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Cold-induced responses in annual bluegrass genotypes with differential resistance to pink snow mold (*Microdochium nivale*)

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ABSTRACT

Greens-type annual bluegrass (*Poa annua* L.) is susceptible to winter stresses including subfreezing temperatures and pink snow mold (SM). To better understand the mechanisms of SM resistance in annual bluegrass, four SM-resistant and four SM-sensitive genotypes were incubated at low temperature with *Microdochium nivale* (Fries) Samuels & Hallett, the causal agent of pink snow mold. We assessed the impact of a 6-week incubation period with SM at 2 °C under high humidity (≥98%) on the accumulation of cold-induced metabolites and on freezing tolerance. Incubation of annual bluegrass inoculated with SM lead to a major decrease in concentration of cryoprotective sugars such as sucrose and HDP (high degree of polymerization) fructans. Conversely, major amino acids linked to stress resistance such as glutamine and arginine increased in crowns of annual bluegrass in response to SM inoculation. One of the major differences between resistant and sensitive genotypes was found in the concentration of HDP fructans, which remained higher in SM-resistant genotypes throughout the incubation period. HDP fructans were also more abundant in freeze-tolerant genotypes, reinforcing their positive impact on winter survival of annual bluegrass. The identification of genotypes that are resistant to both SM and freezing shows the possibility of being able to improve both traits concomitantly.

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1. Introduction

Perennial biotypes of annual bluegrass [*Poa annua* L. forma *reptans* (Hausskn.) T. Koyama] dominate golf green putting surfaces in Canada and northern United States [1]. This unseeded species has been historically considered as a weed rather than as a usable turf-type grass with phenotypic variability reflecting potential sources of useful genes. However, efforts have been undertaken to develop commercial sources of greens-type annual bluegrass suitable for new putting green construction, overseeding or damage repair [2,3]. These breeding programs need to consider adaptation to environmental stresses in cultivar development to ensure the reliability and long-term survival of the seeded material.

Snow molds are the most prevalent and destructive winter diseases affecting cool-season turf in northern United States and in Canada [4]. Snow mold diseases often result in extensive damage to golf greens that significantly decrease the quality of putting surfaces for many weeks in the spring and cause major economic losses associated with green repairs and lost revenues. Although gray snow molds can cause more severe damage than pink snow mold (SM), the former require 3 months of snow cover to cause damage [5]. The psychrophilic fungus *Microdochium nivale* is the most widespread snow mold pathogen, causing damage on turf and forage grasses [6]. SM development is favored by high humidity and ambient temperatures between 0 and 7 °C [7] that can prevail underneath persistent snow cover or impermeable tarps placed on golf greens as protection against freezing damage. Alternatives to existing preventive applications of fungicides to control SM are required to implement reduced pesticide maintenance programs. The development of seed sources more resistant to freezing temperatures and SM is considered an effective and sustainable strategy to improve winter survival and quality of spring regrowth of turfgrass. We recently reported extensive genetic variability for SM resistance among a collection of 32 ecotypes sampled in eastern Canada, and genotypes that showed consistent resistance or sensitivity to SM in independent screenings were identified [8]. In addition, annual bluegrass possesses a large genetic variability for freezing tolerance [9].

The impact of SM on both quantitative and qualitative composition of carbohydrate and amino acids and on the freezing tolerance of cold-acclimated turfgrass is still largely undocumented. It has been reported that cultivars of winter cereals resistant to SM accumulate high levels of carbohydrates, and especially fructans [10]. In addition to being a carbohydrate reserve, fructans are thought

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Table 1
Plant material for assessment of biochemical changes.

Step	Duration (weeks)	Activity	Temperature (°C)	Relative humidity (D/N) (%)	Photon flux density (mol m^{-2} s^{-1})	Photoperiod (h)
Cold acclimation						
1	2	First stage of cold acclimation	2	55/70	250	8
2	2	Second stage of cold acclimation	−2	65/65	0	0
SM incubation						
		Assessment of biochemical changes 1; inoculation with SM				
3	1	Incubation	2	98/98	0	0
		Assessment of biochemical changes 2				
4	1	Incubation	2	98/98	0	0
		Assessment of biochemical changes 3				
5	2	Incubation	2	98/98	0	0
		Assessment of biochemical changes 4				
6	2	Incubation	2	98/98	0	0
		Assessment of biochemical changes 5				

to play important roles in protection against abiotic stresses [11]. Fructan concentrations have been associated with freezing tolerance [12,13] and SM resistance [10,14]. Large accumulations of glutamine and glutamate have also been shown to occur subsequently to infection by a pathogenic fungus in a resistant cultivar of grape [15].

The objectives of this study were to (i) compare changes in carbohydrates and amino acids between greens-type SM-resistant and SM-sensitive genotypes of annual bluegrass to better understand the mechanism of resistance; (ii) assess the relationship between SM resistance and freezing tolerance; and (iii) identify multi-stress resistant genotypes.

2. Materials and methods

2.1. Plant material and SM treatments

Four genotypes of greens-type annual bluegrass with high resistance (#5, 19, 20 and 27) and four genotypes with low resistance (#12, 25, 26 and 31) to SM were selected based on a screening described in Bertrand et al. [8]. Genotypes were vegetatively propagated and maintained in growth chambers at the Agriculture and Agri-Food Canada Research Centre in Québec City, Canada. Briefly, tillers were transplanted individually in a 164 mL-volume Cone-tainer (SC10 super cell low density, Stuewe and Sons, Inc., Corvallis, OR, USA) filled with sand amended with 20% peat (Topdress with peat for greens #02-01, Sols Champlain, Mascouche, QC, Canada) and placed in IPL Rigipots trays (IP110, Stuewe and Sons, Inc., Corvallis, OR, USA). Plants were grown for 5 weeks under the following environmental conditions: photoperiod, 16 h; day-time temperature, 22 °C; and night-time temperature, 16 °C. Artificial lighting was provided by a mixture of high pressure sodium and metal halide 400 W lamps (PL Light Systems, Beamsville, ON, Canada) with a photosynthetic photon flux density of 250 μmol photons m^{-2} s^{-1}. Plants were watered daily and fertilized once a week with 20–20–20 + micronutriments (1 g L^{-1}).

After the 5-week establishment period, plants were incubated for 2 weeks in a growth chamber set to 2 °C with 250 μmol photons m^{-2} $^{-1}$ photosynthetic photon flux density under 8 h photoperiod for acclimation to low temperatures. To induce a second stage of cold acclimation by simulating natural acclimating conditions in frozen soil under snow cover [16], plants were subsequently transferred to a programmable freezer set to −2 °C for an additional period of 2 weeks in the dark. At the end of the cold acclimation period, half of the plants were inoculated with a 0.01 g cm^{-2} mixture of four isolates of *M. nivale* grown for 2 weeks on wheat bran, dried, ground, and kept at −20 °C as described by Bertrand et al. [8]. Sterilized inoculum was similarly applied to control plants. Control and inoculated plants were incubated at 2 °C and ≥98% relative humidity in the dark. To maintain a high level of humidity, the trays containing annual bluegrass were covered with 2 layers of wet paper towels (Kimtuff wiper, Kimberly-Clark, Dallas, TX, USA), sprayed as needed with distilled water and placed in covered plastic boxes. The end parts of the paper towels were in constant contact with water at the bottom of the boxes to maintain wet towels by capillary action. Plant material and SM treatments are summarized in Table 1.

Following 42 days of incubation at 2 °C under 98% of humidity, the percentage of the 11.3 cm^2 area of each Cone-tainer tube covered with *M. nivale* mycelium, and the percentage of injury after 1 week of regrowth at 20 °C under a 16-h photoperiod following 42 days of incubation were assessed visually using a Horsfall–Barratt modified scale [8,17] (Table 2).

2.2. Tissue sampling

Cold acclimated plants (2 weeks at 2 °C followed by 2 weeks at −2 °C) were sampled for molecular analyses prior to inoculation, and after 1, 2, 4 and 6 weeks of incubation at 2 °C under high humidity in the dark, with or without inoculation with SM (Table 1). At each sampling, plants were washed free of soil with cold water and a pooled sample of approximately 0.5 g fresh weight of crown tissue (2 cm above and 1 cm below the transition zone between shoot and root) was harvested and immediately used for extraction. A subsample was oven dried 48 h at 70 °C for dry matter determination. Tissues were ground to a fine powder in liquid nitrogen, 5 mL of deionized water was added, and the samples were heated at 80 °C for 20 min. A 1-mL extract was stored at −80 °C until further analysis by high performance liquid chromatography (HPLC).

2.3. Soluble sugar determination

Carbohydrates were analyzed by HPLC. The HPLC analytic system was controlled by WATERS Empower software (WATERS,

Table 2
Percentage of tube surface covered with *Microdochium nivale* mycelium following 42 days of incubation at 2 °C under 98% humidity, and percentage of injury after 1 week regrowth following 42 days of incubation, both assessed using a Horsfall–Barrat modified scale [17].

Genotype #	Level of resistance	% SM coverage	% Injury
5	R	50.5	12.3
20	R	91.0	17.1
27	R	72.8	28.1
19	R	89.6	30.9
12	S	81.2	36.1
31	S	88.9	47.4
25	S	85.9	53.9
26	S	81.6	57.2

Milford, MA, USA) and was composed of a model 515 pump, a model 717plus autosampler, and a Model 2414 refractometer. Sucrose, glucose, and fructose were separated on a Waters Sugar–Pak column (Waters, Milford, MA, USA) eluted isocratically at 80 °C with deionized water at a flow rate of 0.5 mL min^{-1}. These carbohydrates were detected on a refractive index detector, and peak identity and quantity were determined by comparison to standards. Fructans were quantified as described in Pelletier et al. [18]. Briefly, low degree of polymerization (LDP) fructans was separated on a Bio-Rad HPX-42A column (Bio-Rad, Richmond, CA, USA), while high degree of polymerization (HDP) fructans were quantified with a Shodex KS-804 column (Shodex, Tokyo, Japan). Both columns were eluted isocratically at 25 °C with deionized water at flow rates of 0.5 and 1.0 mL min^{-1}, respectively. Degree of polymerization of HDP fructans was estimated by reference to a standard curve of pullulans (Shodex, P-82) ranging from 0.58×10^4 to 85.3×10^4 MW. The quantity of HDP and LDP fructans were determined by reference to a fructose standard curve and expressed in equivalent fructose units.

2.4. Soluble amino acid determination

Nineteen amino acids (aspartatic acid, glutamic acid, asparagine, serine, glutamine, histidine, glycine, threonine, arginine, alanine, tyrosine, γ-amino butyric acid, α-amino butyric acid, methionine, valine, phenylalanine, leucine, isoleucine, lysine) were analyzed by HPLC. Individual amino acids were separated on a Waters Radial-Pak RESOLVE C18 column (WATERS, Milford, MA, USA) eluted at 40 °C with a gradient of buffer A (0.05 M Na_2HPO_4, 0.05 M $NaCH_3COO$, 2% (v/v) methanol, 2% (v/v) tetrahydrofuran, pH, 7.5) and solution B (65% (v/v) methanol). Precolumn derivatization was made by the autosampler (Waters, model 717^+) by mixing 10 μL of the sample with 10 μL of a solution of 5 mg mL^{-1} of *o*-phtalaldehyde in 0.5 M K borate buffer, pH 10.0. Detection was made using a scanning fluorescence detector (Waters, Model 474) with an excitation wavelength of 338 nm and an emission wavelength of 425 nm [16]. Peak identity and amino acid quantity were determined by comparison to a standard mix containing the nineteen amino acids. Results from amino acid determination were expressed as concentrations on dry weight basis (μmol g^{-1} dry wt.).

2.5. Freezing tolerance

The freezing tolerance of the four SM-resistant and the four SM-sensitive genotypes was tested immediately after cold acclimation, without exposure to SM. In a separate assay, the impact of SM infection on freezing tolerance was determined by measuring the freezing tolerance of one SM-resistant genotype (#20) and one SM-sensitive genotype (#25) after 3 weeks of incubation at 2 °C and high relative humidity with or without SM inoculum. We choose to evaluate freezing tolerance after 3 weeks because this duration of incubation allows for significant mycelium development without apparent extensive SM damage [8].

Freezing tests were performed in a programmed freezer following a 16-h equilibration period at −2 °C according to a procedure described previously by Castonguay et al. [19]. Temperature was lowered by 2 °C per 30-min period followed by a 90-min plateau at each test temperature. Cold acclimated plants that did not undergo incubation at 2 °C were exposed to eight test temperatures ranging from −12 to −24 °C. Cold acclimated plants that were incubated with SM at 2 °C under high humidity, and their respective controls were tested at temperatures ranging from −6 to −18 °C. At the end of each temperature plateau, 12 Cone-tainers of each ecotype were withdrawn from the freezers and thawed at 2 °C for 24 h. Plants were then transferred to initial growth conditions.

Percentage of plant injury over the 11.3 cm^2 area of each Cone-tainer tube was assessed visually after 1 week of regrowth using a modified Horsfall and Barratt visual scale [17]. The 0–11 scale was expanded to accommodate one additional injury category at both ends of the scale, whereby 0.5 was used for injury from 0 to 1.5% and 10.5 for injury from 98.5 to 100%. Injury scores were transformed to their corresponding mid-percentage values in data analysis to estimate the 50% killing temperature (LT_{50}). The data were analyzed using a probit regression model with the independent variables "TSOL" and "genotypes", and the interaction between the two factors:

$$\text{Model}: P(y = 1) = \Phi(x'b)$$

where Φ = cumulative distribution function of the standard normal distribution and $x'b_i = b0_i + b1_i \times \text{Tsol}$ for the *i*th genotype.

The genmod procedure of the SAS program [20] was used to estimate the parameters of the model. An estimation of the values of "TSOL" that yields a response rate of 50% for each genotype was then calculated using these parameters ($TL50_i = -b0_i/b1_i$). The variance attributed to this TL50 was estimated by the delta method. The comparisons of TL50 of the p genotypes were made with the following Chi-square statistic:

$$\chi^2_{\text{obs}} = \sum_{j=1}^{p} \frac{(\text{TL50}_j - \text{TL50}_0)^2}{\sigma^2(\text{TL50}_j)},$$

where

$$\text{TL50}_0 = \frac{\sum_j \text{TL50}_j/\sigma^2(\text{TL50}_j)}{\sum_j 1/\sigma^2(\text{TL50}_j)}.$$

2.6. Statistical analysis

A two-way analysis of variance model with nested factors, with four replicates, was performed to study the effect of inoculation treatments and levels of resistance of the genotypes on biochemical compounds. Data were subjected to an analysis of variance using the MIXED procedure of SAS [20]. Inoculation treatments, level of resistance and sampling dates were considered as fixed factors. LS means comparisons were used to statistically compare inoculation treatments and levels of resistance.

3. Results

After 42 days of incubation at 2 °C under 98% humidity, *M. nivale* mycelium was covering between 50 and 91% of the surface of each tube of annual bluegrass, which resulted in an average of 22.1% of injury for resistant genotypes and of 48.7% of injury for sensitive genotypes (Table 2).

3.1. Carbohydrates

Changes in carbohydrate composition in crowns of annual bluegrass were monitored before inoculation in plants cold acclimated at −2 °C, and after 1, 2, 4, or 6 weeks of incubation at 2 °C in the presence (I) or absence (NI) of SM inoculum. Results show a progressive decrease in the concentrations of total soluble carbohydrates (TSC) in both controls (NI) and SM-inoculated (I) plants following their transfer from −2 °C to 2 °C in the dark (Fig. 1A). TSC reduction was in part due to a marked decrease in sucrose concentration during the first week of incubation at 2 °C (Fig. 1B). After 4 and 6 weeks of incubation, TSC concentrations were significantly lower in SM-inoculated plants than in uninoculated controls. SM-resistant genotypes generally maintained higher levels of TSC than SM-sensitive genotypes. A marked effect of inoculation was

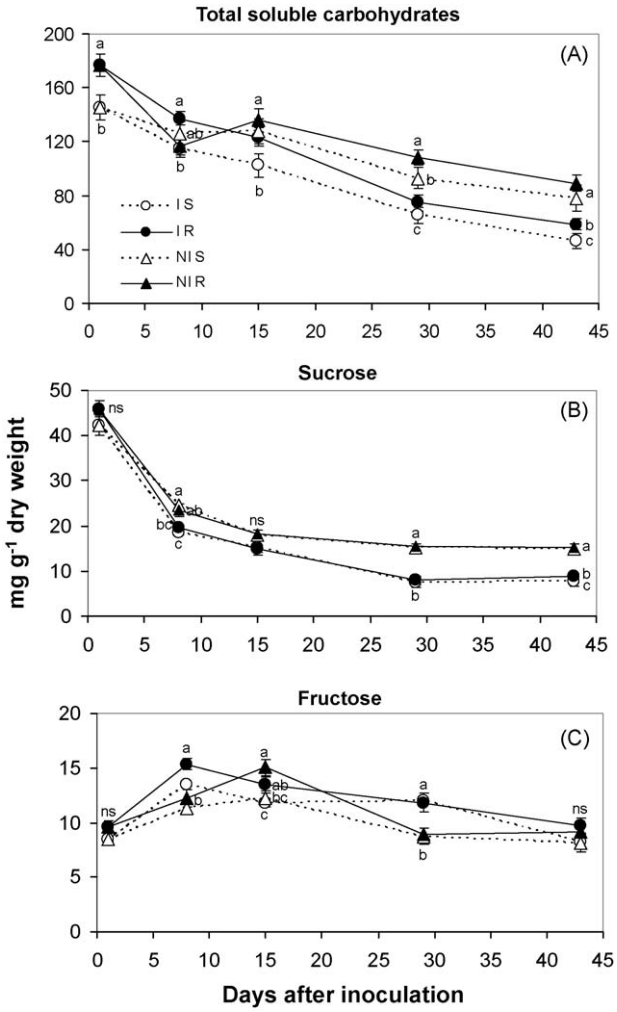

Fig. 1. Changes in total soluble carbohydrates (A), sucrose (B), fructose (C) in crowns of four SM-resistant and four SM-sensitive annual bluegrass genotypes incubated with or without *M. nivale*. All plants were cold acclimated at −2 °C followed by incubation at 2 °C under high relative humidity (≥98%). Carbohydrate concentrations were expressed as mg g^{-1} dry weight at 1, 2, 4, and 6 weeks of incubation. Data are the means of four independent samples ± standard error. Means with the same letters on a given sampling date are not statistically different at $P = 0.05$. IS: inoculated SM-sensitive; IR: inoculated SM-resistant; NIS: non-inoculated SM-sensitive; NIR: non-inoculated SM-resistant; ns: not significantly different.

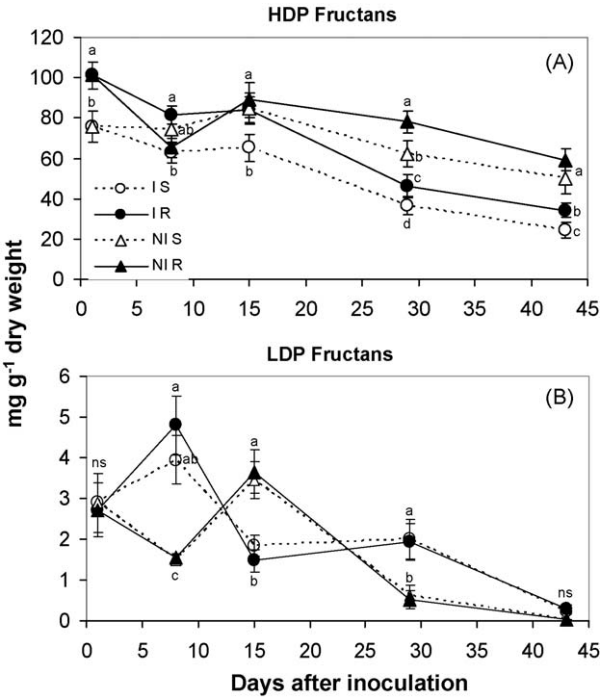

Fig. 2. Changes in HDP fructans (A) and LDP fructans (B) in crowns of four SM-resistant and four SM-sensitive annual bluegrass genotypes incubated with or without *M. nivale*. All plants were cold acclimated at −2 °C followed by incubation at 2 °C under high relative humidity (≥98%). Carbohydrate concentrations were expressed as mg g^{-1} dry weight at 1, 2, 4, and 6 weeks of incubation. Data are the means of four independent samples ± standard error. Means with the same letters on a given sampling date are not statistically different at $P = 0.05$. IS: inoculated SM-sensitive; IR: inoculated SM-resistant; NIS: non-inoculated SM-sensitive; NIR: non-inoculated SM-resistant. ns: not significantly different.

also observed with sucrose, whose concentrations were lower at the end of the incubation period in inoculated (8 mg g^{-1} dry wt.) than in control plants (15 mg g^{-1} dry wt.). Conversely, we observed a rapid increase in fructose concentrations during the first week of incubation at 2 °C that was of higher magnitude in inoculated (from 9 to 14.4 mg g^{-1} dry wt. on average) than in control plants (from 9 to 11.8 mg g^{-1} dry wt. on average). Interestingly, inoculated genotypes resistant to SM accumulated significantly higher fructose levels at the end of the first week of incubation, than SM-sensitive genotypes. Significant differences in fructose concentrations between inoculated and non-inoculated controls were also observed 4 weeks after inoculation, however, without significant differences between SM-resistant and SM-sensitive genotypes (Fig. 1C).

The chromatographic system we used allowed discrimination between fructans of high degree of polymerization (HDP) and fructans of low degree of polymerization (LDP). The major fraction of fructans in annual bluegrass was HDP fructans, with an average DP of 30 (mean of 68 mg g^{-1} dry wt.). LDP fructans, ranging from 3 to 9 fructose units, were much less abundant (mean of 2 mg g^{-1} dry wt.) in crowns of cold-acclimated annual bluegrasses (Fig. 2). Moreover, initial levels of HDP fructans were higher in SM-resistant than in SM-sensitive genotypes. Throughout the incubation period at 2 °C, we observed a gradual decrease in fructan concentrations which, however, remained significantly higher in uninoculated controls than in SM-inoculated plants. Furthermore, HDP fructans were more abundant in SM-resistant than in SM-sensitive genotypes regardless of the treatment. There was a significant increase of LDP fructans in inoculated plants in the first week of incubation that was followed by a rapid decrease afterwards (Fig. 2B). A similar response was also observed in uninoculated controls but was delayed by 1 week. At the end of the 6-week incubation period, LDP fructans were no longer detectable in crowns of annual bluegrass.

3.2. Amino acids

In contrast with what was observed with TSC, the transfer of plants from −2 to 2 °C promoted a large increase in the concentration of total free amino acids (TAA) from ≈100 to ≈250 μmol g^{-1} dry wt. at the end of the incubation period. TAA concentrations were significantly higher in inoculated plants than in controls at the first and the fourth week of incubation (Fig. 3A). Inoculated plants of SM-resistant genotypes had higher concentration of TAA than sensitive genotypes after 4 weeks of incubation with the inoculum (295 μmol g^{-1} dry wt. vs. 264 μmol g^{-1} dry wt.). Glutamine and asparagine concentrations significantly increased during the incubation and were by far the most important contributors to the observed increase in TAA concentrations (Fig. 3B and C). Glutamine was more abundant in inoculated than in control plants and, after 4 weeks of incubation up to the end of the experiment, its concentrations significantly differed between inoculated SM-resistant

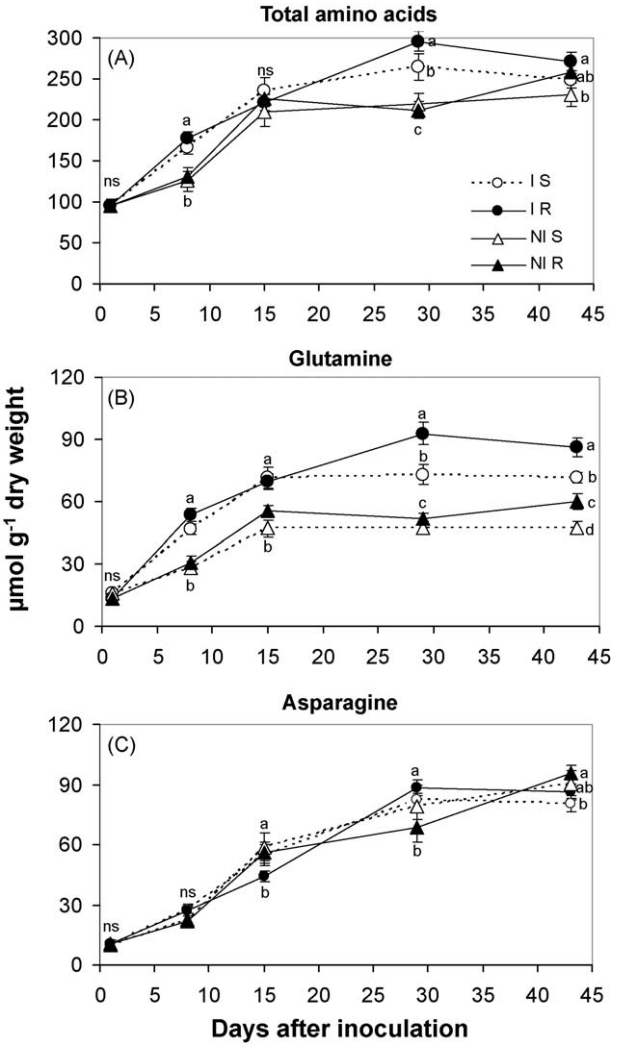

Fig. 3. Changes in total amino acids (A), glutamine (B), and asparagine (C) in crowns of four SM-resistant and four SM-sensitive annual bluegrass genotypes incubated with or without *M. nivale*. All plants were cold acclimated at −2 °C followed by an incubation period at 2 °C under high relative humidity (≥98%). Free amino acids concentrations were expressed as μmol g^{-1} dry weight at 1, 2, 4, and 6 weeks of incubation. Data are the means of four independent samples ± standard error. Means with the same letters on a given sampling date are not statistically different at $P = 0.05$. IS: inoculated SM-sensitive; IR: inoculated SM-resistant; NIS: non-inoculated SM-sensitive; NIR: non-inoculated SM-resistant. ns: not significantly different.

(93 μmol g^{-1} dry wt.) and SM-sensitive (73 μmol g^{-1} dry wt.) genotypes (Fig. 3B). Although asparagine concentrations markedly increased from ≈10 μmol g^{-1} dry wt. to nearly 100 μmol g^{-1} dry wt. at the end of the incubation period, they did not significantly differ between treatments and levels of resistance (Fig. 3C).

For all treatments, proline concentrations progressively decreased from 11.1 to 3.2 μmol g^{-1} dry wt. following inoculation and incubation at 2 °C (Fig. 4A). On the other hand, arginine concentrations were markedly contrasted between inoculated (10.1 μmol g^{-1} dry wt.) and control plants (2.1 μmol g^{-1} dry wt.) after 2 weeks of incubation (Fig. 4B). Moreover our results show an accumulation of the aromatic amino acids, tyrosine and phenylalanine that occurred earlier in inoculated than in control plants (Fig. 4C and D). Afterwards, tyrosine concentrations decreased progressively while the level of phenylalanine remained stable and did not differ between treatments except for a significant decline observed at the last sampling in the inoculated plants only.

3.3. Relationship between freezing tolerance and snow mold resistance

SM-resistant genotypes were on average significantly more tolerant to freezing than SM-sensitive genotypes. As shown in Fig. 5A, mean LT_{50} reached −15.4 °C for the four SM-resistant genotypes tested while the mean LT_{50} was −13.9 °C for the four SM-sensitive genotypes tested. There were however noticeable exceptions such as the SM-sensitive genotype #31 (−15.3 °C) which was significantly more tolerant to freezing than SM-resistant genotypes #5 (−12.9 °C) and #27 (−13.9 °C). To assess the impact of SM infection on freezing tolerance, we measured the LT_{50} for both inoculated and uninoculated plants of the most SM-resistant genotype (#20) and the most SM-sensitive genotype (#25). Our results show that after 3 weeks of incubation at 2 °C, SM infection induced a decrease of freezing tolerance (Fig. 5B) with a significant lower level of freezing tolerance for SM-sensitive genotype #25 as compared to SM-resistant genotype #20. Conversely, both SM-resistant and SM-sensitive genotypes acclimated to cold at subzero temperature (−2 °C) were more tolerant to freezing than plants incubated at 2 °C.

4. Discussion

In northern climates, overwintering grasses and cereals must survive injury caused by subfreezing temperatures and snow mold development under long-lasting snow cover [21]. Inoculation with *M. nivale* has revealed significant genetic variation in SM resistance in both forage grasses and winter rye [22]. Genotypic variation in snow mold resistance has also been documented in winter wheat [23,24]. Casler et al. [25] demonstrated that genetic variation exists within creeping bentgrass for pest resistance and stress tolerance. A recent assessment of genetic diversity for SM resistance among genotypes of annual bluegrass collected in eastern Canada revealed large genetic diversity for that trait within this species [8]. These findings indicate that improvement of SM resistance through selection is a practical approach that could be extended to turfgrass species.

It is already known that some plants exhibit a phenomenon called cross-adaptation whereby exposure to a given stress confers tolerance to other stresses [26]. In cocksfoot, however, the increased freezing tolerance of a polycross group selected for improved freezing tolerance did not show a parallel increase in resistance to SM [27]. In an attempt to decipher the link between freezing tolerance and snow mold resistance, Yoshida et al. [14] investigated distinct patterns of accumulation of metabolites typically associated with cold hardening in autumn and early winter between freezing tolerant and snow mold-resistant cultivars of winter wheat. To identify molecular traits associated with SM resistance and the potential impact of these low temperature pathogens on freezing tolerance, we measured several cold-induced metabolites in SM-inoculated genotypes of annual bluegrass of contrasted resistance to SM as described in the following paragraphs.

4.1. Carbohydrates and SM resistance

We observed significantly lower levels of TSC in SM-inoculated plants of annual bluegrass than in the respective controls. A similar decrease in soluble sugars has been observed in tomatoes after infection with *Botrytis cinerea* [28] and with sunflower following infection with *Sclerotinia sclerotiorum* [29]. In accordance with the early observations of Fiyomoto and Bruehl with wheat [30], the concentration of soluble sugars remaining at the end of the experiment was higher in non-inoculated than in inoculated annual bluegrass. Furthermore, the concentration of soluble sugars remaining at the end of a 6-week incubation with SM was higher in SM-resistant

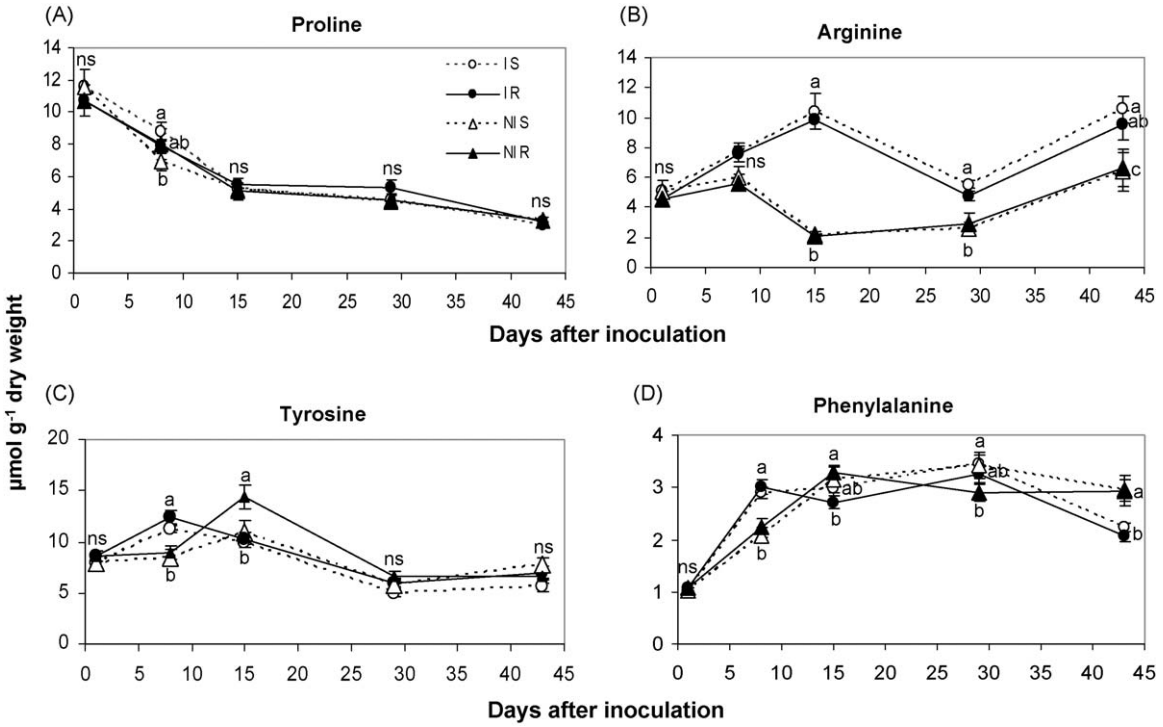

Fig. 4. Changes in proline (A), arginine (B), tyrosine (C), and phenylalanine (D) in crowns of four SM-resistant and four SM-sensitive annual bluegrass genotypes incubated with or without *M. nivale*. All plants were cold acclimated at −2 °C followed by an incubation period at 2 °C under high relative humidity (≥98%). Free amino acids concentrations were expressed as µmol g^{-1} dry weight at 1, 2, 4, and 6 weeks of incubation. Data are the means of four independent samples ± standard error. Means with the same letters on a given sampling date are not statistically different at $P = 0.05$. IS: inoculated SM-sensitive; IR: inoculated SM-resistant; NIS: non-inoculated SM-sensitive; NIR: non-inoculated SM-resistant. ns: not significantly different.

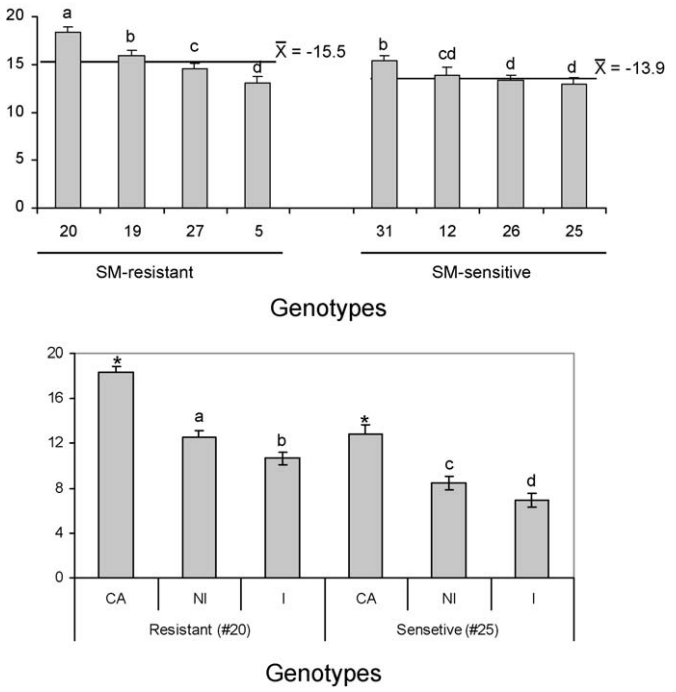

Fig. 5. Relationship between the lethal temperature killing 50% of the plants (LT_{50}) and snow mold resistance. (A) Four SM-resistant and four SM-sensitive genotypes were tested. The mean LT_{50} values for SM-resistant or sensitive genotypes were significantly different (−15.4 and −13.9 °C respectively). (B) Effect of inoculation on freezing tolerance (LT_{50}) of a SM-resistant and SM-sensitive genotype. Plants were tested immediately after a 2 weeks acclimation period at −2 °C and after a subsequent incubation at 2 °C and high relative humidity (≥98%) in the presence (I) or absence (NI) of SM inoculum. Calculated upper and lower 95% confidence limits for LT_{50} values are indicated. LT_{50} means with confidential limits that overlap are not statistically different at $P \leq 0.05$ and are represented by the same letters.

than in SM-sensitive genotypes as was previously observed with genotypes of *Festolium* contrasting in their SM resistance [31]. Sugar depletion during infection could be explained by an increased use of sugar both by the plants, for the activation of a cascade of defense reactions that requires additional energy, and by the fungi which divert plant sugars for their own growth.

A detailed analysis of individual carbohydrates revealed a rapid decrease of sucrose concentrations that was more pronounced in SM-inoculated plants than in controls. This initial decline of sucrose was accompanied by an accumulation of fructose that reached higher concentrations in SM-inoculated plants after the first week of incubation at 2 °C. A similar response has previously been observed in other plant-pathogen interactions and has been attributed to the induction in invertase activity that catalyses the hydrolytic cleavage of sucrose into hexoses [32]. Such induction of cell wall invertase activity following infection could trigger the up-regulation of sugar-responsive *PR* genes [33]. Our observation of an increase in fructose concentrations of higher amplitude in inoculated plants of SM-resistant genotypes is in agreement with its putative involvement as a signalling molecule regulating gene expression [34]. Even though the concentration of HDP fructans decreased during the long-term incubation of annual bluegrass at 2 °C, their depletion was significantly greater in inoculated plants than in controls.

It is noteworthy that regardless of the inoculation treatment, SM-resistant genotypes retained more HDP fructans than SM-sensitive genotypes at the last two sampling dates. A positive correlation between snow mold resistance and fructan concentrations content retained after incubation under a snow cover has also been observed with wheat [14] and *Aegilops cylindrica* [35]. Our results are in agreement with the suggestion by Gaudet et al. [10] that grasses resistant to snow molds metabolize fructans at a lower rate allowing them to maintain higher concentrations. Whether maintenance of higher HDP fructans confers direct pro-

tection against SM or if it is merely a reflection of the SM-resistance trait is still unknown. Snow mold fungi could be less able to metabolize fructans polymers as compare to mono- and disaccharides. Perhaps, genotypes that maintain a higher proportion of TSC in the form of fructans have more carbohydrate reserves available for regrowth. A dual role has been proposed for fructans in stress resistance: a source of carbon and energy for the synthesis of osmoprotectans and spring regrowth, and an intermediate in a sugar-sensing mechanism of disease resistance [10]. This dual role might occur in annual bluegrass based on our observations of the differential accumulation of HDP and LDP fructans in response to SM infection.

Similarly to what we observed with fructose, the concentration of LDP fructans increased rapidly, up to four fold, during the first week following infection with SM, and reached higher levels in resistant genotypes. There is evidence that soluble sugars can act as metabolic activators of the sugar-sensing hexokinase, which initiates signal transduction and activation of genes including host defense genes [36,37]. The observations that oligosaccharides involved as signalling molecules contain 8–16 monosaccharides units [38] and accumulate rapidly following inoculation, support a possible role for LDP fructans as signalling molecules. Higher LDP fructan concentrations in SM-resistant than in SM-sensitive genotypes in the early stage of infection may promote an earlier and more sustained activation of defense mechanisms while higher levels of HDP fructans remaining after SM incubation would further support spring regrowth of resistant genotypes.

4.2. Amino acids and SM resistance

In agreement with the general metabolic responses of fungus-infected plants [39], we observed a progressive increase in the concentrations of free amino acids during the first 4 weeks of incubation. This has been attributed to increased respiration rates and proteolysis in response to infection [39]. Glutamine was a major contributor to the observed increase in the pool of free amino acids. In an early experiment, Sadler and Scott [40] observed a large increase in glutamine in barley leaves infected with powdery mildew. It has been shown that plants remobilize and export nutrients away from the infection site by N recycling into glutamine through the action of glutamine synthetase. Recent observations of an increased induction of glutamine synthetase-encoding gene (*GS1*) in *Nicotiana tabacum* by avirulent strains of *Pseudomonas* led Pageau et al. [41] to conclude that *GS1* could function as a defense gene in a metabolic safeguard form of resistance. Another major contributor of the increased pool of amino acids is asparagine, whose concentration increased from 10 to 100 μmol g^{-1} dry wt. during the incubation period at 2 °C. Like glutamine, asparagine is a major form for the redistribution of N reserves within the plant. Thus, the steady increase in both glutamine and asparagine in all plants following the transfer from −2 to 2 °C is indicative of extensive N recycling under these conditions. However, glutamine increase was significantly higher in SM-inoculated plants than in controls, and in SM-resistant than in SM-sensitive genotypes. These differential responses could be reflective of extensive de novo protein synthesis required to withstand pathogen infection.

We also observed markedly higher accumulations of arginine in inoculated than in non-inoculated plants. Arginine is a precursor of polyamines that have been involved in the regulation of plant development and stress tolerance [42,43]. The increase in arginine concentrations in response to inoculation is another indication of the deployment of plant defense mechanisms. The observation of a rapid increase of phenylalanine, an aromatic amino acid which concentration has been shown to be correlated with phenylalanine ammonia lyase activity [44] and which acts as precursor for the biosynthesis of phenylpropanoid compounds [45], also points out at an up-regulation of plant defense capabilities in response to infection with SM. Tobacco plants overexpressing the enzyme phenylalanine ammonia lyase, the first enzyme in the pathway leading to the transformation of phenylalanine into phenylpropanoids, showed less sensitivity to infection with the fungal pathogen, *Cercospora nicotianae* [46]. The pattern of increase of tyrosine, another aromatic amino acid related to defense mechanisms against biotic stress, was similar to phenylalanine in the first week following SM infection. Both phenylalanine and tyrosine are end products of the shikimate pathway and are precursors in the production of secondary defense metabolites including phenylpropanoid compounds [47]. Proline concentrations significantly declined throughout the incubation period but did not respond to SM infection nor did it noticeably differ between SM-resistant and SM-sensitive genotypes. Although proline has been related to freezing tolerance in turfgrass [48], our results do not support a determinant role of proline with regard to SM resistance.

4.3. Relationship between SM resistance and freezing tolerance

Our results show that, among the genotypes tested, the SM-resistant genotypes were, on average, more freezing resistant and accumulated more HDP fructans than the SM-sensitive genotypes. Recently, the concentration of HDP fructans has been shown to be closely related to the level of freezing tolerance of annual bluegrass [9]. Our results extend their adaptive value in overwintering plants to their contribution to SM resistance. Yoshida et al. [14] also observed that SM-resistant cultivars of winter wheat maintained a higher proportion of soluble carbohydrate as fructans.

The capacity of fructans to stabilize membranes by interacting with lipid headgroups [13,49] and their contribution as a source of energy for regrowth could be key traits to confer long-term survival under snow cover. After their transfer to 2 °C, turfgrasses progressively loss key cryoprotective metabolites such as HDP fructans, sucrose and proline. Sucrose reached lower concentrations in inoculated than in uninoculated plants which were also less freezing tolerant, an observation in accordance with its role as a cryoprotectant that prevents freeze-induced membrane lesions [50]. Proline depletion during the time course of the experiment when plants were partially loosing their cold acclimation confirms the association between proline concentration and the level of freezing tolerance achieved by the plants [51,52]. Enhancement of SM resistance by cold acclimation has been reported for cereals and grasses [10,53,54]. Conversely we report in the current study, a SM-induced reduction of freezing tolerance in annual bluegrass. This is similar to the observation of Gaudet and Chen [55] that sublethal damage by cottony snow mold decreased the freezing tolerance of winter wheat. It also supports the conclusion of Gaudet et al. [10] that any stress that adversely affects the plant metabolism is also likely to reduce its resistance to subsequent stress.

4.4. Conclusion

This study demonstrates that several cold-induced metabolic changes are affected by SM infection in annual bluegrass. For instance, concentrations of cryoprotective sugars such as sucrose and HDP fructans decreased, whereas amino acids like glutamine and arginine accumulated following infection with SM. Taken together, our results indicate that HDP fructans are closely associated with both snow mold resistance and freezing tolerance in annual bluegrass and that they could be used as indicator of resistance. We conclude that it is possible to identify and select for genotypes that possess cross-resistance to both freezing and pink snow mold.

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