Components of Pink Snow Mould Resistance in Winter Wheat are Expressed Prior to Cold Hardening and in Detached Leaves

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Keywords: detached leaf assay, developmental stage, *Microdochium nivale*, partial disease resistance, *Triticum aestivum*

Abstract

Resistance to pink snow mould, caused by *Microdochium nivale*, was investigated in four resistant winter wheat lines from the USDA World Cereal Collection (CI9342, CI14106, PI173440 and PI181268) and three Nordic wheat lines (Bjørke, Rida and V1004). Pink snow mould resistance was tested in non-hardened and cold-hardened plants incubated under artificial snow cover and in detached leaf segments mounted on water agar and incubated at either 3°C in darkness or at room temperature with light during the day. The wheat lines CI9342, CI14106 and PI181268 were more resistant than the Nordic lines, both before and after cold hardening. Thus, although cold hardening strongly increases the level of snow mould resistance in all the wheat lines, some resistance mechanisms are also present prior to cold hardening in some of the resistant lines. CI9342, CI14106 and PI181268 also had a higher level of resistance than the other lines in the detached leaf assay, indicating that these lines have some resistance mechanisms acting in the leaves. The resistance of PI173440 was expressed only in intact hardened plants and not in non-hardened plants or in detached leaves. This indicates that this line relies on cold hardening-related changes in the crown for its resistance. In the detached leaf assay the rate of lesion development varied greatly between leaves of different order. The highest correlation with the whole plant test was obtained when using secondary leaves and incubation at 3°C in the dark.

Introduction

*Microdochium nivale* (pink snow mould) is one of several fungi that can cause snow mould on cereals and grasses covered by snow. *Microdochium nivale* can also cause other diseases, e.g. fusarium head blight, seedling blight and crown rot. Resistance to snow moulds in cereals and grasses increases dramatically during cold hardening (Ärsvoll, 1977; Tronsmo, 1984a; Gaudet and Chen, 1987; Nakajima and Abe, 1996). In addition, snow mould resistance is dependent on the developmental stage of the plants. In wheat, young seedlings at the pretillering stage have a certain level of snow mould resistance. This level drops transiently and then increases with the development of the plants (Gaudet and Chen, 1987; Gaudet and Kozub, 1991; Nakajima and Abe, 1996). The increase in snow mould resistance with both developmental stage and cold hardening is thought to be related to the accumulation of carbohydrate reserves (Bruehl and Cunfer, 1971; Kiyomoto, 1987; Bengtsson, 1989; Gaudet et al., 1999, 2000, 2001) and particularly the increase in the proportion of fructan (Abe and Yoshida, 1997; Gaudet et al., 2001), which is a main carbohydrate storage polymer in Poaceae species in the temperate and cold zones (Meier and Reid, 1982). A large carbohydrate storage increases the ability of plants to sustain longer under snow cover, may enable plants to tolerate the presence of snow mould fungi in their tissues and/or may ensure the necessary resources for defence reactions against infection at the end of the winter, the time when snow moulds usually develop. How fructan in particular may play a role in snow mould resistance is unclear, but snow mould fungi might be less able to metabolize fructan polymers compared to mono- and disaccharides (Gaudet et al., 1999). Cold hardening of plants also induces resistance to fungal pathogens that infect during the growing season, suggesting that some general defence-related trait is present in cold hardened plants (Tronsmo, 1984b; Tronsmo et al., 1993). Accumulation of defence-related proteins is thought to be involved in cold hardening-induced disease resistance (Tronsmo et al., 1993; Hon et al., 1995; Ergon et al., 1998; Hiilovaara-Teijo et al., 1999; Kuwabara et al., 2002; Gaudet et al., 2003a,b). Reduced cell water potential may also play a role (Bruehl and Cunfer, 1971; Tronsmo, 1986).
In the 1960s a number of snow mould resistant winter wheat lines in the USDA World Cereal Collection were identified (Bruehl, 1982). The resistance of these lines is thought to be related to a very rapid and strong accumulation of carbohydrates, particularly fructan, during hardening as well as a low rate of consumption during winter (Bruehl and Cunfer, 1971; Kiymoto and Bruehl, 1977; Kiymoto, 1987; Bengtsson, 1989; Abe and Yoshida, 1997; Yoshida et al., 1998; Gaudet et al., 2000, 2001). Resistance also appears to be somewhat correlated with a higher degree of polymerization of fructan (Gaudet et al., 2001). To our knowledge, resistance mechanisms of the resistant lines have only been studied in cold hardened plants. In addition to hardening-dependent resistance mechanisms, resistant material may also depend on mechanisms present prior to hardening. Such mechanisms could be the same as, or different from, those induced by cold hardening.

Whole plant tests of snow mould resistance in controlled environments are particularly time-consuming and costly, since the plants have to be incubated for many weeks at low temperature. Although faster screening at higher temperatures may be feasible (Nakajima and Abe, 1990) it is still difficult to hit the appropriate incubation length for the differentiation between genotypes. Several incubation periods are therefore commonly used in parallel, increasing the size of the experiments. Detached leaf assays for pathogen resistance are faster and cheaper than assays using intact plants. A detached leaf assay for detection of components of resistance to fusarium head blight (Diamond and Cooke, 1999; Browne and Cooke, 2004, 2005; Browne et al., 2005).

The aim of the present study was (1) to test if some of the snow mould resistant winter wheat lines identified in the USDA World Cereal Collection partly rely on resistance mechanisms present prior to cold hardening and (2) to investigate whether hardening-dependent, age-dependent and genotype-dependent pink snow mould resistance expressed in intact plants are also expressed in detached leaves. Information on this will clarify to what extent the different forms of resistance are dependent on the crown, and will indicate whether there is a potential for the use of detached leaf assays in practical breeding of resistance to snow moulds.

Materials and Methods

Plant material and inoculum

Seven winter wheat (Triticum aestivum L.) lines were used in this study; the Norwegian cultivar Rida and breeding line V1004 (Graminor AS, Ilseng, Norway), the Swedish cultivar Bjørke (Svalöf Weibull AB, Svalöv, Sweden), and four snow mould resistant lines selected from the USDA World Cereal Collection; CI9342 (originating from Russia), PI173440 (originating from Turkey), PI181268 (originating from Afghanistan) and CI14106 (unknown origin) (Bruehl, 1982). Seeds (three per pot) were sown at 2–3 cm depth in 10 cm pots containing soil (‘P-jord’; L.O.G., Oslo, Norway). Four experiments were performed. In experiments 1 and 2, seeds were sown directly, while in experiments 3 and 4, seeds were pregerminated for 2 days in petri dishes on the laboratory bench prior to seeding. Three different growth regimes produced young non-hardened plants (Y), older non-hardened plants (O), and cold hardened plants (H). In experiments 1 and 2, plants were grown under non-hardening conditions in a growth chamber for two (Y, H) or three (O) weeks at 18°C day/12°C night, 16 h photoperiod and a light intensity of 250 µmol/m²/s (HPI bulbs, Philips, the Netherlands). H plants were subsequently placed at 2°C with a 16 h photoperiod and a light intensity of 180 µmol/m²/s (Philips HPI) for 2 weeks. In experiments 3 and 4, plants were grown in a greenhouse at Ås (59°51’N, 10°40’E), Norway, in June and August, respectively, with 16 h additional light of approximately 100 µmol/m²/s (Powerstar HQ1-BT, Osram GmbH, Munich, Germany). The plants grew faster in experiments 3 and 4 than in the first two experiments, and they were therefore grown for only 8 (Y, H) or 13 (O) days. The additional cold hardening treatment of H plants was as in experiments 1 and 2. Prior to inoculation of whole plants and detached leaves, 3–5 random pots of each cultivar and growth regime were sampled for the measurement of fresh and dry weights of shoots, including the crown. The developmental stage (number of leaves) was also recorded (Table 1). Inoculum of M. nivale (Fr.) Samuels and Hallet (teleomorph Monographella nivealis (Schaffnit) E. Müller] isolate 5/93 (Plant Protection Centre, Ås, Norway) was prepared from mycelial PDA disks stored at ~80°C. A mycelial suspension containing gelatine (2 g/l) was prepared as described by Tronsmo (1993).

Whole plant test

A whole plant test of resistance to pink snow mould was performed in all four experiments. The pots were inoculated into four inoculated and four non-inoculated blocks per incubation period. In experiments 1 and 2 the blocks consisted of one pot of each wheat line and growth regime, giving a total of 21 pots. This design allows for a comparison of both growth regimes and wheat lines. In experiments 3 and 4, plants of different growth regimes were placed in separate blocks, giving four inoculated and four non-inoculated blocks per growth regime, each with one pot per wheat line. With this design, plants from different growth regimes cannot be compared. Inoculated blocks were sprayed with the mycelial suspension (0.5 ml per pot), while the non-inoculated blocks were sprayed with gelatine (2 g/l) only. The pots were incubated under moist cellulose wadding and plastic sheets at 2°C in the dark, the pots belonging to the same block being under one common ‘snow cover’. In an attempt to obtain maximum differentiation between treatments, incubation
periods were chosen according to the disease progression observed. In experiments 1 and 2, two incubation periods were used: 5 and 7 weeks in experiment 1, and 6 and 9 weeks in experiment 2. In experiments 3 and 4, only one incubation period was used, and the length of this period differed between plants from the three growth regimes. The blocks were incubated for 6 (Y), 6.5 (O) and 15.5 (H) weeks in experiment 3 and for 4.5 (Y), 5 (O) and 10 (H) weeks in experiment 4. After incubation, pots were transferred to the greenhouse, where the temperature was approximately 16°C. Additional light (100 μmol/m²/s) was given 16 h a day. Plants were cut at 7 cm above soil level and allowed to regrow for 1 week. Plants were then cut at soil level, and the fresh and dry weight of shoot material in each pot was recorded. Resistance was calculated as the relative regrowth, that is, the ratio between the fresh weight of inoculated and non-inoculated plants.

Detached leaf assays
Detached leaf assays were performed in experiments 3 and 4 only. Leaves were sampled from three replicate pots from each growth regime and wheat line (other pots than those used for the whole plant test). The first, second and third (if present) leaf blade were cut off at the base and kept between moist tissue paper. The term 'leaf class' will be used for the growth regime and leaf number combinations. Four leaf segments, one from each of four leaves, were placed on 0.5% water agar in petri dishes with the adaxial side facing up. Four petri dishes per leaf class and wheat line were inoculated with an 8 μl droplet of inoculum, placed on the middle of each segment. Two petri dishes per leaf class and wheat line were mock inoculated with an 8 μl droplet of gelatine (2 g/l). Half of the petri dishes were incubated at room temperature (RT) on the lab-bench with light during the day and the other half were incubated at 3°C in darkness. Observations of the length of water-soaked lesion, the length of mycelial colony on the leaf surface (under incubation at RT in light only) and the presence of water-soaked background was made at 3 days after inoculation (d.a.i.) in petri dishes incubated at RT in light and at 7, 9 and 13 and d.a.i. in petri dishes incubated at 3°C in darkness. Average lesion and colony length was calculated for each petri dish and used in the statistical analysis.

Data analysis
Shoot weight prior to inoculation, relative regrowth, length of water-soaked lesion and length of mycelial colony were subjected to analysis of variance and correlation analysis in the statistical program package SAS 8.2 (SAS Institute Inc., Cary, NC, USA), using PROC GLM and PROC CORR.

Results
Whole plant test
The effect of inoculation on regrowth of plants after incubation depends on the length of the incubation period. In experiment 1, inoculated plants had a significantly lower regrowth than non-inoculated plants after the 7 weeks incubation period, but not after the 5 weeks incubation period. The effect of inoculation after the long incubation period was present only in Y plants and not in O or H plants (Table 2). In the other experiments the effect of inoculation was significant after all incubation periods and within all growth regimes. H plants had a significantly better relative regrowth, and thus a higher degree of pink snow mould resistance, than Y plants while the relative regrowth of O plants differed between experiments (Table 3). In experiment 1, in which there was a low disease pressure, O plants were similar to H plants. In experiment 2, in which there was a higher disease pressure, O plants were similar to Y plants or intermediate, depending on the length of incubation and the disease pressure obtained.

Variation in resistance among wheat lines was studied only in data sets in which there was a significant effect of inoculation. This was the case for Y plants exposed to the long incubation period in experiment 1, all growth regimes and incubation periods in experiments 2–4. However, Y plants in experiment 3 was excluded from analysis because all inoculated plants died and therefore this data set did not provide any information about resistance. Thus, three experiments with interesting data remained per growth regime.

Table 1
Size and developmental stage prior to inoculation

<table>
<thead>
<tr>
<th>Growth regime</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
<th>Experiment 3</th>
<th>Experiment 4</th>
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<tbody>
<tr>
<td></td>
<td>DW&lt;sup&gt;c&lt;/sup&gt;</td>
<td>DW</td>
<td>FW&lt;sup&gt;d&lt;/sup&gt;</td>
<td>NL&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Y</td>
<td>0.06 c&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0.04 c</td>
<td>0.41 b</td>
<td>2-2.5</td>
</tr>
<tr>
<td>O</td>
<td>0.15 b</td>
<td>0.14 a</td>
<td>1.29 a</td>
<td>3-6.5</td>
</tr>
<tr>
<td>H</td>
<td>0.20 a</td>
<td>0.08 b</td>
<td>0.45 b</td>
<td>2-2.5</td>
</tr>
</tbody>
</table>

<sup>a</sup>The values are averages of seven winter wheat lines.
<sup>b</sup>Y, young non-hardened plants; O, older non-hardened plants; H, Y plants cold hardened for an additional 2 weeks.
<sup>c</sup>Dry weight (g).
<sup>d</sup>Fresh weight (g).
<sup>e</sup>Number of leaves.
<sup>f</sup>Figures from the same experiment not followed by the same letter are significantly different according to the Student–Newman–Keul test (P ≤ 0.05).
Components of Snow Mould Resistance in Wheat

(average values of the two incubation periods in experiment 2 were used as one of the three data sets). Statistical analysis was performed on each growth regime separately. There were significant differences between wheat lines in pink snow mould resistance (relative regrowth) among H plants and Y plants. Among O plants the effect of wheat line was not significant (P = 0.07). Among the Y plants, CI9342, PI181268 and CI14106 had higher relative regrowth and thus higher levels of pink snow mould resistance than Bjørke, Rida, V1004 and PI173440 (Fig. 1). Among O plants, CI9342 had the highest relative regrowth. In experiment 3, where the average relative regrowth in O plants was as low as 0.02, only plants of CI9342, CI14106 and PI181268 survived and among these CI14106 had the highest relative regrowth. Among H plants, CI9342 had a better relative regrowth than Rida and Bjørke, while the other lines were intermediate. In experiment 3, where the average relative regrowth of H plants was 0.3, a very good separation between lines was obtained. CI9342, PI181268, PI173440 and CI14106 had higher relative regrowth values between 0.4 and 0.55, while the relative regrowth of Bjørke, Rida and V1004 were between 0.05 and 0.1. Shoot weight of wheat lines prior to inoculation was not correlated with relative regrowth of wheat lines in any of the growth regimes (data not shown).

**Detached leaf assays**

**Symptoms** A distinct water-soaked lesion developed from the inoculation point on inoculated leaf segments, but not on mock-inoculated leaf segments, during incubation at 3°C in darkness. The mycelium developing on the leaf surface was very sparse and difficult to observe with the naked eye. In some inoculated leaves a dark brown necrosis appeared at the inoculation point. At RT and daylight the symptoms developed much faster and the water-soaked lesions were less distinct and often brownish. The mycelium

<table>
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<th>Table 2 Analyses of variance of plant regrowth after inoculation with Microdochium nivale and incubation under artificial snow cover</th>
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<tbody>
<tr>
<td>Source</td>
</tr>
<tr>
<td>Experiment 1</td>
</tr>
<tr>
<td>Y</td>
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<tr>
<td>O</td>
</tr>
<tr>
<td>H</td>
</tr>
<tr>
<td>I</td>
</tr>
<tr>
<td>B</td>
</tr>
<tr>
<td>WL</td>
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<tr>
<td>IW</td>
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<th>Table 3 Resistance to Microdochium nivale in winter wheat plants grown under different growth regimes</th>
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<tr>
<td>Growth regime</td>
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<tr>
<td></td>
</tr>
<tr>
<td>Y</td>
</tr>
<tr>
<td>O</td>
</tr>
<tr>
<td>H</td>
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</table>

*Resistance was measured as the relative regrowth; the ratio between the fresh weight of inoculated plants and mock inoculated plants after incubation and regrowth. The average values of seven winter wheat lines are shown.

bY, young non-hardened plants; O, older non-hardened plants; H, Y plants cold hardened for an additional 2 weeks.

cIncubation period.

dDisease pressure obtained, calculated as (1 – average relative regrowth).

Numbers not followed by the same letter are significantly different at the 5% level according to the Student–Newman–Keul test (within experiment and incubation period).
was not as sparse and there was a higher frequency of necrotic reactions. Under RT and daylight, the average lesion length at 3 days after inoculation (d.a.i.) was around 1 mm, corresponding to the average lesion length obtained somewhere between 9 and 13 d.a.i. under incubation at 3°C in darkness.

Water-soaked background appeared across some leaf segments in response to detachment and/or incubation (present on both inoculated and mock-inoculated leaf segments). This occurred mainly on primary leaves of H plants and to some extent on secondary leaves of H plants and primary leaves of Y plants. There was no consistent variation between wheat lines in the frequency of water-soaked background.

**Water-soaked lesion** At 7 and 9 d.a.i. (3°C and darkness, experiment 4 only) there were significant differences between leaf classes in the length of the water-soaked lesion, but there were no significant differences between wheat lines (Table 4). At 13 d.a.i., however, there were effects of both leaf class and wheat line in both experiments. Under incubation at RT and light there was also an effect of leaf class. A significant effect of wheat line was present only in experiment 4, presumably due to the higher number of samples in this experiment. There were no significant interactions between leaf class and wheat line under any of the incubation conditions.

The major variation in lesion length was due to differences between leaves of different order. In general, leaves of a higher order were more resistant than leaves of a lower order (Fig. 2). When comparing leaf classes across unhardened plants of different ages (Y and O plants), the older secondary leaves from O plants had larger lesions than the younger secondary leaves from Y plants under both incubation conditions. However, among primary leaves this relationship was opposite, particularly in the early stages of incubation (data not shown). Thus it appears that old primary leaves are more resistant than young primary leaves, while old secondary leaves are more susceptible than young secondary leaves. The primary leaf of H plants had smaller lesions than the primary leaf of Y plants under both incubation conditions (Fig. 2). The lesions developing on the secondary leaves of H plants were similar to those developing on the secondary leaves of Y plants.

CI9342, PI181268 and CI14106 had the smallest lesions under both incubation treatments (Fig. 3). PI173440, which was resistant in the whole plant experiments when cold hardened, did not exhibit any resistance in the detached leaf assays, not even when looking at hardened leaves only (data not shown). Lesion length on the secondary leaf incubated at 3°C in darkness was negatively correlated with snow mould resistance of intact plants in all the growth regimes, the strongest correlation being among H plants ($r = -0.91$) (Table 5). Under incubation at RT with daylight a significant correlation was only obtained in the primary leaf of Y plants, and this correlation was weaker than that of the secondary leaf incubated at 3°C in darkness.

**Mycelial colony** The length of the mycelial colony was recorded under incubation at RT with daylight only. There were significant differences between leaf classes (Table 6). In both experiments, the length of the mycelial colony was larger in hardened plants than in non-hardened plants (Fig. 4). A significant effect of
wheat line and an interaction between wheat line and leaf class was present in experiment 4. CI9342, PI181268 and CI14106, which had the smallest lesions, also had the smallest mycelial colonies.

Discussion

Whole plant experiments

The three Nordic wheat lines were less resistant to pink snow mould than the four lines from the USDA World Cereal Collection. Among Y plants, CI9342, PI181268 and partially CI14106, were more resistant than the Nordic lines, but PI173440 were not. Among O plants the only significant difference revealed by the SNK-test was the difference between CI9342 (resistant) and V1004 (sensitive). However, in the experiment with the highest disease pressure only plants of CI9342, CI14106 and PI181268 survived, indicating that these had a higher resistance level than the other lines. Among H plants, CI9342 was more resistant than Bjørke and Rida, and PI181268, CI14106 and PI173440 also tended to be more resistant than the Nordic lines. The disease pressure obtained for H plants were low in two of the experiments, but it was high in one experiment and in this experiment there was a very clear difference between the Nordic lines (relative regrowth = 0.4 to 0.55) and the other four lines (relative regrowth = 0.05 to 0.1).

The snow mould resistance of CI14106, CI9342, PI181268 and PI173440 have been demonstrated earlier (Bruehl, 1967a,b,c; Litschko et al., 1988; Gaudet and Kozub, 1991), but these studies have only involved cold hardened material. Our results demonstrate that pink snow mould resistance is also present at a low level in some of the resistant lines (CI9342, PI181268 and CI10406, but not PI173440) prior to hardening and that hardening-independent mechanisms
of resistance therefore exist. It cannot be ruled out that some mechanisms may be induced by low temperature during incubation, but such mechanisms must then be independent of light and will therefore not involve carbohydrate accumulation.

Gaudet and Kozub (1991) compared frost resistance and resistance to cottony snow mould in winter wheat lines in a study including some of the resistant lines observed as relative regrowth in the whole plant test and as water-soaked lesion developing on leaf segments inoculated with Microdochium nivale. Lesion length was measured after 13 days of incubation at 3°C in darkness (a) or 3 days of incubation at room temperature with daylight (b). Each column represents the average of five leaf classes (Y1, Y2, O2, H1 and H2) across experiments 3 and 4 (a) or the average of all leaf classes in experiment 4 (b) (no significant differences between wheat lines in experiment 3). No common letters above two columns indicate that the average lesion size of the wheat lines were significantly different according to the Student-Newman-Keul-test (P ≥ 0.05).

**Table 5**

<table>
<thead>
<tr>
<th>Growth regime</th>
<th>13 d.a.i.</th>
<th>3 d.a.i.</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>First leaf</td>
<td>Second leaf</td>
</tr>
<tr>
<td>Y</td>
<td>NS</td>
<td>−0.89**</td>
</tr>
<tr>
<td>O</td>
<td>NS</td>
<td>−0.85*</td>
</tr>
<tr>
<td>H</td>
<td>−0.76*</td>
<td>−0.91**</td>
</tr>
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</table>

aResistance of winter wheat lines to Microdochium nivale was measured as relative regrowth in the whole plant test and as water-soaked lesion length in the detached leaf assays. Correlations were tested within each growth regime and leaf number. NS, not significant; **0.001 < P < 0.01; *0.01 < P < 0.05.

bDays after inoculation.

cDetached leaf assay performed in experiment 4 only.

dDetached leaf assay performed in experiment 4 only.

eThe length of the mycelial colony were analysed using the model $Y_{ij} = LC + WL + LC_i \times WL_j + Error$, where $Y_{ij}$ length of mycelial colony; $LC_i$, leaf class; $WL_j$, wheat line; $F$-values are given; NS, not significant; ***0.0001 < P < 0.001; **0.001 < P < 0.01; *0.01 < P < 0.05.

bDegrees of freedom.

days after inoculation.

Table 6 Analyses of variance on mycelial colony length in detached leaf assays of resistance to Microdochium nivale

<table>
<thead>
<tr>
<th>Source</th>
<th>Experiment 3</th>
<th>Experiment 4</th>
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<tr>
<td></td>
<td>d.f.</td>
<td>3 d.a.i.</td>
</tr>
<tr>
<td>LC</td>
<td>2</td>
<td>15.68***</td>
</tr>
<tr>
<td>WL</td>
<td>6</td>
<td>NS</td>
</tr>
<tr>
<td>LC × WL</td>
<td>11</td>
<td>NS</td>
</tr>
</tbody>
</table>

from the USDA World Cereal Collection. They found a close association between cultivar ranking on locations with snow mould stress and locations with freezing stress. The study by Pulli et al. included mainly Nordic cultivars and none of the resistant lines. Norstar, which is a relatively susceptible cultivar compared to some of the resistant lines (Gaudet and Kozub, 1991), appeared to be more resistant than many of the Nordic cultivars in a snow mould chamber test performed by Pulli et al. (1996). Together this indicates that the resistant lines selected from the world collection have some specific snow mould resistance components not present in the Nordic material and that variation in snow mould resistance in Nordic cultivars may be largely due to variation in the ability to cold harden.

**Detached leaf assays**

**Differences between leaf classes** Leaf class had a major effect on the development of water-soaked lesions in response to inoculation. Most of the variation was due to leaf position and/or leaf developmental stage; among plants grown under the same growth conditions the primary leaves were more susceptible than the secondary leaves, and the secondary leaves more susceptible than the tertiary leaves. As water-soaked background was observed mostly in primary leaves, the observed differences between leaves of different order could be due to a lower level of tolerance to detachment and incubation in the primary leaves.

Unlike the situation in the whole plant experiments, growth regime had less effect on the resistance expressed in the detached leaf assays. This supports the idea that the higher resistance of older and hardened plants relative to young non-hardened plants depends on carbohydrate reserves in the crown, as this type of resistance will not show up in detached leaf assays. However, as H leaves appeared to be more susceptible to detachment and incubation, they may be more resistant to pink snow mould than what is apparent in this study. The mycelial colonies on the leaf surface developed faster on hardened leaves than
on non-hardened leaves, in spite of the fact that the hardened leaves tended to have smaller water-soaked lesions. Cold hardening is known to increase the rigidity and thickness of cell walls (reviewed by Fujikawa et al., 1999). This may make it harder for fungal hyphae to digest the cells of hardened plants and cause the fungus to grow on the surface of the leaves rather than inside the leaf. This suggests that changes in the cell wall rigidity, thickness and composition during cold hardening could be one of the mechanisms by which cold hardened plants become more resistant to pathogens.

**Differences between wheat lines and correlation with whole plant tests** At time points where there was a significant effect of wheat line, PI181268, CI9342 and CI14106 always had the smallest lesions. This shows that these lines possess not only resistance mechanisms that are independent of cold hardening as shown in the whole plant experiments, but also mechanisms that are expressed in detached leaves. The resistance of PI173440, however, is dependent of cold hardening and is also not expressed in detached leaves. This indicates that PI173440 relies on cold hardening-induced changes in the stem and crown and does not have additional hardening-independent mechanisms or mechanisms expressed in leaves. In the detached leaf assay the length of the mycelial colonies also tended to be smaller on leaves of PI181268, CI9342 and CI14106 than on the other lines. Together the results indicate that these lines are able to inhibit growth of *M. nivale* both inside the leaf as well as on the leaf surface, possibly due to a higher activity of defence-related proteins (Ergon, 1999; Gaudet et al., 2000, 2003a,b). This mechanism could represent some of the hardening-independent resistance possessed by these lines.

Results from the detached leaf assay correlated well with results from the whole plant test, but only for some leaf classes. Water-soaked lesion length on secondary leaves incubated at 3°C in darkness correlated well with whole plant resistance, particularly among hardened plants. The strong effect of leaf order on lesion length has implications for screening of resistance to fusarium head blight and other diseases as well, and may pose a problem if the material under testing varies in developmental rate. However, since the variation between leaves of different order could be due to variation in tolerance to incubation, it may be reduced with the addition of kinetin (a senescence retarder) to the water agar, as done by Diamond and Cooke (1999) and Browne and Cooke (2004). A detached leaf assay is not likely to pick up variation in snow mould resistance related to the carbohydrate storage or other characteristics specific to the crown, but detects resistance components present in leaves. In the breeding of partial disease resistance against snow mould the detached leaf assay could be useful in combination with tests for carbohydrate content in the crown.

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