. Molecular analysis would help reveal the causes for regional differences observed in the resistance of ecotypes, as well as possible genetic influences on resistance and susceptibility.

Table 1.1: Origin of ecotype collection of *Poa annua* var. *reptans*. Canadian ecotypes were collected from putting greens during the summer of 2002. Quebec ecotypes were collected by Louis Simard. Ontario ecotypes were submitted by golf course turf managers. US ecotypes were obtained from the annual blue grass collection of Dr. David Huff at The Pennsylvania State University.

Ecotype	***Province***	***Origin***
FB6H	Copetown, Ontario	Flamborough Hills Golf
WM2	Kitchener, Ontario	Westmount G.C.C.
WM9	Kitchener, Ontario	Westmount G.C.C.
CV11	Mississauga, Ontario	Credit Valley Golf Club
RH18	Richmond Hill, Ontario	Richmond Hill Golf Club
TG5	Milton, Ontario	Trafalgar Golf and Country Club
GTI	Guelph, Ontario	Guelph Turfgrass Institute
B14[2]	Rimouski, Quebec	Club de Golf de Bic
B17[1]	Rimouski, Quebec	Club de Golf de Bic
CH12	Chicoutimi, Quebec	Club de Golf Chicoutimi
HD17	St. Therese, Quebec	Golf Hillsdale
IS8BE	St. Dorothee, Quebec	Club de Golf Islemere
LE15	Levis, Quebec	Club de Golf Lévis
LO16	Louiseville, Quebec	Club de Golf Louiseville
MB11	Montebello, Quebec	Club de Golf Montebello
MCC18	Montreal, Quebec	Country Club de Montréal
PM18	Montreal, Quebec	Club de Golf Piedmont
PRO12	St. Hyacinthe, Quebec	Club de Golf la Providence
RC6	Charlesbourg, Quebec	Club de Golf Royal Charbourg
RC17	Charlesbourg, Quebec	Club de Golf Royal Charbourg
SH3	Magog, Quebec	Club de Golf Sherbrooke
SM10	St. Michel de Bellechasse, Quebec	Club de Golf St-Michel
SY2	St. Hyacinthe, Quebec	Club de Golf St-Hyacinthe
WH13	Hudson Heights, Quebec	Club de Golf Whitlock
Q98-6-18[1]	Long Island, NY	Penn State University
Q98-3-30	Long Island, NY	Penn State University
Q98-3-12	Long Island, NY	Penn State University
Q98-4-21	Long Island, NY	Penn State University
Q98-6-30[3]	western Pennsylvania	Penn State University
Q98-3-6	Long Island, NY	Penn State University
Q97-1-10	Maryland shore	Penn State University
Q98-4-6[2]	Long Island, NY	Penn State University

[1] Field study only.
[2] Cold chamber study only.
[3] The western Pennsylvania ecotype was grouped with the Ontario rather than the US Coastal ecotypes because of climatic similarity.

Table 1.2: Percent (%) injury observed on inoculated ecotypes at the Vic West field plot on March 22, 2003. Ecotypes are ranked from least to most affected.

Ecotype	***Injury (%)***
WM2	47.9 a*
Q97-1-10	50.5 ab
WM9	60.0 abc
LO16	60.2 abc
RH18	60.4 abc
Q98-3-6	61.4 abcd
FB6H	61.5 abcd
Q98-6-30	62.2 abcde
TG5	63.7 abcde
WH13	66.4 bcdef
IS8BE	67.5 cdef
SY2	67.4 cdef
SH3	67.6 cdef
GTI	68.2 cdef
PRO12	71.0 cdefg
Q98-3-12	71.6 cdefgh
HD17	71.8 cdefgh
LE15	71.9 cdefgh
B17	74.1 cdefghi
CV11	77.5 defghi
Q98-6-18	78.6 efghi
RC6	80.8 fghi
PM18	81.4 fghi
MB11	81.6 fghi
Q98-4-21	81.8 fghi
SM10	82.9 fghi
Q98-3-30	84.9 ghi
RC17	84.9 ghi
MCC18	87.8 hi
CH12	89.8 i

* Means followed by the same letter are not significantly different from each other at p=0.05 according to Duncan's multiple range test.

Table 1.3: Percent (%) injury observed on inoculated ecotypes at the Vic West field plot on April 18, 2003. Ecotypes are ranked from least to most affected.

Ecotype	***Injury (%)***
FB6H	20.9 a*
Q97-1-10	21.3 a
LO16	29.4 ab
SY2	29.8 ab
SH3	32.2 abc
WM2	32.6 abc
GTI	33.8 abcd
TG5	34.9 abcde
PRO12	40.8 abcde
RH18	40.9 abcde
IS8BE	42.1 abcde
B17	42.8 abcdef
Q98-3-6	43.7 abcdef
Q98-3-12	43.8 abcdef
Q98-6-30	45.0 abcdef
HD17	45.8 abcdef
MB11	50.4 bcdefg
WM9	50.9 bcdefgh
CV11	51.7 bcdefgh
LE15	51.9 bcdefgh
WH13	52.2 bcdefgh
RC6	55.5 cdefghi
PM18	58.5 defghi
Q98-6-18	59.6 efghi
SM10	67.6 fghij
Q98-4-21	71.1 ghij
RC17	71.6 ghij
CH12	75.9 hij
Q98-3-30	80.3 ij
MCC18	85.9 j

* Means followed by the same letter are not significantly different from each other at p=0.05 according to Duncan's multiple range test.

Table 1.4: Mean percent (%) injury scores of inoculated ecotypes collected in Ontario, Quebec and the US assessed immediately after snowmelt on March 22, 2003 and after a recovery period on April 18, 2003.

Region (ecotypes)	***Mean Injury after Snowmelt (%)***	***Mean Injury after Recovery Period (%)***
US (7)	72.4 b	49.3 b
Quebec (16)	76.8 b	49.3 b

*Means within a column followed by the letter are not significantly different at p = 0.05 in a Duncan's multiple range test. (March 22, 2003, p=0.0001; April 18, 2003, p=0.0074)
** The single collection from western Pennsylvania was grouped with the Ontario collections since the climate there was considered to be most similar to that of southern Ontario rather than the U.S. east coast.

Table 1.5: Percent (%) injury observed on uninoculated ecotypes at the Vic West field plot on March 22, 2003. Ecotypes are ranked from least to most affected.

Ecotype	***Winter Injury (%)***
Q98-3-6	15.7 a*
WM2	21.7 ab
WM9	27.7 abc
Q98-3-12	28.5 abcd
TG5	30.3 abcde
HD17	37.8 abcdef
RH18	39.3 abcdefg
CV11	44.5 abcdefgh
Q98-6-30	48.3 abcdefghi
LO16	52.0 bcdefghij
CH12	52.3 bcdefghij
SH3	53.7 bcdefghij
SM10	59.8 cdefghij
FB6	60.5 cdefghij
GTI	64.3 defghij
SY2	64.3 defghij
Q98-3-30	64.5 defghij
RC6	65.7 efghij
B17	67.0 fghij
PM18	67.2 fghij
Q97-1-10	68.3 fghij
WH13	68.5 fghij
PRO12	71.7 fghij
IS8BE	72.3 fghij
RC17	74.3 ghij
Q98-4-21	78.3 hij
MB11	78.7 hij
LE15	80.5 hij
MCC18	83.0 ij
Q98-6-18	86.5 j

*Means followed by the same letter are not significantly different from each other at p=0.05 according to Duncan's multiple range test. (p=0.0037)

Table 1.6: Percent (%) injury observed on uninoculated ecotypes at the Vic West field plot on April 18, 2003. Ecotypes are ranked from least to most affected.

Ecotype	***Winter Injury (%)***
CV11	5.5 a*
WM2	5.5 a
HD17	9.0 ab
Q98-3-6	9.5 ab
LO16	12.2 ab
TG5	16.8 abc
SH3	19.7 abcd
CH12	21.2 abcd
FB6H	25.7 abcde
RH18	25.3 abcde
WM9	27.2 abcde
Q98-3-12	29.2 abcdef
GTI	36.0 abcdef
SM10	39.3 abcdef
SY2	43.8 abcdef
Q98-6-30	50.7 abcdefg
PM18	52.3 bcdefg
MB11	58.2 cdefg
B17	58.3 cdefg
Q98-4-21	58.3 cdefg
RC6	59.7 cdefg
Q98-3-30	60.0 cdefg
PRO12	65.0 defg
WH13	66.7 efg
LE15	67.8 efg
Q97-1-10	69.3 efg
RC17	70.5 efg
IS8BE	73.0 fg
MCC18	74.5 fg
Q98-6-18	94.0 g

* Means followed by the same letter are not significantly different from each other at p=0.05 according to Duncan's multiple range test. (p=0.0024)

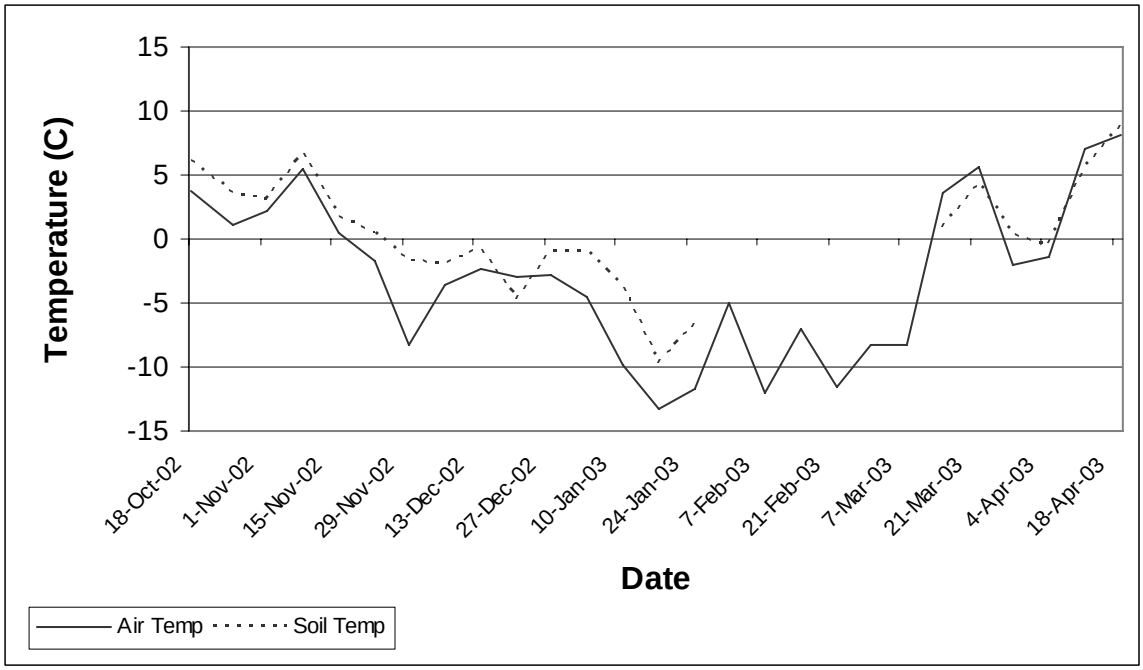

Figure 1.1: Air and soil temperature fluctuations during winter 2002 - 2003 at GTI field plot. Soil temperature was measured at a depth of approximately 7 cm. Data is missing from February to March due to equipment malfunction.

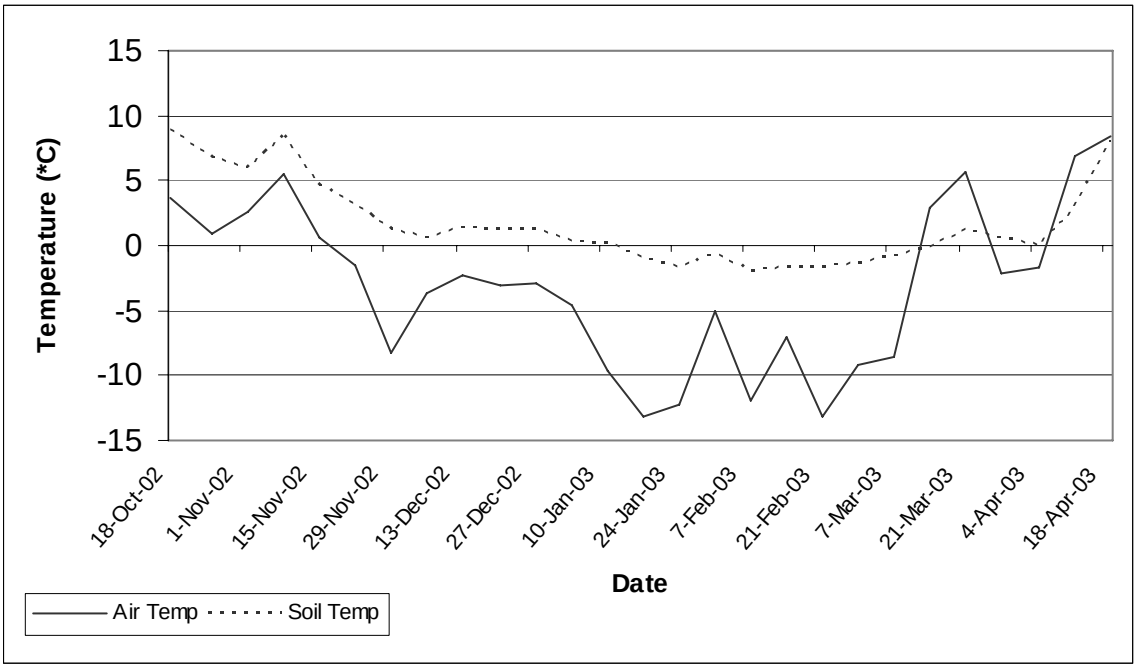

Figure 1.2.: Air and soil temperature fluctuations during winter 2002 - 2003 at Vic West field plot. Soil temperature was measured at a depth of approximately 7 cm. Snowcover lasted from mid-December, 2002 to early March, 2003

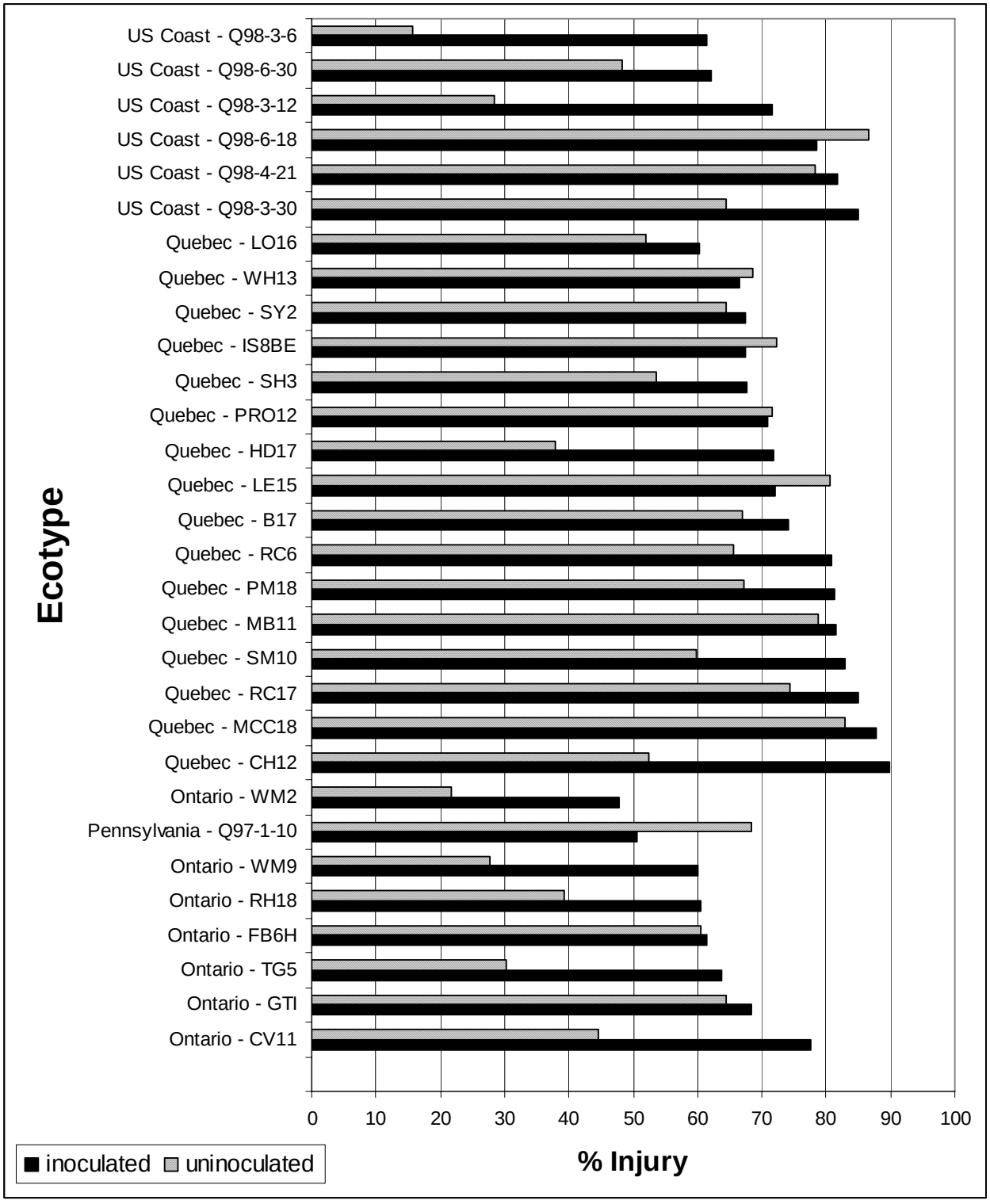

Figure 1.3: Percent (%) injury levels observed on inoculated and uninoculated-fungicide-treated plots ranked from least to most susceptible to attack by pink snow mould. Observations were made on March 22, 2003.

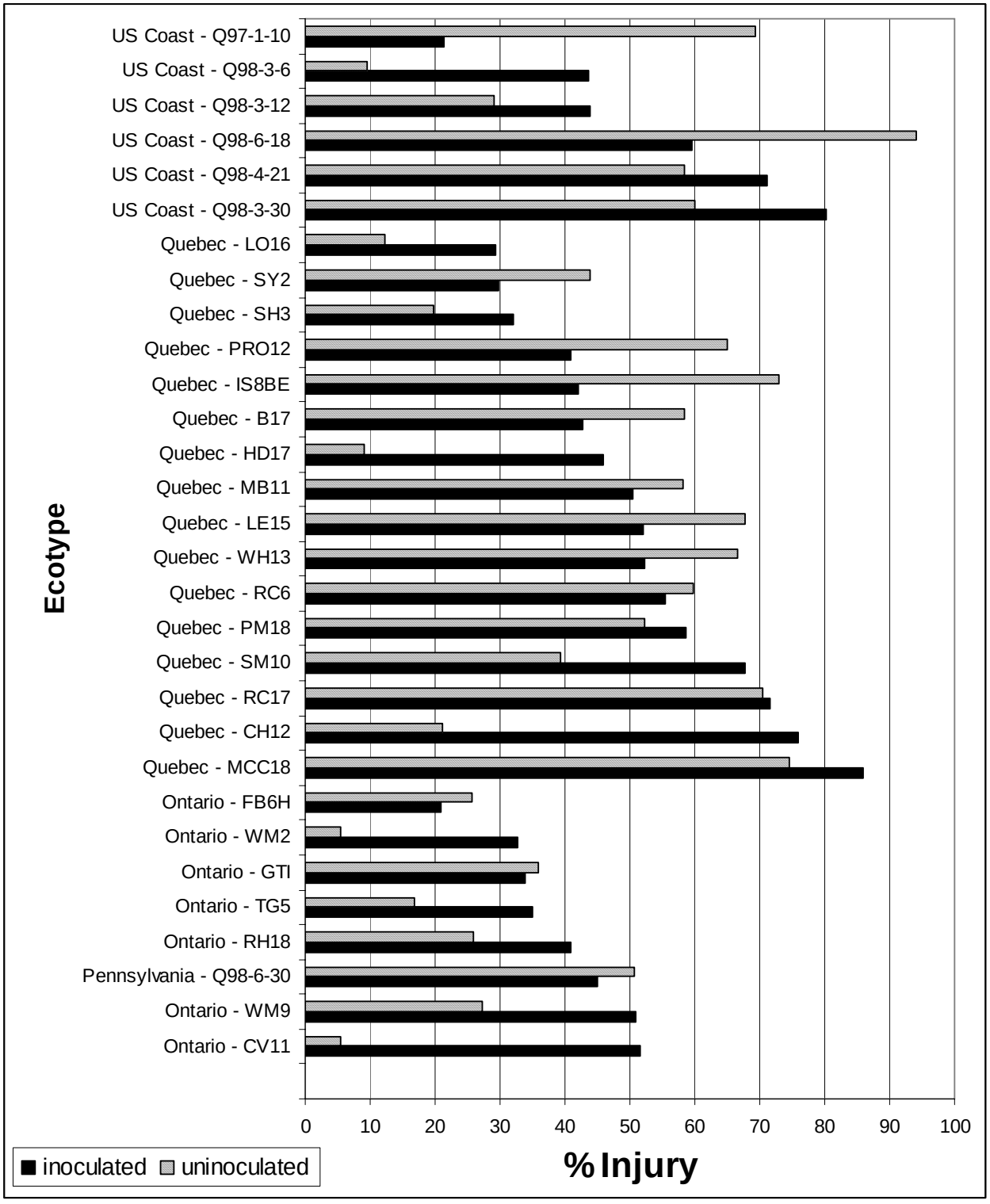

Figure 1.4: Percent (%) injury levels observed on inoculated and uninoculated-fungicide-treated plots ranked from least to most susceptible to attack by pink snow mould. Observations were made on April 18, 2003.

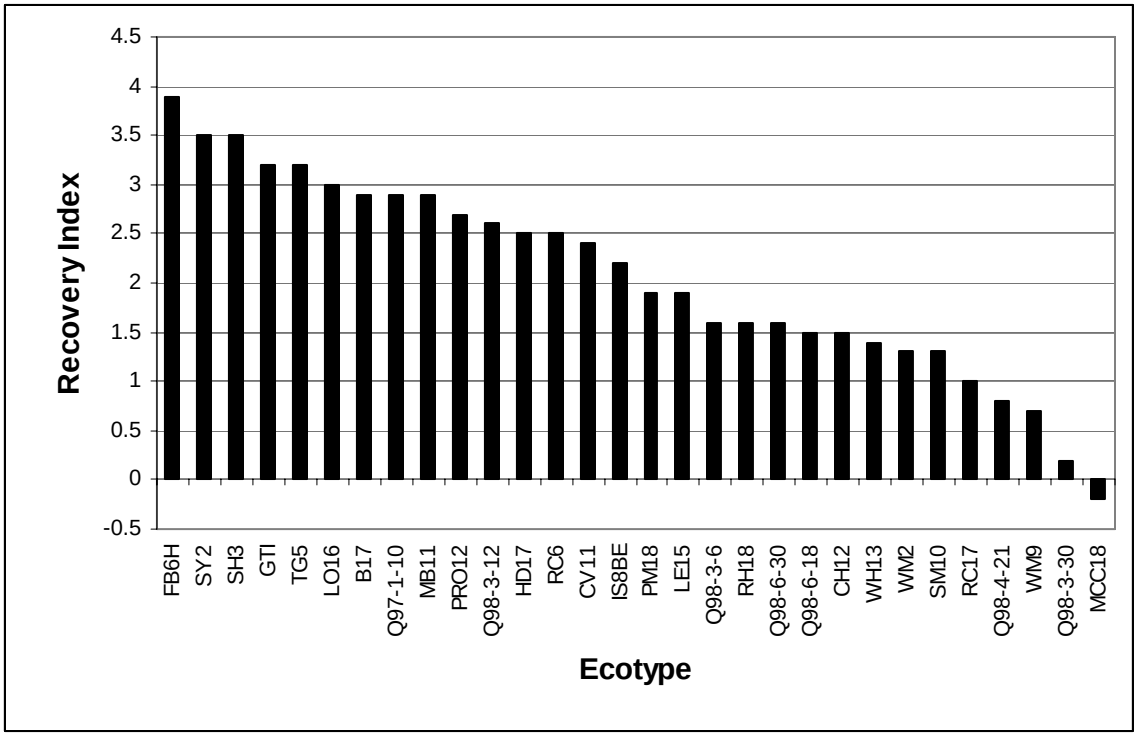

Figure 1.5: Rate of recovery of inoculated and uninoculated ecotypes based on a recovery index calculated by subtracting injury ratings scored after the recovery period (March 22, 2002) from injury ratings scored after snowmelt (April 18, 2002) and determining the mean score.

Chapter 2

Genetic Variability for Resistance to Pink Snow Mould Among Ecotypes of *Poa annua* var. *reptans* Assessed Under Controlled Conditions

2.1 Introduction

Perennial ecotypes of annual bluegrass (*Poa annua* var. *reptans* (Hauskins) Timm) are commonly found on golf greens in temperate zones throughout North America (Beard, 1970; Mitich, 1998). Annual bluegrass is generally considered a weed and most of the research in the past has emphasized control (Beard, 1970). More recently, the literature has focused on the cultural requirements of *P. annua* to provide turf managers with guidelines for better management of that species (Beard, 1970; Beard et al., 1978).

Annual bluegrass is commonly attacked by the pink snow mould pathogen, *Microdochium nivale* (Fries) Samuels and Hallett (Smith et al., 1989). Damage occurs under snow cover and is not apparent until the spring when snow recedes (Hsiang, 1996). Snow mold blemishes may take months to recover and disrupt the aesthetic quality and playability of the golf green. Fungicide is applied to turf in late autumn to early winter to control pink snow mould (Anonymous, 2002). In order to reduce the need for pesticide use on the golf course, turf managers would like to use disease resistant turf cultivars as part of an integrated pest management (IPM) program (Fermanian et al., 2003). At present, there is no commercial seed source for *P. annua* adapted to the golf green environment available to turf managers for greens repair or overseeding (Huff, 1996). However, due to the adaptability and genetic variability of *P. annua,* it may be possible to identify ecotypes with superior pink snow mould resistance.

In Chapter 1, we presented the results from a field screening of *P. annua* ecotypes collected from golf course putting greens for their resistance to pink

snow mould. Although we were able to identify ecotypes that differ significantly with regard to their levels of resistance, variable winter conditions during the study may have affected the sensitivity of the screening assay. Screening performed in growth chambers attempts to simulate winter temperature conditions while avoiding other winter stresses such as ice injury, desiccating winds, and dehardening temperatures which were experienced in the field assay. The closed environment study also offered the opportunity to observe the interaction with pink snow mould during the epidemic rather than after the damage had already occurred. Although there are a number of studies on winter cereals inoculated with snow moulds in controlled environments (Nakajima and Abe, 1990; Meidener et al., 1993; Hommo, 1994; Maurin et al., 1996), we did not find any report on the assessment of pink snow mould disease on turfgrass under controlled environments.

Hommo (1994) used a liquid culture of *M. nivale* isolates collected from Finland, which was sprayed on winter rye cultivars for resistance screening in a cold chamber. Winter rye plants were sown in boxes with a peat-soil mixture and also in beakers containing a nutrient solution. Test plants were covered with moist cellulose wadding and plastic sheeting to mimic the protected environment under the snow. Plants in nutrient solution were incubated for 7 weeks and those in peat soil were incubated for 9 weeks. Plants were evaluated for damage after the incubation period, and then placed in a greenhouse for recovery and assessment of the final survival rate. Significant differences in the amount of snow mould damage among cultivars were found in both the nutrient solution and the peat soil mixture. Significant differences in final survival rate were found in the nutrient solution only.

Meidener et al. (1993) also used a liquid culture which was mixed with sterilized vermiculite for application to winter rye plants to assess the optimal conditions for an infection test in a growth chamber. Four isolates of *M. nivale* from different geographic regions were propagated separately and mixed in equal volumes of

inoculum prior to inoculation. The vermiculite-mycelium mixture was spread evenly on the soil surface of pots containing the winter rye plants. Three different cold hardening methods were used to assess the effects of environment on resistance. Plants were inoculated after the cold hardening treatments, and after incubation at 1^{o}C for a period of 5, 8, 11, or 14 days, plants were first evaluated for snow mould injury, and then placed in a greenhouse for recovery. After the recovery period, plants were evaluated for fresh weight and fungal protein content. Plant age and incubation temperatures were critical factors affecting resistance in this type of screening. It was possible to assess genotypic variation between winter rye cultivars for all resistance traits.

Maurin et al. (1996) used two different inoculum sources to study pink snow mould on winter wheat. An agar disk of inoculum was placed at the base of the stem to produce stem lesions and plants were then transferred to a vernalization chamber. Stem lesions were assessed after a 2 week incubation period by calculating the stem disease index. The second inoculum source was a conidial suspension that was sprayed on flowers at anthesis to reproduce head blight symptoms. Spikes were individually covered with plastic bags to maintain high relative humidity, placed in a growth chamber for 2 weeks, and subsequently transferred to a greenhouse for recovery. Disease severity was expressed as a percentage of diseased spikelets per spike. Significant differences were detected in the reaction of winter wheat cultivars to infection by *M. nivale*.

Nakajima and Abe (1990) used inoculated wheat bran mixed with sterilized vermiculite to inoculate winter wheat cultivars in a screening experiment for pink snow mould pathogen resistance. The inoculum was spread on the soil surface at a rate of 0.06 g cm^{-2} and plants were covered with moistened cotton to simulate snow cover. Growth chamber temperatures ranged from 5^{o} to 18^{o}C to explore the relationship between snow mould development and incubation temperature. Incubation periods ranging between 7 and 18 days at 15^{o}C were tested to develop a rapid laboratory screening technique to identify resistance to

M. nivale among winter wheat cultivars. Varietal differences in resistance to *M. nivale* were detected among winter wheat cultivars. Resistance expressed by wheat cultivars in the growth chamber were consistent with that observed in plants incubated in boxes outdoors with natural snow cover. Incubation at higher temperatures reduced the length of time required to complete the screening to a period of weeks.

The various inoculation protocols outlined above were able to detect significant variations in pink snow mould resistance in winter cereals in growth chambers. Although some of the procedures would not be appropriate for inoculation of turfgrass with *M. nivale*, infested wheat bran or agar are probably suitable inoculum sources and have been used in prior field studies locally (Cook and Hsiang, 2004). It was necessary to test these two modes of inoculation using various rates in order to establish protocols for infecting turfgrass with *M. nivale*.

The objectives of this study were to 1) establish protocols for the inoculation of turfgrass with *Microdochium nivale* under controlled conditions; and 2) screen ecotypes of *Poa annua* collected from golf greens that were 15+ years old for resistance to pink snow mould under controlled conditions. To increase the chances of finding a naturally occurring population of *P. annua* with resistance to pink snow mould, it is important to start with a population that has adapted to golf course maintenance practices in a cold temperate climate. Samples collected from populations assumed to have existed for long periods of time on old golf greens were screened for snow mould resistance in this study.

2.2 Materials and Methods

2.2.1 Preliminary Study

I) Plant material

Two turf species were tested in the preliminary experiment. One *Poa annua* ecotype from a golf green in Kitchener, ON was collected by Louis Simard with a 10.5-cm-diameter cup cutter and then propagated by tillers into 3.8-cm-diameter forestry tubes containing 1 L of an 80:20 v/v sand:soil media mix. The golf green was established in 1937 and had not been heavily renovated since that time. The other species was Providence creeping bentgrass (*A. stolonifera*) which was seeded into the same type of tubes and media as the *P. annua* ecotype and grown for 11 weeks before inoculation. The grasses were placed in a growth chamber where they were watered daily. Tubes were fertilized with 20-8-20 NPK fertilizer via irrigation lines at a concentration of 200-250 mg L^{-1} (approximate rate 475 L m^{-2}, 4-5 g N m^{-2} $week^{-1}$) and the grass plants were cut back to 1 cm height once per week. Average irradiance levels in the growth chamber were 146.7 µmol photons m^{-2} s^{-1}; average temperature was 19.3^{o}C; and average relative humidity was 76.6%.

II) Inoculum preparation

Two strains of *Microdochium nivale* were collected from Kentucky bluegrass (*Poa pratensis* L.) turf from the Guelph Turfgrass Institute in 1999 and from Aylmer, Quebec in April, 2002 courtesy of Dr. T. Hsiang. Leaf blades were placed in a 0.1% Tween-20 solution for 10 s, then surface-sterilized in a 1% $NaClO_3$ solution for 60 s. The tissue was then rinsed twice in autoclaved distilled water and plated on potato dextrose agar (PDA) amended with streptomycin at 100 ppm to discourage growth of bacteria. The plates were incubated for 1 to 2 weeks at room temperature (20 to 25^{o}C). *Microdochium nivale* was isolated from the leaf

tissue by removing a sample of hyphae from the colony margin with a surface-sterilized scalpel and transferring it to a fresh PDA plate. Plates were incubated at room temperature (20 to 25^{o}C) in indirect sunlight until sporodochia were formed, after approximately 2 weeks. With exposure to light, the pink pigmentation of the hyphae and sporodochia intensified. Sporodochia were removed from each colony with a sterilized needle and placed in a 1.5 mL tube with 1 mL of autoclaved water. The tube was agitated with a vortex mixer for 10 s and the spore suspension was poured into a plate containing PDA. The solution was spread evenly over the surface of the PDA with a surface-sterilized glass rod. After incubation at room temperature in indirect sunlight for 2-3 days, a single-spore colony was selected from each sample and replated on PDA for incubation at 4^{o}C for approximately 2 weeks.

To produce wheat bran inoculum, 500 mL mason jars containing 50 g of wheat bran (Co-op, Guelph, ON) with 45 mL of water were autoclaved at 120^{o}C for 20 minutes 3 times at 24 hour intervals. The wheat bran was stirred in a flow hood after the first autoclaving. After the third autoclaving, the sterilized wheat bran was inoculated with six 5-mm-diameter plugs of PDA containing *M. nivale*. The liner lid of each Mason jar was inverted so the rubber gasket did not contact the glass to allow for some gas exchange. Many attempts at growing inoculum were unsuccessful until this modification in protocol. The inocula were allowed to grow for two weeks at room temperature (25^{o}C). Wheat bran cultures were opened occasionally and stirred in a flow hood during incubation to break up any clumps and increase the growth of the fungi through the medium. Each isolate was dried separately in a fumehood for 24 hours and then ground into a powder using a blender. The ground wheat bran was easier to apply to turf and provided more uniform coverage than wheat bran that had not been ground.

III) Inoculation and Incubation

Five treatments were applied to turf samples: 1) 5-mm-diameter plug of PDA from *M. nivale* culture; 2) 0.05 g cm^{-2} of infected wheat bran; 3) 0.01 g cm^{-2} wheat bran; 4) 0.001 g cm^{-2} wheat bran; and 5) 0.0001 g cm^{-2} wheat bran. Turf was misted with water before inoculum was applied. Tubes containing turf were inserted through holes in the lids of plastic containers. Containers were 5 L rectangular Rubbermaid boxes measuring approximately 27.5 x 40 x 14 cm. A drill press with a 3.5-cm-diameter circular bit was used to drill 24 holes spaced approximately 5 cm apart into the lid of the plastic container (Figure 2.1). Each container represented one block. Each block contained two repetitions of each treatment applied to each turf species. Two uninoculated controls of each turf species were also included in each block. Another plastic box was inverted over the block and misted with water periodically throughout the experiment to maintain humidity. The boxes were then incubated for six weeks in a cold chamber with an average temperature of 3.4^{o}C. Weekly ratings were taken using an adjusted Horsfall - Barrett scale (Green et al., 1998) to assess disease progression and severity. The scale was applied as follows: 0 = 0% injury, 1 = 1 to 4% injury, 2 = 5 to 9%, 3 = 10 to 16%, 4 = 17 to 25%, 5 = 26 to 36%, 6 = 37 to 50%, 7 = 51 to 63%, 8 = 64 to 74%, 9 = 75 to 83%, 10 = 84 to 90%, 11 = 91 to 95%, 12 = 96 to 99%, and 13 = 100% injury.

IV) Statistical Analysis

The ratings were transformed to percentage values from rating scores before analysis. The data were subjected to analysis of variance using the Proc GLM procedure of SAS v. 8.0 (SAS Institute, Cary, NC) to determine treatment and concentration effects and to detect host/isolate effects and interactions.

2.2.2 Cold Chamber Experiment

I) Plant Material

Naturally occurring ecotypes of *Poa annua* var. *reptans* were collected from golf course putting greens in Ontario and Quebec during the summer of 2002 (May – July) by Louis Simard. To ensure that the ecotypes collected were of the subspecies *reptans* (greens-type *P. annua*), samples were selected from greens that had been established more than 25 years ago without extensive renovation since establishment. This time period would allow evolution of ecotypes in response to golf course management practices and traffic stress, so that breeding for these traits would be unnecessary. A list of collected specimens and the origin of each collection are found in Chapter 1, Table 1.1.

A cup cutter with a 10.5-cm-diameter was used to remove a 10 cm deep plug of turf and soil from one or two greens at each golf course. Individual turf plants were separated from the collected sample and then transplanted into 3.8 cm diameter forestry tubes containing 1 L of an 80:20 v/v sand:soil media. Ecotypes of *P. annua* collected by Dr. David Huff at Penn State University were grown by seed into the same type of forestry tubes using the same media. Two bentgrass species, creeping bentgrass (*Agrostis stolonifera* Huds.) and velvet bentgrass (*A. canina* L.), were also included in the cold chamber study for comparison purposes. The creeping bentgrass was the cultivar 'Providence' and the velvet bentgrass was the cultivar 'Vesper'. The bentgrasses were grown from seeds as described previously.

Starting on January 13, 2003, the transplanted annual bluegrass and seeded bentgrasses were grown in a greenhouse where they were watered daily. Average daily temperature in the greenhouse was $20.4^{o}C \pm 2.4^{o}C$ from January 13 until April 2, 2003. Average daily (6am to 6pm) light levels were 1284 KJ m^{-2} according to weather data collected by the University of Guelph weather station.

No supplementary light was supplied. The ecotypes were fertilized with 20-8-20 fertilizer provided by the greenhouse through irrigation lines at a concentration of 200-250 ppm (approximate rate 475 L m^{-2}, 4-5 g N m^{-2} $week^{-1}$) and the grass was cut back to 1 cm height twice per week.

II) Inoculum Preparation

Three strains of *Microdochium nivale* used in this study were collected from Kentucky bluegrass (*Poa pratensis* L.) turf at the Guelph Turfgrass Institute (isolates MN96074 and MN96103) and from a golf course in Aylmer, Quebec (isolate O2123) by Dr. T. Hsiang. The inoculum was prepared and applied to the plants following the procedures described previously in section 2.2.1

III) Experimental Design

Tubes of *P. annua* were arranged in trays according to a randomized complete block design. The design contained 7 blocks of inoculated turf and 3 blocks of uninoculated controls. Two replications of turf samples were cold acclimated together in a cold chamber for two weeks to mimic cold hardening conditions in the field. The average temperature was 3.7^{o}C and supplementary light was supplied during cold acclimation (<5 μmol photons $m^{-2}s^{-1}$).

Following cold acclimation, turf was misted with water and inoculated with infected wheat bran at a rate of 0.01 g cm^{-2} (from initial experiment). One group was transferred to a different cold chamber (CC1 at 6^{o}C) and the other replication remained in the original cold acclimation chamber set at 2^{o}C (CC2). Plastic boxes were inverted over the replicate blocks and the turf was misted periodically to maintain humidity. Turf was incubated in the cold chambers for 6 weeks. Weekly ratings of disease progression were performed using the adjusted Horsfall - Barrett scale (Green et al., 1998) that was described in the preliminary study.

IV) Statistical Analysis

Ratings of the ecotypes were transformed into a percent injury value from rating scores and were analyzed using the General Linear Model procedure of SAS ver. 8.0 (SAS Institute, Cary, NC) to assess the statistical significance of the data. Duncan's multiple range test was calculated to assess significant differences between ecotypes. Regional differences in resistance were assessed using Duncan's multiple range test to analyze injury observed on ecotypes in both cold chambers. Correlation analysis was conducted between inoculated and uninoculated ecotypes and between cold chamber experiments to detect any relationships. Results from the cold chamber study were also compared to results from the field study using correlation analysis.

2.3 Results

I) Preliminary Study

The preliminary study was performed without any cold hardening treatments of the turf plants prior to inoculation. The purpose of this study was to identify an optimum inoculum source and rate of application for the cold chamber study. Therefore, an initial cold acclimation treatment was not considered necessary. Results indicated that there was no significant host x isolate interaction ($p=0.9889$), so both isolates behaved the same on both turf species. The highest rates of infection were obtained with 0.01 g cm^{-2} and 0.05 g cm^{-2} of wheat bran, which did not differ significantly (Table 2.1). The inoculum application rate of 0.01 g cm^{-2} was chosen for further experiments in the cold chamber.

II) Ecotype Susceptibility to Pink Snow Mould

Ecotypes inoculated with *M. nivale* differed significantly for injury levels in both cold chambers after six weeks of incubation ($p<0.0001$) (Tables 2.2, 2.3). Table

2.2 presents the mean injury observed on the inoculated ecotypes in cold chamber 1 during evaluation on August 5, 2003 and Table 2.3 shows the data from cold chamber 2 during evaluation on August 6, 2003. Ecotypes with the lowest levels of injury were assumed to be the least susceptible to pink snow mould and the ones with the highest levels of injury were assumed to be the most susceptible. The most susceptible ecotypes were significantly different from the least susceptible ecotypes, but the ecotypes in the mid-range were not significantly different from each other (Tables 2.2, 2.3).

The annual bluegrass ecotypes that showed the least *M. nivale* injury in both cold chambers were WM9 (Ontario) and RH18 (Ontario), (Figures 2.2, 2.3) with average injury ratings of 66.7% and 60.4% injury (CC1, CC2) for WM9 and 65.3% and 63.6% injury for RH18. Although some ecotypes were less susceptible than others to pink snow mould, injury levels sustained by these ecotypes during this study were considered unacceptable for use as turf selections for putting greens. However, the injury levels were significantly lower than that of the most susceptible ecotypes; FB6H (Ontario) (CC1=97.6%, CC2=89.3%), LE15 (Quebec) (CC1=96.6%, CC2=92.4%), and TG5 (Ontario) (CC1=98.9%, CC2=84.1%); or the two bentgrasses with injury scores of 96.1% (CC1) and 97.4% (CC2) injury (velvet bentgrass) and 94.6% (CC1) and 99.3% (CC2) injury (creeping bentgrass) (Figures 2.2, 2.3).

III) Incubation Temperature Effect

The two cold chambers were set at different temperatures, which resulted in different levels of injury between the two chambers. The average temperature during incubation in cold chamber 1 (CC1) was $6.7^{o} \pm 0.4^{o}$C and in cold chamber 2 (CC2) it was $3.1^{o} \pm 0.2^{o}$C (Figure 2.4). Of the inoculated ecotypes, the least susceptible ecotypes in CC1 had injury ratings between 65.3% and 75.0% injury (Figure 2.2) and the least susceptible ecotypes in CC2 between 60.4% and 68.4% injury (Figure 2.3). Similar differences in injury of the uninoculated

ecotypes was found between the two incubators with averages of 31.8% to 39.3% injury of the least susceptible ecotypes in CC1 (Figure 2.2) and 13.7% to 11.0% injury of the least susceptible ecotypes in CC2 (Figure 2.3). There was a significant incubator by ecotype interaction for both inoculated and uninoculated ecotypes ($p = 0.0083$, inoculated, Table 2.4); $p = 0.0027$, uninoculated, Table 2.5) so the results from the two chambers were analyzed separately. Injury ratings between the two cold chambers were significantly and positively correlated for both the inoculated ($r^2 = 0.24$, $p = 0.0001$) and uninoculated ecotypes ($r^2 = .0.29$, $p = 0.0001$), but the low values imply a weak to moderate relationship between injury observed on ecotypes in the two chambers.

Correlation analysis of cold chamber results with field results indicated no significant correlation of inoculated ecotypes (snowmelt, $r^2 = 0.09$, $p = 0.12$; recovery, $r^2 = 0.006$, $p = 0.71$) or uninoculated ecotypes (snowmelt, $r^2 = 0.05$, $p = 0.23$; recovery, $r^2 = 0.002$, $p = 0.81$)

IV) Regional Differences

Ecotypes received from Penn State University were significantly less susceptible to pink snow mould than ecotypes from Quebec or Ontario ($p=0.0001$) with average injury values of 79.9%, 86.0%, and 86.9% respectively (Table 2.6). Bentgrass species were the most susceptible with an average injury value of 98.3%.

V) Uninoculated Ecotypes

Uninoculated ecotypes were used as controls to assess potential injury attributable to low temperature. Ecotypes were significantly different from each other for injury due to cold stress in both cold chambers ($p<0.0001$, Tables 2.7, 2.8). Figures 2.2 and 2.3 show the comparison between injury levels of the ecotypes on inoculated and uninoculated plots in CC1 and CC2 respectively.

The injury levels of inoculated plots were generally higher than the injury levels of uninoculated plots, implying that injury was cumulative with abiotic and biotic components. The levels of injury observed between inoculated and uninoculated ecotypes were significantly, but weakly, correlated in both CC1 ($r^2 = 0.27$, $p = 0.0001$) and CC2 ($r^2 = 0.07$, $p = 0.0072$). Although there is a statistically significant relationship between winter injury of inoculated plots and uninoculated plots, the low correlations imply a weak relationship.

2.4 Discussion

The central objective of this study was to assess whether there is enough genetic variability among *Poa annua* var. *reptans* ecotypes to select for more resistant types. Although the ecotypes tested here experienced high levels of injury that would be unacceptable under putting green conditions, the existence of significant genetic variability suggests the possibility of using this type of assay to discover resistant types with continued screening.

Cold chamber assessment of ecotype resistance to pink snow mould was undertaken to achieve a better control of the environmental conditions to which the plants were exposed during their incubation. This approach significantly reduces uncontrolled variability and should allow a more precise evaluation of the variations in ecotype resistance. Additional advantages of conducting ecotype screening in a cold chamber are that: 1) it takes much less time than a field study; 2) it enables researchers to repeat their experiment many times during the season; 3) resistance to pink snow mould can be assessed under varying cold acclimation or incubation conditions and; 4) ecotypes collected from other sources may be readily included in an assay to discover new sources of resistance.

However, a major disadvantage of cold chamber studies is that conditions in the incubator cannot exactly replicate natural conditions. For example, temperature

fluctuations, natural snow cover, and additional winter stresses such as ice damage, hail, or desiccation by cold winds and their interactions are not reflected. Since these conditions may influence the hardiness of the turf and/or growth of the pathogen, it is often not feasible to apply results obtained under growth chamber conditions to make reliable inferences about field performance. Indeed the results from the field for ecotype injury were not significantly correlated with lab results for either inoculated or uninoculated tests.

Another possible explanation for the discrepancy between field and cold chamber results is that the temperatures tested in the cold chamber (3^o and 7^oC) were optimal for Fusarium patch development in the field, but not pink snow mould. Furthermore there was no artificial covering on the grass in the growth chambers to simulate that provided by snow cover. Perhaps resistance to Fusarium patch differs from that of pink snow mould, and this interesting observation should be studied further.

The comparisons between inoculated and uninoculated values showed that the growth chambers had less variability ($r^2 = 0.07$, $p=0.0001$ for growth chambers, and $r^2 = 0.28$, $p = 0.0029$ for field data). In the growth chamber, the inoculated injury values were consistently greater than the uninoculated injury values (Figures 2.2 and 2.3) while in the field, many of the ecotypes showed higher injury for uninoculated ecotypes compared to their inoculated counterparts (Figure 1.3 and 1.4). The growth chamber may allow a more direct assessment of the potential resistance or susceptibility of particular cultivars under controlled conditions without the confounding effects of abiotic winter injury, and offers the advantage of conducting experiments throughout the year rather than once a year. However, because of the necrotrophic nature of the attack by *M. nivale*, the environmental conditions prior to infection may be critical in the success of the infection.

Cold acclimation conditions that were used in this study were not optimal and may have affected the capacity of the plant to withstand aggression by the pathogen allowing high levels of injury to occur. The temperature of 3.7^{o}C in the cold chamber during cold hardening was theoretically adequate to induce metabolic changes experienced under natural conditions (Levitt, 1980). Light is also important for cold hardening during acclimation at above 0^{o}C in the fall (Levitt, 1980), and is required to induce snow mould resistance in winter wheat (Nakajima and Abe, 1996; Gaudet et al., 1999). Irradiance levels available in the cold chamber were probably not adequate to induce full expression of cold hardening and may have influenced the resulting injury observed among the ecotypes. According to Dionne et al. (2001a, b), cold acclimation under environmentally-controlled conditions should proceed in the following sequence to simulate natural hardening conditions in frozen soil under snow cover: an initial acclimation period of two weeks at a constant temperature of 2^{o}C under 8h photoperiod and a photosynthetic photon flux density (PPFD) of approximately 150 μmol $m^{-2}s^{-1}$ followed by a transfer to a freezer at -2^{o}C in the dark for an additional 2 weeks. The additional cold hardening at freezing temperatures is believed to increase freezing tolerance (Levitt, 1980), and the absence of light mimics conditions under snow cover. Cold hardening conditions in the current experiment did not include a second-phase hardening at below 0^{o}C in the dark, and light levels during the initial phase of cold hardening at above 0^{o}C were significantly lower (<5 μmol $m^{-2}s^{-1}$) than those recommended.

Injury levels observed on inoculated plants kept at ~7^{o}C (CC1) were consistently higher than those maintained at ~3^{o}C (CC2). Exposure to warmer temperatures after cold hardening results in a loss of freezing tolerance in plants (Levitt, 1980). Therefore, transferring ecotypes to a cold chamber with a higher temperature may have caused partial dehardening of the ecotypes making them more susceptible to cold stress and pathogen attack. The positive correlation of the injury level of both the inoculated and uninoculated ecotypes between the two

cold chambers suggests that the ecotypes were showing similar behavior in both incubators with respect to susceptibility.

The injury ratings of ecotypes provided by Penn State were significantly lower than those of the ecotypes collected from golf courses in Canada. The regional differences in ecotype susceptibility illustrate genetic variability resulting from varying evolutionary pressures based on climatic and environmental differences between the regions. The bentgrasses had the highest levels of injury in the cold chamber, but showed little injury compared to annual bluegrass ecotypes in the field. The creeping bentgrass in the field was established on the putting green prior to plot establishment (i.e. placement of the annual bluegrass plugs in the plots), whereas the cold chamber study included the bentgrasses as plugs grown in tubes. This difference in methods of establishment may explain some of this discrepancy. Testing of creeping bentgrass plugs alongside annual bluegrass plugs in the field would be required to assess this possibility.

The ecotypes differed in their ability to tolerate cold stress, which indicates genetic variability among ecotypes. However, some injury to uninoculated plants may be attributable to physiological problems (e.g. etiolated growth) or to cross-contamination of snow mould from inoculated plants. Correlation analyses indicated that the ability of ecotypes to withstand cold temperatures was related to how well they resisted pink snow mould, although the relationship was only weakly correlated. The effects of cold hardening have been shown to influence both cold tolerance and pink snow mould resistance in winter wheat (Gaudet, 1994; Gaudet et al., 1999). Cold hardening has also been shown to influence the level of cold tolerance expressed in *P. annua* var. *reptans* ecotypes (Dionne et al., 2001b). Further examination of pink snow mould resistance mechanisms in *P. annua* ecotypes is required to assess any biochemical relationship with cold tolerance. The cold chamber offers the advantage of conducting biochemical analysis of the ecotypes during the pink snow mould epidemic, which is more

difficult in the field due to snow cover. Metabolic changes triggered by events during cold hardening and incubation may be more easily observed.

The ecotypes screened in this study should be evaluated under varying cold acclimation conditions so that the actual level of pink snow mould resistance may be characterized under different cold hardening regimes. Biochemical analysis of the most and least susceptible ecotypes should be conducted during cold acclimation and incubation to assess how cold hardening influences the expression of pink snow mould resistance as well as metabolic differences between ecotypes of varying susceptibility. The use of cold chamber assays offers many advantages in that respect. The environmental conditions can be modified to mimic key factors in the field without the interference of additional stresses imposed by the environment. Tissue samples can be taken from leaves, crowns, and roots at regular intervals during the incubation. A recovery period in a greenhouse or growth chamber would also offer the opportunity to observe the ability of ecotypes to recover after pathogen attack. Future experiments should look at varying cold hardening conditions to assess the optimum climate for full expression of snow mould resistance in *P. annua* ecotypes. Comparison of ecotypes from different regions using molecular techniques may help reveal genetic differences associated to snow mould resistance. Molecular comparison of ecotypes may reveal genetic traits that are important for breeding *P. annua* cultivars with improved pink snow mould resistance.

Table 2.1: Effect of inoculum source and rate of application of two *M. nivale* isolates on average pink snow mould injury observed on one ecotype of annual bluegrass and one cultivar of creeping bentgrass in a cold chamber. There were 12 replications of each treatment (isolate by host by inoculum level combination). Ecotypes were assessed weekly for six weeks following their incubation in a growth chamber.

Inoculum Source	***Rate***	***% Injury***
Wheat Bran	0.05 g cm^{-2}	99.8 a*
Wheat Bran	0.01 g cm^{-2}	93.8 a
Wheat Bran	0.001 g cm^{-2}	64.3 b
Wheat Bran	0.0001 g cm^{-2}	46.4 c
Agar	5 mm diameter plug	35.9 c

* Means followed by a letter in common are not significantly different at p=0.05.

Table 2.2: Percent (%) injury observed on inoculated ecotypes under controlled conditions for six weeks with no light in cold chamber 1 set at 7°C. Ecotypes are ranked from least to most affected.

Ecotype	***Injury (%)***
RH18	65.3 a*
WM9	66.7 ab
Q98-3-12	75.0 bc
WH13	76.1 c
IS8BE	77.3 cd
Q98-3-6	78.1 cd
CV11	79.6 cde
Q97-1-10	79.6 cde
LO16	80.4 cdef
Q98-4-21	80.4 cdef
Q98-6-30	81.0 cdef
SH3	83.4 cdefg
SY2	84.8 defgh
CH12	85.0 defgh
SM18	86.7 efghi
B14	87.1 efghij
MCC18	88.4 fghijk
MB11	88.9 fghijk
RC6	90.2 ghijkl
PM18	90.7 ghijklm
Q98-4-6	91.7 ghijklm
WM2	92.8 hijklm
HD17	93.2 hijklm
PRO12	94.1 ijklm
Creeping Bentgrass	94.6ijklm
Q98-3-30	94.8 ijklm
RC17	95.4 jklm
GTI	95.6 jklm
Velvet Bentgrass	96.1 klm
LE15	96.6 klm
FB6H	97.6 lm
TG5	98.9 m

* Means followed by the same letter are not significantly different from each other at p=0.05 according to Duncan's multiple range test based on seven replicates for each mean.

Table 2.3: Observed injury (%) on inoculated ecotypes under controlled conditions for six weeks with no light in cold chamber 2 set at 3°C ranked from least to most susceptible.

Ecotype	***Injury (%)***
WM9	60.4 a*
RH18	63.6 ab
Q98-6-30	65.0 abc
IS8BE	65.6 abc
Q98-4-21	68.4 abcd
Q98-3-12	71.0 bcde
MB11	71.0 bcde
HD17	72.4 bcdef
Q98-3-6	73.0 bcdefg
Q97-1-10	73.6 cdefg
WM2	73.6 cdefg
SY2	73.9 cdefgh
WH13	75.9 defghi
CV11	76.7 defghij
LO16	79.6 efghijk
Q98-4-6	80.5 efghijkl
SH3	81.9 fghijkl
CH12	82.4 ghijkl
Q98-3-30	83.3 hijklm
RC17	83.6 ijklm
RC6	83.9 ijklm
MCC18	84.8 ijklmn
SM18	85.1 ijklmn
PRO12	85.6 jklmn
PM18	86.6 klmn
GTI	87.4 klmn
B14	87.6 klmn
FB6H	89.3 lmno
LE15	92.4 mnop
TG5	94.1 nop
Velvet Bentgrass	97.4 op
Creeping Bentgrass	99.3 p

* Means followed by the same letter are not significantly different from each other at p=0.05 according to Duncan's multiple range test based on seven replicates for each mean.

Table 2.4: Mean squares, degrees of freedom (DF) F values, and levels of probability of F from the analysis of variance (ANOVA) of the model for % injury of turfgrass ecotypes when inoculated with *M. nivale* in a cold chamber.

Source	DF	Mean Square	F Value	Pr > F
Model	69	652.7	8.61	<0.0001
Ecotype	31	1098.5	14.49	<0.0001
Block	6	278.3	3.67	0.0015
Incubator	1	5170.7	68.2	<0.0001
Ecotype*Incubator	31	133.6	1.76	0.0083
Error	378	75.8		

Table 2.5: Mean squares, degrees of freedom (DF) F values, and levels of probability of F from the analysis of variance (ANOVA) of the model for % injury of uninoculated turfgrass ecotypes in a cold chamber.

Source	DF	Mean Square	F Value	Pr > F
Model	65	1760.9	9.55	<0.0001
Ecotype	31	2006.3	10.88	<0.0001
Block	2	40.1	0.22	0.8050
Incubator	1	40382.5	219.04	<0.0001
Ecotype*Incubator	31	380.7	2.07	0.0027
Error	126	184.4		

Table 2.6: Regional and species variation in injury ratings (%) of *Poa annua* ecotypes and two *Agrostis* spp. inoculated with *M. nivale* after incubation in a cold chamber in the dark. Ecotypes were assessed weekly for six weeks during incubation.

Turf Origin	***% Injury***
US (7)	79.9 a*
Quebec (16)	86.9 b
Bentgrasses (2)	98.3 c

* Means followed by the same letter are not significantly different at p=0.05 according to Duncan's multiple range test. ($p<0.0001$).
** The single collection from western Pennsylvania was grouped with the Ontario collections since the climate there was considered to be most similar to that of southern Ontario rather than the U.S. east coast

Table 2.7: Percent (%) injury observed on uninoculated ecotypes under controlled conditions for six weeks with no light in cold chamber 1 set at 7°C. Ecotypes are ranked from least to most affected.

Ecotype	***Injury (%)***
WM9	31.8 a*
IS8BE	36.3 ab
Q98-4-21	36.3 ab
CV11	39.3 abc
SY2	43.8 abcd
Q98-6-30	43.8 abcd
Q97-1-10	52.5 bcde
Q98-3-12	56.5 cdef
Q98-3-6	56.5 cdef
SH3	60.5 defg
B14	62.5 defg
RH18	64.3 efgh
CH12	64.3 efgh
SM18	65.0 efghi
WM2	68.3 fghij
MCC18	68.3 fghij
Q98-4-6	69.0 fghij
Q98-3-30	69.8 fghij
Creeping Bentgrass	71.0 efghijk
GTI	72.3 fghijk
WH13	75.7 ghijkl
LO16	78.3 ghijkl
PM18	78.3 ghijkl
TG5	81.7 hijkl
HD17	81.7 hijkl
RC17	83.0 hijkl
PRO12	83.0 hijkl
FB6H	83.7 ijkl
RC6	86.3 jkl
MB11	89.0 kl
Velvet Bentgrass	89.0 kl
LE15	92.5 l

* Means followed by the same letter are not significantly different from each other at p=0.05 according to Duncan's multiple range test based on three replicates for each mean.

Table 2.8: Percent (%) injury observed on uninoculated ecotypes under controlled conditions for six weeks with no light in cold chamber 2 set at 3°C. Ecotypes are ranked from least to most affected.

Ecotype	***Injury (%)***
CV11	11.0 a*
Q98-4-21	13.0 ab
Q98-6-30	13.0 ab
IS8BE	13.7 ab
WM9	13.7 ab
Q97-1-10	15.7 abc
Q98-3-12	15.7 abc
Q98-3-6	15.7 abc
RH18	16.3 abc
Q98-4-6	18.3 abc
MCC18	18.3 abc
Q98-3-30	21.2 abc
SM18	27.2 abcd
PRO12	27.7 abcd
Creeping Bentgrass	28.5 abcde
SH3	33.0 abcdef
MB11	36.0 bcdefg
SY2	36.0 bcdefg
CH12	36.3 bcdefg
PM18	39.7 cdefg
WM2	46.3 defgh
HD17	50.5 defgh
RC17	52.3 efghi
TG5	53.8 fghi
FB6H	55.7 fghij
RC6	59.8 ghijk
B14	65.0 hijk
LO16	68.3 hijk
GTI	68.3 hijk
WH13	75.7 ijk
LE15	79.0 jk
Velvet Bentgrass	81.8 k

* Means followed by the same letter are not significantly different from each other at p=0.05 according to Duncan's multiple range test based on three replicates for each mean.

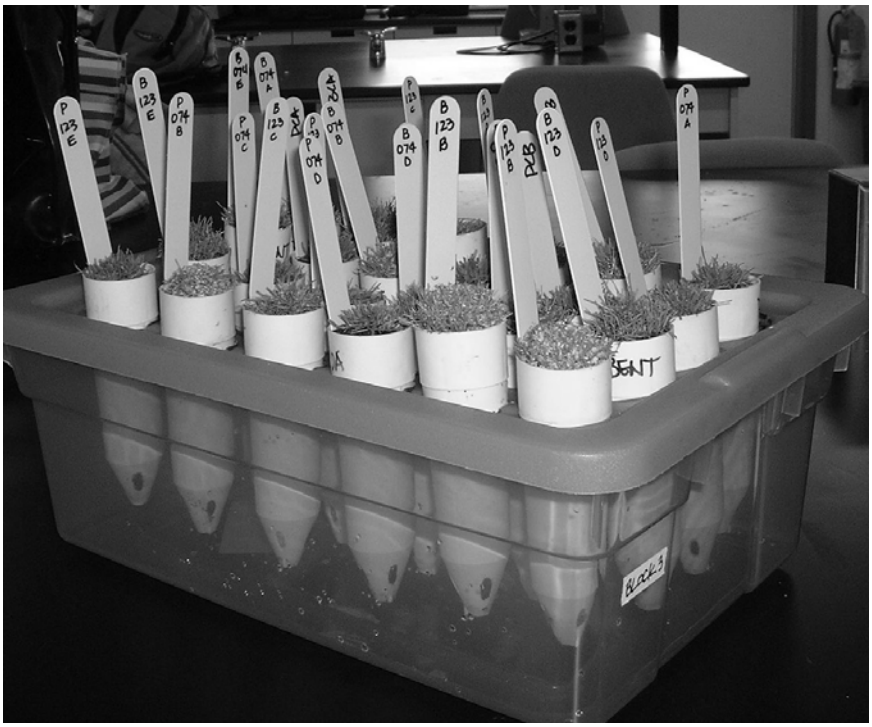

Figure 2.1: Example of block construction and arrangement for cold chamber protocol experiment.

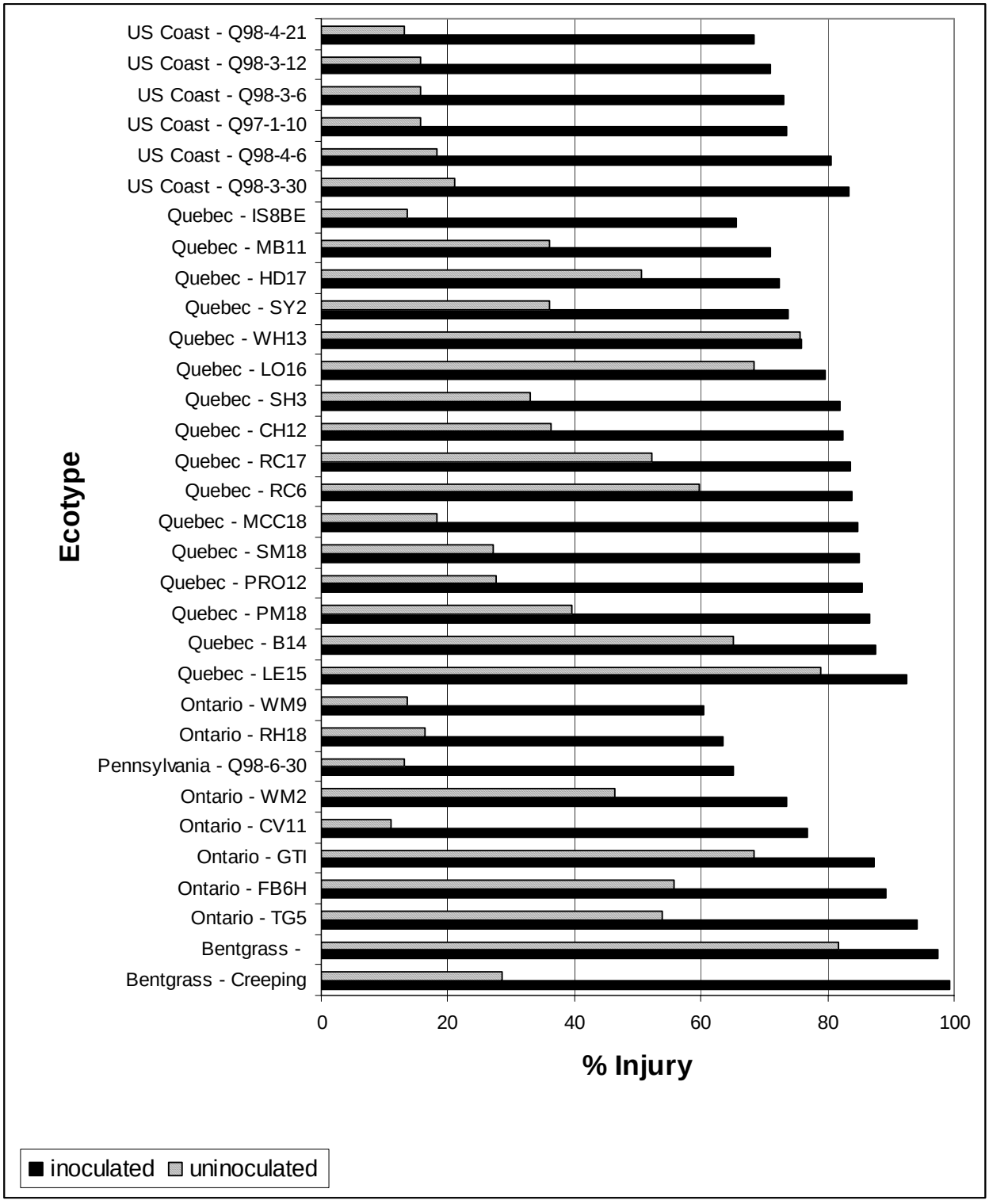

Figure 2.2: Percent (%) injury levels observed on inoculated and uninoculated plots in cold chamber 1 set at 7°C. Ecotypes are ranked from least to most affected by pink snow mould attack. Details about the ecotypes can be found in Table 1.1.

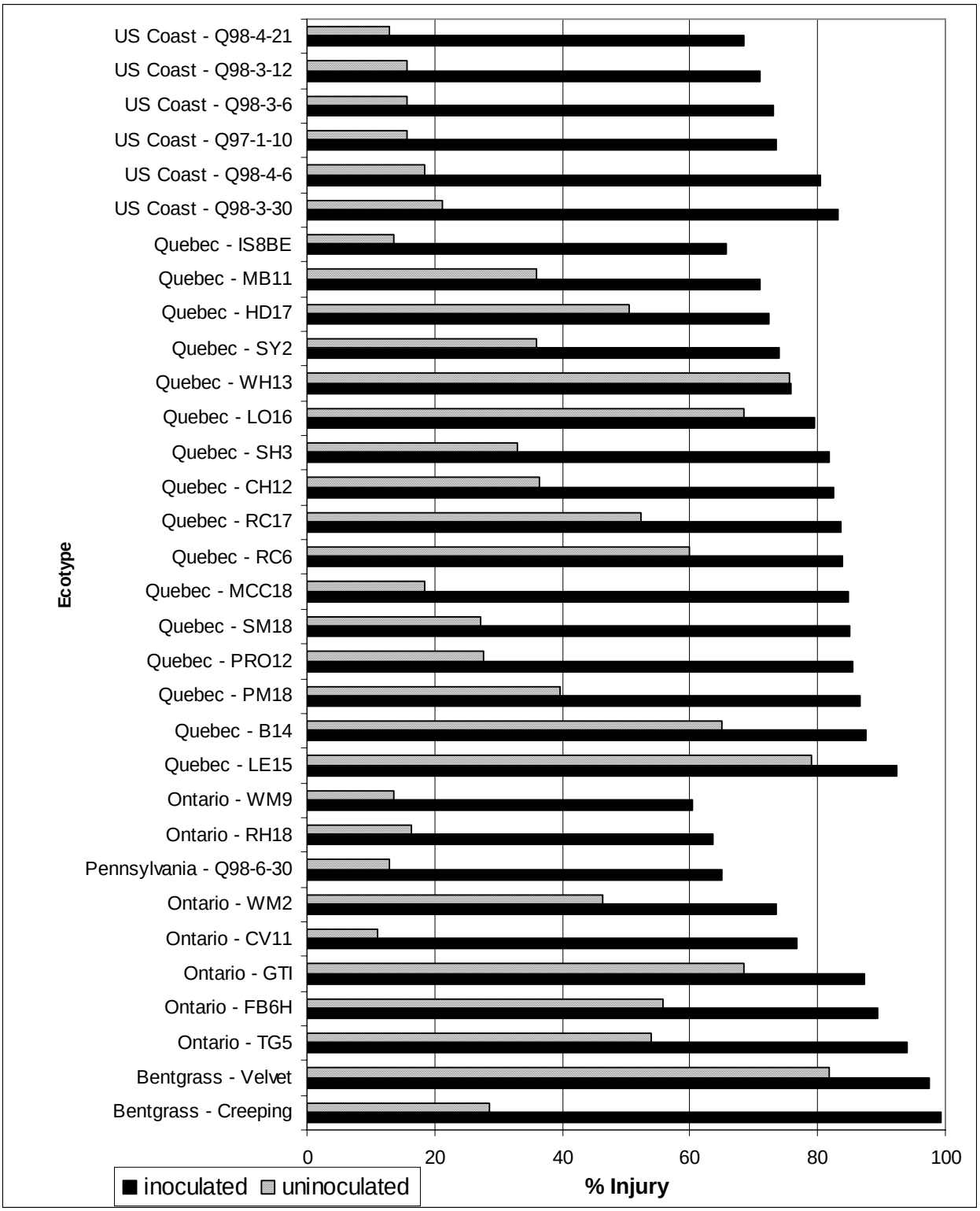

Figure 2.3: Percent (%) injury levels observed on inoculated and uninoculated plots in cold chamber 2 set at 3^{o}C. Ecotypes are ranked from least to most affected by pink snow mould attack. Details about the ecotypes can be found in Table 1.1.

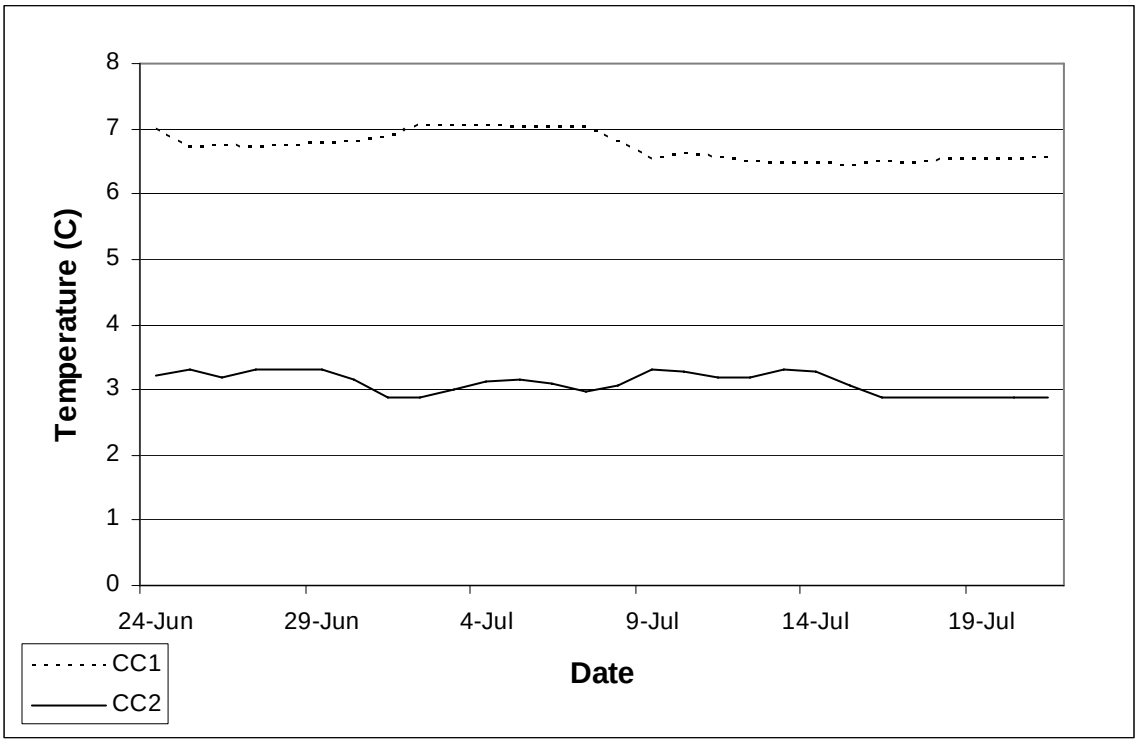

Figure 2.4: Temperature fluctuation in cold chamber 1 (CC1) and cold chamber 2 (CC2) during incubation of ecotypes.

General Discussion

The objectives of this study were to determine 1) if the level of resistance to pink snow mould was variable among ecotypes of *Poa annua* var. *reptans*; 2) how the resistance was manifested; 3) if levels of resistance vary between different regions of collection; and 4) if low temperature acclimation and incubation conditions affect the level disease resistance.

The results of both the field study and the cold chamber study indicated that there were significant differences in the level of injury apparent among ecotypes although none of the ecotypes were found to be fully resistant. The injury levels found in this study suggest that the tested ecotypes would not be suitable as seed sources for golf green maintenance, although these results are based on only one season of observation. Ecotypes may show varied performance under varying climatic conditions during the fall and winter. The performance of the most and least susceptible ecotypes confirms the presence of significant genetic variability among *P. annua* ecotypes that may allow the identification of highly resistant types with further screening. Many factors could have influenced the resulting injury levels of the ecotypes including cold hardening and environmental conditions. The full potential of the ecotypes will become more apparent with further study.

Results from our experiments concur with observations made in previous studies on pink snow mould resistance of winter cereals which found varietal differences based on visual assessment, biochemical analysis, and recovery ability (Nakajima and Abe. 1990, 1994, 1996; Meidener et. al., 1993; Hommo, 1994; Maurin et. al., 1996; Yoshida et. al., 1998). Significant differences were found in the level of injury expressed by the annual bluegrass ecotypes after snowmelt and in their speed of recovery.

Variation in injury levels immediately after snow receded may reflect genetic variability in susceptibility to *Microdochium nivale* among ecotypes. The use of

P. annua ecotypes with lower susceptibility on putting greens would help reduce the amount of damage found in the spring. The mechanisms that render certain ecotypes less susceptible are unknown since no biochemical analyses were conducted in this study. Environmental and genetic effects on the expression of pink snow mould resistance could be explored in future studies through biochemical and molecular analysis of ecotypes that have contrasted levels of resistance.

Variation also exists in the ability of ecotypes to recover after the epidemic. Recovery capacity is the ability of an ecotype to produce new growth after being infected with pink snow mould and is an important resistance mechanism (Meidener et al., 1993). A recovery period was used to determine survival rate for screenings of winter cereal cultivars. Nakajima and Abe (1990) assessed winter wheat plants for percent necrosis and percent survival after three weeks of recovery in a greenhouse under natural conditions. Meidener et al. (1993) compared snow mould injury among winter rye cultivars three weeks after snowmelt in the field and one week after being removed from the cold chamber. The maintenance of organic reserves in wheat crowns following snow mould infection may be an important aspect of snow mold resistance (Yoshida et al., 1998). The level of reserves available for spring growth is dependent on the amounts that were accumulated during cold hardening and their rate of utilization during winter (Gaudet et al., 1999). The ability of turfgrass to produce new growth in the spring despite being infected with pink snow mould is an important characteristic for putting green turf.

Generally, the ecotypes that had the lowest injury levels after snowmelt in the field study had the lowest injury levels after the recovery period (Q97-1-10, WM9, and LO16). This may indicate that the less susceptible ecotypes were more vigorous after snowmelt, which led to lower levels of injury after the recovery period. This may also indicate that these ecotypes were tolerant of the weather conditions during the recovery period, as well as any new *M. nivale* infections

that may have arisen. Similar statements could be made about the ecotypes that were most susceptible to the pathogen (RC17, MCC18, CH12, and Q98-3-30). After the recovery period, these ecotypes had the highest levels of injury. This may indicate that these ecotypes were significantly weakened by the disease, which influenced the condition of the ecotypes after recovery. There is also the possibility that these ecotypes had poor tolerance to abiotic stresses or subsequent *M. nivale* infections that occurred during the recovery period. The results of this study indicate that low susceptibility of *P. annua* ecotypes to the pathogen during the winter generally resulted in lower levels of injury after the recovery period. However, this was not true for all ecotypes. Ecotype WM9 was among the least susceptible after snowmelt, but injury levels after the recovery period were average. The ecotypes with the highest recovery rates (FB6H, SY2) were among the ecotypes with the lowest levels of injury after the recovery period, but were among the average for susceptibility to *M. nivale* after snowmelt.

Low susceptibility to *M. nivale* and quick recovery are both desirable traits for putting green turf. Combining these traits through breeding may help develop a superior seed source for turf managers. More resistant cultivars of annual bluegrass would help reduce fungicide use on the greens and also reduce the labour required to repair damage found in the spring in order to make the greens playable.

The area of collection also influenced the level of susceptibility of the ecotypes. Significant differences in snow mould resistance were observed between ecotypes originating from different regions. Ecotypes from Ontario exhibited the lowest levels of susceptibility in the field assay while the ecotypes from Penn State University were the least susceptible in the cold chamber experiment. The reason for this disparity is unknown. Genetic variation between ecotypes arises from the evolutionary pressures to which they were exposed. Natural selection occurs as a result of long term exposure to repeated stress that progressively eliminates unfit individuals. Observed differences in snow mold resistance

between regions of collections indicate that both macro- and micro-environments contribute to the selection pressures leading to snow mould resistance.

Disease resistance expressed by the annual bluegrass ecotypes screened (inoculated tests) was weakly correlated with the expression of resistance to winter stresses (uninoculated tests). This may imply that susceptibility and resistance to both types of stresses share common bases of adaptation. Studies on winter wheat found a relationship between cold tolerance and pink snow mould resistance in certain cultivars (Gaudet, 1994; Gaudet et al., 1999). Resistance to these stresses is believed to be influenced by carbohydrate accumulation during cold hardening and the depletion of reserves over the winter (Yoshida et al., 1998; Gaudet, 1994; Gaudet et al., 1999). Cold tolerance of annual bluegrass ecotypes has been linked to the accumulation of certain proteins during cold hardening (Dionne et al., 2001b). No biochemical analysis was conducted in this study, so the effects of cold hardening on susceptibility to pink snow mould are unknown.

The presence of genetic variability among the ecotypes in this study is very promising. Further study of these and other ecotypes of *P. annua* var. *reptans* may lead to important discoveries on the sources of disease resistance. Aesthetically unacceptable levels of injury were observed on all ecotypes in both the field and the cold chamber studies. Certain environmental conditions, such as cold hardening conditions present in the cold chamber and severe climatic conditions in the field during this study may have influenced the level of injury observed.

Weather conditions during the winter may have influenced winter hardiness levels of the ecotypes. It is likely that any stress that adversely affects the plant's metabolism is also likely to increase the plant's susceptibility to other stresses (Gaudet et. al., 1999). The more severe weather conditions experienced by the ecotypes in the field may have influenced their ability to withstand winter

stresses, including pink snow mould. Partial dehardening of ecotypes during brief warm periods during the winter or spring can also affect susceptibility of the ecotypes to pink snow mould. This would explain the high injury rates of both inoculated and uninoculated ecotypes in the field.

Results obtained from ecotypes studied in the field were not necessarily similar to those obtained from ecotypes in a cold chamber. Both approaches have certain advantages, and perhaps both should be used in combination in order to fully assess the genetic potential for resistance. Thus, further study with these ecotypes should be pursued both in the field and in the cold chamber to accurately evaluate the performance of the ecotypes. Field studies should include various microenvironments to highlight differences in susceptibility under different cold hardening and incubation conditions. On the other hand cold chamber experiments should try to mimic as much as possible the environmental conditions of interest in the field (Hommo, 1994). For instance, suboptimal conditions for cold hardening in the cold chamber study may have influenced the expression of pink snow mould resistance of *P. annua* ecotypes. Cold hardening in the cold chamber should follow the protocols established by Dionne et al. (2001a, b) to ensure maximum expression of resistance.

In future studies, a time course analysis of symptom development should be considered to help determine the level of disease that leads to significant injury to turf plants and how this threshold varies among ecotypes. Tissue sampling during the disease progress should be made to allow biochemical and molecular analyses. Comparative analysis of biochemical composition of the most and least susceptible ecotypes could allow the identification of cold- or disease-induced metabolic changes that are linked to ecotype susceptibility. These molecular analyses may also help identify resistance genes that determine the level of resistance. The identification of molecular markers could help hasten the screening process by quickly eliminating highly susceptible ecotypes that lack adaptive genes. Both the field study and the cold chamber study can examine

the ability of ecotypes to survive under prolonged stress conditions (Hommo, 1994) and should be used together to ensure that various attributes contributing to resistance or susceptibility of the ecotypes are taken into consideration. The existence of variability in the responses to pink snow mould attack reveals the genetic potential for pink snow mould resistance within the species. The analysis of additional sources of *Poa annua* var*. reptans* from various regions of North America will help uncover natural variants with superior levels of resistance.

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