Evaluation of Spray and Point Inoculation Methods for the Phenotyping of *Puccinia striiformis* on Wheat

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Abstract

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The fungus *Puccinia striiformis* causes yellow (stripe) rust on wheat worldwide. In the present article, new methods utilizing an engineered fluid (Novec 7100) as a carrier of urediniospores were compared with commonly used inoculation methods. In general, Novec 7100 facilitated a faster and more flexible application procedure for spray inoculation and it gave highly reproducible results for virulence phenotyping. Six point inoculation methods were compared to find the most suitable for assessment of pathogen aggressiveness. The use of Novec 7100 and dry dilution

with *Lycopodium* spores gave an inoculation success rate of 100% in two independent trials, which was significantly higher and more consistent than for spore suspension in Soltrol 170, water, water + Tween 20, and Noble agar + Tween 20. Both Soltrol 170 and Novec 7100 allowed precise quantification of inoculum, which is important for the assessment of quantitative epidemiological parameters. New protocols for spray and point inoculation of *P. striiformis* on wheat are presented, along with the prospect for applying these in rust research and resistance breeding activities.

Yellow (stripe) rust caused by the fungal pathogen *Puccinia striiformis* is one of the most important diseases on wheat (*Triticum aestivum* L.) (Hovmøller et al. 2011; Wellings 2011). The pathogen is an obligate biotroph which primarily infects the leaves, although the glumes and awns can also become diseased in epidemic situations. High humidity and relatively cool temperature (10 to 15°C) are optimal for infection, which occurs through stomata. Fungal colonies grow semisystemically in the intercellular space between the mesophyll cells (Cartwright and Russell 1981) and individual colonies on a leaf may merge to form hyphal networks. Under optimal conditions, disease symptoms appear as chlorotic areas 6 to 7 days after infection, and yellow to orange urediniospore-bearing pustules emerge after 10 to 14 days. The semisystemic growth and the emergence of several pustules from each colony restrict the distinction of pustules from different infection sites, in contrast to other wheat rust fungi such as *P. graminis* and *P. tritricina*, where each infection site results in only one pustule.

Yellow rust can be controlled by fungicides or host resistance, the latter often preferred for both economic and environmental reasons. The durability and efficiency of host resistance in agriculture depend on pathogen diversity, where the individual isolates can be grouped into races based on selected host cultivars (differential sets) (Allison and Isenbeck 1930; Hungerford and Owens 1923). Modern differential sets are based on cultivars with resistance (*R*) genes of major effect on individual isolates following the principles of the gene-for-gene hypothesis (Flor 1955; Wan and Chen 2014). Differential sets are an important tool in surveillance programs, e.g., for the detection of new virulence rendering wheat cultivars susceptible or exotic incursions which may change the characteristics of the entire pathogen population (Hovmøller and Henriksen 2008; Hovmøller et al. in press; Wan and Chen 2014). Isolates with a well-characterized virulence phenotype are also useful in plant breeding to provide information about the resistance characteristics of the breeding material

and the vulnerability of the material to races of variable origin (Hovmøller 2007).

It is recommended to carry out yellow rust experiments under controlled conditions because the disease symptoms are sensitive to environmental factors such as light and temperature (Bariana and McIntosh 1994; de Vallavieille-Pope et al. 2002; Hickey et al. 2012). Furthermore, standardized methods for inoculation are important to ensure sufficient and homogenous distribution of disease symptoms on plants. Commonly used methods are direct application of spores by rubbing with infected plants, spraying with spores diluted in mineral oil such as Soltrol 170 or water with an added surfactant, or dusting of spores dry diluted in *Lycopodium* spores (Dhingra and Sinclair 1995).

In addition to *R* genes with major effects, many breeding programs now also focus on *R* genes with minor effects, which are often considered more durable (McDonald and Linde 2002). The efficiency of genes with minor effects can be assessed based on epidemiological parameters such as infection efficiency, latent period, lesion growth, and spore production capacity (de Vallavieille-Pope et al. 2000) i.e., the parameters which are also used to characterize pathogen aggressiveness (Lannou 2012; Pariaud et al. 2009). The latest developments in the epidemiology of *P. striiformis* indicate that variation in these traits may be a significant driver in pathogen population dynamics (Hovmøller et al. 2008; Milus et al. 2009). Reliable assessment of quantitative traits requires a highly standardized experimental setup due to random variation caused by, for example, inoculation density and plant growth stage (Pariaud et al. 2009).

The main objective of this study was to evaluate commonly used *P. striiformis* inoculation methods on wheat and to develop a simple and reliable point inoculation method allowing quantification of inoculum and minimizing the risk of isolate admixture. The methods are discussed in relation to the development of more effective and flexible systems for the phenotyping of disease resistance and for assessment of pathogen aggressiveness.

Materials and Methods

Plant material. Differential sets consisting of 8 to 10 seeds of 34 differential wheat genotypes were sown in pots (7 × 7 × 8 cm) filled with Pindstrup peat-based substrate with slow-release nutrients optimized for wheat seedlings (Pindstrup Mosebrug A/S) (Thach et al. 2015). The plants were grown in spore-proof greenhouse cabins with 16 h of natural light supplemented with sodium light at 100 μE m⁻² s⁻¹ when daylight was <10,000 lux. Temperature conditions were climate controlled to 17°C (day) and 12°C (night). Plants were

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generally inoculated when 12 days old, with the second leaf halfway unfolded. Growth-regulated wheat seedlings (maleic hydrazide, Antergon MH 180; Crompton Registrations Ltd.) of 'Cartago', 'Morocco', and 'Anja' were used to multiply *P. striiformis* urediniospores at the same growth stage and under the same conditions as above. Subsets of the 34 wheat differential genotypes were used in specific experiments to optimize spore concentration, effect of airbrush cleaning procedures, and evaluation of inoculation methods.

Fungal material. The following *P. striiformis* isolates with different virulence phenotypes were used: AR00-05 (2000) from Arkansas (Milus et al. 2006), DK12/07 (2007), and DK09/11 (2011) from Denmark, and GB75/30 (1975) from the United Kingdom (Table 1). Spores were retrieved from liquid nitrogen (N₂, -196°C); isolate DK12/07 and DK09/11 were multiplied on Morocco, isolate AR00-05 on Cartago, and GB75/30 on Anja. The multiplication pots were covered with cellophane bags (Helmut Schmidt Verpackungsfolien GmbH) to prevent cross contamination between isolates. Spores were harvested by shaking the plants inside the cellophane bag, after which they were transferred to cryo vials, dried, and kept in a desiccator at room temperature until experimental use the following day.

Experiment 1. Procedures for spray and rub inoculation. Different spore carriers for spray inoculation and rub inoculation were evaluated on the 34 differential wheat genotypes using DK12/07, DK09/11, and AR00-05 (Supplementary Fig. S1; Table 1). Three methods were investigated: (i) rubbing each differential set with 20 heavily infected seedlings; (ii) 25 mg of freshly harvested spores suspended in 5 ml of Novec 7100 (3M) applied with an airbrush spray gun (standard class; Revell GmbH), and (iii) the same procedure as in (ii) but using Soltrol 170 mineral oil (Phillips Petroleum Co.). The latter method was followed by a minimum of 10 min for oil evaporation. Between each spray inoculation, the airbrush spray gun was cleaned using 70% ethanol for 5 s followed by spraying with Novec 7100 or Soltrol 170 for another 5 s. After inoculation, the plants were misted with water and placed in darkness at 10°C for 24 h and transferred to the greenhouse cabins with automatic watering. Assessment of infection types (IT) was done 17 to 19 days after inoculation using a 0 to 9 scale (McNeal et al. 1971). Photos of infected leaves were obtained by a DSLR Canon EOS 7D fixed on a camera stand equipped with a 100-mm Canon macro lens.

Experiment 2. Optimization of spore concentration and cleaning procedures. Seedlings of 'Chinese 166', 'Opata', 'Compare', 'Heines Peko', and Cartago wheat were used to optimize the spore concentration for spray inoculation using Novec 7100. The plants were inoculated with AR00-05 using three spore concentrations: spores at 1.5, 5, and 20 mg ml⁻¹ of Novec 7100. Inoculation and incubation were carried out as in experiment 1. Nineteen days after inoculation, the numbers of plants with macroscopic visible infection sites were recorded for each plant. The number of plants with symptoms and signs relative to the total number of plants in the pot was used to evaluate the inoculation success rate. Symptoms were recorded separately for leaf 1 and leaf 2, leaves were detached and mounted on a plastic frame, and photos were taken as described in experiment 1.

The cleaning efficiency for Novec 7100 spray inoculation was tested in experiment 1. Six pots, each with nine plants of Morocco, were mock inoculated between inoculating differential sets. This was repeated six times; plants were incubated for 20 h, transferred to greenhouse cabins, and examined for disease symptoms after 12 and 16 days.

Experiment 3. Evaluation of spore carriers for point inoculation. Twenty seeds of Morocco wheat were sown in trays (55 by 45 by 7 cm, length by width by height) along acrylic pedestals (22.5 × 15 × 15 cm, length × width × height) placed in Pindstrup substrate. The second leaf of 16- to 17-day-old seedlings was fixed on the acrylic pedestals prior to inoculation using double-sided tape followed by overlay with surgical tape (Leukofix; BSN Medical GmbH). Six different methods were evaluated for point inoculation using DK09/11 (Supplementary Fig. S2; Table 2): (i) spores at 2 mg ml⁻¹ of Novec 7100, 2 µl of suspension applied to the center of each leaf with a pipette (Finnpipette 2 to 20 µl; ThermoLabsystems) (method 1), (ii) spores at 2 mg ml⁻¹ of Soltrol 170, 2 µl of suspension applied to the center of each leaf with a pipette (method 2), (iii) spores at 2 mg ml⁻¹ of deionized water with 0.05% Tween 20 (Sigma-Aldrich), 5 µl of suspension applied to the leaf center with a pipette (method 3), (iv) spores at 2 mg ml⁻¹ of tap water, 5 µl of suspension applied to the center of the leaf (method 4), (v) spores at 2 mg ml⁻¹ of Noble agar (Milus et al. 2009), 5 µl applied to the center of each leaf with a pipette (method 5), and (vi) spores mixed with *Lycopodium* spores (1:9, wt/wt) and applied to the center of the leaf with an artist brush, size 1 (method 6). The experimental setup consisted of three blocks (trays) with three replications of each

Table 1. Virulence phenotypes of *Puccinia striiformis* isolates used in this study

Isolate ^y	Virulence for <i>Yr</i> genes ^x																	
	1	2	3	4	5	6	7	8	9	10	15	17	25	27	32	Sd ^z	Sp ^z	Su ^z
AR00-05	–	(2)	–	–	–	6	7	8	9	–	–	–	25	–	–	(Sd)	–	–
DK12/07	1	2	3	4	–	–	–	–	9	–	–	17	25	–	32	Sd	–	Su
DK09/11	1	2	3	4	–	6	7	–	9	–	–	(17)	25	–	32	Sd	Sp	Su
GB75/30	–	–	–	–	–	–	–	–	–	–	–	–	25	–	32	Sd	Sp	–

^x Avirulence is indicated by – and virulence was defined by infection type (IT) above 6 on a 0-to-9 scale (McNeal et al. 1971). Parentheses indicate intermediate IT scores of 5 to 6.

^y Isolates DK12/07 and DK09/11 were collected in Denmark in 2007 and 2011, respectively. Isolate AR00-05 was collected in 2000 in Arkansas by Professor Gene Milus (Milus et al. 2006) and GB75/30 was collected in the United Kingdom in 1975 and provided by Dr. Lesley Boyd, National Institute of Agricultural Botany (NIAB), UK.

^z Resistance specificities in Strubes Dickkopf (Sd), Spalding Prolific (Sp), and Suwon/Omar (Su), respectively.

Table 2. Inoculation success rate (Inoc) and the mean latent period in hours (LP ± standard deviation [SD]) and lesion length in mm (Length ± SD) for six different methods for point inoculation of wheat seedling leaves with spores of *Puccinia striiformis*^z

Method	Suspension	Experiment 1 (n = 9)			Experiment 2 (n = 9)		
		Inoc (%)	LP (h)	Length (mm)	Inoc (%)	LP (h)	Length (mm)
1	Spores + Novec 7100	100	237 (±8) a	31 (±11) a	100	268 (±28) a	29 (±6) a
2	Spores + Soltrol 170	66	272 (±29) b	21 (±3) ab	33	352 (±39) c	17 (±2) bc
3	Spores + Water + Tween 20	33	284 (±25) b	26 (±14) ab	44	339 (±32) bc	17 (±5) bc
4	Spores + Water	100	259 (±15) ab	21 (±3) ab	88	302 (±52) abc	15 (±4) bc
5	Spores + Agar + Tween 20	33	264 (±12) ab	20 (±6) b	88	294 (±25) abc	13 (±3) c
6	Spores+ <i>Lycopodium</i> spores	100	253 (±14) ab	22 (±5) ab	100	281 (±24) ab	22 (±7) ab

^z In each experiment, n = number of leaves inoculated per method (replicates). Values followed by the same letters within columns are not significantly different (Tukey-Kramer, P < 0.05).

inoculation method within a block. The experiment was repeated twice. Inoculated leaves were observed for 25 days and a leaf with no sign of pustules (and chlorosis) was recorded as a failed inoculation. The inoculation success rate was then calculated for each method as the percentage of inoculated leaves with sporulating pustules (lesions). Latent period was recorded for each leaf as the time from inoculation until the first appearance of open pustules with emerging urediniospores (Milus et al. 2009). Beginning 9 days after inoculation, inoculation sites were examined using a $\times 10$ hand lens at 24-h intervals until urediniospores had been observed for all successful sites. At each time point,

Fig. 1. Effects of inoculation method on disease symptoms on the second leaf of wheat seedlings. From left to right of each photo, the plants were inoculated with spores rubbed from infected plants, spores suspended in Novec 7100, and spores suspended in Soltrol 170. The effect of method is presented for a range of expected infection types: **A**, 0 to 1; **B**, 2 to 3; **C**, 3 to 4; **D**, 5 to 6; **E**, 7; and **F**, 8. Cultivar (first)–isolate (second) interactions are shown on the pictures.

the pustules were classified into three categories: (i) visible urediniospores, (ii) open without visible urediniospores, and (iii) closed. Latent period was adjusted to the nearest 12-h interval based on the progress of the urediniospore production at the time of assessment. The edges of the developing lesion, defined as the leaf area with sporulating pustules, were marked with a felt pen 4 days after the latent period. The distances between the markings were measured with a ruler to the nearest millimeter and used as an estimate for lesion size.

Experiment 4. Point inoculation for assessment of IT and pathogen aggressiveness. Point inoculation using Novec 7100 was evaluated in relation to virulence phenotyping and assessment of pathogen aggressiveness. IT was assessed on the second leaf of 18 seedlings of Heines Peko and ‘Spaldings Prolific’, which were grown in separate trays with the leaves fixed on acrylic pedestals, as described above. Each leaf was inoculated with one of the isolates DK09/11, AR00-05, and GB75/30 in a random order, resulting in six replications of each isolate within a tray. The leaves were scored for IT 18 days after inoculation using the 0 to 9 scale (McNeal et al. 1971).

Pathogen aggressiveness was studied based on fixed leaves of ‘Croplan Genetics 514W’, where inoculation with each of the three isolates was replicated 12 times. Results were measured as latent period and lesion growth, the latter estimated by marking the edges of the developing lesion with 4-day intervals. The distance between consecutive markings was measured to the nearest millimeter and used as lesion growth.

The risk of unintentional spread of spores during point inoculation of adjacent plants was evaluated by inoculating DK09/11 on every second fixed leaf of 20 seedlings of Morocco. The experiment was replicated three times. Noninoculated leaves were regularly observed for infection until 24 days after inoculation.

Fig. 2. Setup for evaluation of six spore carriers for point inoculation of second leaf of seedlings with spores of *Puccinia striiformis*. Plants within the same tray are shown at three time points: **A**, 24 h after inoculation; water-soaked leaf areas were observed for treatment with Soltrol 170; **B**, 14 days after inoculation; sporulating pustules were observed at most inoculation sites, few leaves with only chlorosis and four leaves with no visible symptoms; and **C**, 18 days after inoculation; black lines mark the edges of the lesion 4 days after latent period. The size of the lesion to the far right (method 5) represented an outlier compared with other lesions.

Data analysis and image editing. Data for latent period and lesion length were analyzed in general linear models (GLM) using the procedure Proc GLM in SAS statistical software (version 9.2; SAS Institute Inc.). Variance structure and normal distribution of data were assessed for all models by plots of model residuals. Lesion length data were log-transformed for normalization of residuals.

The effect of repeating the experiment was analyzed in a model which included experiment, method, block nested in experiment, and their interactions as fixed factors. Due to a significant effect of

experiment, data from each experiment were further analyzed in separate models with method, block, and their interaction as fixed factors. The variance homogeneity of method treatments was further tested for lesion length by the use of Levene's test. Treatment comparisons for both latent period and lesion length were based on least square means due to unbalanced data caused by failed infections. Tukey-Kramer adjustments for multiple comparisons were applied (Kramer 1956; Tukey 1953).

Data for comparison of isolates for latent period and lesion growth rate were analyzed as a one-way analysis of variance (ANOVA) using

Fig. 3. Infection types (IT) produced by isolate AR00-05, GB75/30, and DK09/11 on two wheat differential cultivars. **A**, Heines Peko: AR00-05, IT = 6, GB75/30, IT = 1, and DK09/11, IT = 8; **B**, Spalding Prolific: DK09/11, IT = 6, AR00-05, IT = 0, and GB75/30, IT = 7.

Fig. 4. Latent period and lesion growth for isolates GB75/30, AR00-05, and DK09/11 after point inoculating the second leaf of Croplan Genetics 514W seedlings. **A**, GB75/30 had a significantly longer latent period than AR00-05 and DK09/11, resulting in fewer sporulating pustules 13 days after inoculation. **B**, Edges of each lesion marked with a felt pen 4 and 8 days after latent period for the individual treatment.

the procedure PROC ANOVA in SAS statistical software. Isolates were compared with a *t* test without adjustment for multiple comparisons.

Images were edited in Windows Photo Gallery (build 16.4.3528.331, 2012) and Fiji (<http://fiji.sc/Fiji>) (Schindelin et al. 2012). Editing in Photo Gallery included cropping to region of interest and adjustment of exposure, color, and sharpness. Editing in Fiji included color thresholding in order to select and fill in black background.

Results

Experiment 1. Procedures for spray and rub inoculation. Comparison of spray inoculation using either Novec 7100 or Soltrol 170 and rub inoculation with yellow-rust-infected plants on wheat differential sets showed that the spore carrier had no influence on IT and the symptoms were similar for all plants within a pot (Fig. 1). The second leaf gave more consistent results than the first leaf, which occasionally showed the first indication of senescence at the time of assessment. The rub-inoculation method resulted in more variation in number of infections per leaf compared with spray inoculation using Novec 7100 or Soltrol 170. No effect of method was detected with respect to percentage of leaves without symptoms (escape).

Experiment 2. Optimization of spore concentration and cleaning procedures. This experiment was carried out to find the optimal spore concentration using Novec 7100. The inoculation success rate for the low spore concentration (1.25 mg ml⁻¹) was 48% on leaf 1 and 77% on leaf 2. The medium concentration (5 mg ml⁻¹) resulted in 75% inoculation success on leaf 1 and 99% on leaf 2, and the high concentration (20 mg ml⁻¹) resulted in 87 and 99% on leaf 1 and 2, respectively. The amount and distribution of symptoms were similar for inoculation with medium and high spore concentrations whereas a low concentration resulted in fewer symptoms for low IT. This was also observed for high IT (above 7), where the lesions were often located toward the tip of the leaves.

Cleaning the airbrush spray gun with 70% ethanol and Novec 7100 was effective and no disease symptoms were observed on mock-inoculated control plants.

Experiment 3. Evaluation of spore carriers for point inoculation. Inoculation using Novec 7100 (method 1) and spores mixed with *Lycopodium* spores (method 6) gave the highest inoculation success rate (100%) in both experiments (Table 2; Fig. 2). Inoculation using spores suspended in water (method 4) gave inoculation success rates of 100 and 88%, whereas spores suspended in water + Tween 20 (method 3) resulted in only 33 and 44% successful infections, respectively. A significant effect of experiment and method was found for both latent period ($P < 0.0001$) and lesion growth ($P = 0.0025$) whereas the interaction between the two factors was nonsignificant. In both experiments, the latent period recorded for Novec 7100 was significantly shorter than for methods where spores were suspended in either Soltrol 170 or water + Tween 20 (Table 2). Inoculation using Novec 7100 resulted in the longest lesions whereas the shortest lesions were found for Noble agar (method 5). No significant effect of method on homogeneity of variance was found for lesion length in experiment 2 ($P = 0.923$) whereas, in experiment 1, a low *P* value was found ($P = 0.054$) due to the large variance of method 3. In a few cases, infection sites were observed outside the inoculation sites and these were not considered in the assessments. In one case, the size of the lesion on a leaf inoculated with method 5 was clearly different from others (Fig. 2C), most likely due to cross contamination from an adjacent leaf inoculated using *Lycopodium* spores as a spore carrier and, therefore, not considered.

Experiment 4. Point inoculation for assessment of virulence phenotype and pathogen aggressiveness. The suitability of using Novec 7100 for assessment of the host–pathogen interaction phenotype was done by inoculation of two differential wheat cultivars with three different isolates. In all cases, the expected IT was observed (Fig. 3).

Pathogen aggressiveness was measured by latent period and lesion growth on Croplan Genetics 514W using three isolates (Fig. 4). The latent period of GB75/30 was significantly longer than for AR00-05 and DK09/11, whereas GB75/30 had the largest lesion growth (Fig. 4B). Overall, the results indicated that this experimental setup can be used to assess aggressiveness of *P. striiformis*.

The reliability of the point inoculation method using Novec 7100 was initially tested in a setup where every other fixed leaf was inoculated. Approximately one single infection site (lesion) was observed per 10 noninoculated leaves after careful application of the inoculum and indirect misting of plants.

Discussion

In this study, commonly used methods for the inoculation of wheat seedlings with urediniospores of *P. striiformis* were compared with a new method using an engineered fluid, Novec 7100. In general, the results obtained with Novec 7100 were similar to results obtained with Soltrol 170 mineral oil or rubbing plants using a pot of severely infected seedlings. Both Soltrol 170 and rubbing by infected plants have been used in rust epidemiological studies (de Vallavieille-Pope et al. 2012; Li et al. 2006; Singh and Huerta-Espino 2003). Novec 7100 and Soltrol 170 had the advantage that the amount of inoculum could be adjusted, which was considered important, particularly for the assessment of epidemiological parameters (Pariaud et al. 2009). Novec 7100 was used recently for the successful recovery of historic samples of *P. striiformis* from the ‘Stubbs collection’ after up to 45 years of storage in liquid N₂ (Thach et al. 2015). None of the three methods caused any adverse effect on either the plants or the pathogen, although water-soaked areas could be observed 1 to 3 days after inoculation with Soltrol 170. These water-soaked symptoms gradually disappeared later on.

Novec 7100 evaporated almost instantly after inoculation, in contrast to Soltrol 170, which required a minimum of 10 min of drying before incubation. In previous studies using Soltrol 170 as a rust spore carrier, 15 to 60 min were allowed for oil evaporation (de Vallavieille-Pope et al. 2012; Ordoñez and Kolmer 2009; Rouse et al. 2011), and up to 2 h has been reported (Boshoff et al. 2002). Such a time delay between subsequent inoculations may be inconvenient when processing many spore samples in rust diagnostic laboratories. The spore suspension using Novec 7100 was quickly prepared and applied, and we observed no effects on spore viability when spores were left in the suspension for up to 2 h. In the present study, approximately 10 min were required for the inoculation of a differential set of 34 pots using Novec 7100, including cleaning of the airbrush spray gun and the fume hood.

Novec 7100 belongs to a group of engineered fluids mainly used for cleaning purposes in a range of industries and has a good environmental, health, and safety profile, with low acute toxicity (3M 2009, 2015). The compound was easy to store and handle, and the necessary equipment should be accessible from most retailers. The Novec 7100 inoculation method required only a minimum of training and, therefore, was easy to implement and use. Other inoculation systems involved the use of settling towers or engineered spraying devices (Andres and Wilcoxson 1984; Eyal et al. 1968; Mortensen et al. 1979). This may be very useful in situations where only one or a few isolates are used and where the inoculation density needs to be monitored precisely. Due to the easy and fast cleaning of the airbrush spray gun, Novec 7100 may be preferred in situations where many isolates are handled within a short time period. However, it is recommended to test efficiency of cleaning procedures for each host–pathogen system before implementation. Novec 7100 has also been successfully applied in our laboratory for inoculation of urediniospores of other rust fungi, i.e., *P. graminis* on wheat, rye, ryegrass, Cock’s-foot, and Kentucky bluegrass; *P. tritici* on wheat; *P. coronata* on rye grass; and *P. hordei* on barley, and powdery mildew on barley.

The low and high spore concentrations resulted in similar disease symptoms and signs, although less disease and more escapes were observed for the low concentration. Therefore, the important aspect was to ensure a sufficient number of infections on a sufficient number of plants (i.e., to minimize ‘escapes’ which could be misinterpreted as IT 0). Novec 7100 (5 ml) with the spore concentration of 5 mg ml⁻¹ was optimal for the inoculation of one differential set consisting of 34 pots. Such an amount of spores was easily obtained from one multiplication cycle on one pot of susceptible wheat seedlings. For other rust species such as *P. tritici*, the required amount of inoculum may be lower due to a higher germination rate and infection efficiency

(de Vallavieille-Pope et al. 1995). In general, disease symptoms and signs may vary depending on the environmental conditions, in particular for cultivars carrying *R* genes such as *Yr2* or *Yr17* (Bariana and McIntosh 1994; Calonnet et al. 1997). Therefore, inoculated plants should be kept under controlled and well-defined experimental conditions.

A flexible setup for point inoculation of fixed leaves was developed where the number of leaves and the plant growth stage could be adjusted. The spore suspension media affected all three parameters: inoculation success rate, latent period, and lesion length. The highest inoculation success rate and the shortest latent period were observed for spores suspended in Novec 7100 and for spores mixed with *Lycopodium* spores. Novec 7100 is preferred over dry dilution for two reasons: *Lycopodium* spores may be difficult to quantify and inoculation using dry spores represents a risk of unintentional spread of spores in a setup, where different isolates are tested side by side to reduce the impact from random environmental factors.

Point inoculation has been applied in many host–pathogen systems, and different methods have been suggested (Chaerani et al. 2007; Clemow et al. 2011; Jia et al. 2003). Some methods involved the use of detached-leaf assays, which may not be representative for disease development on intact leaf material. For *P. striiformis*, a few point inoculation methods have been reported for adult plants; for example, the method of Broers and Lopez-Atilano (1994), which was used by Næstoft et al. (2000) on seedlings, the latter with a low inoculation success rate. Milus et al. (2009) used a method where spores were suspended in a low-concentration agar solution and applied to flag and flag-1 leaves with an inoculation success rate of 72% on a highly susceptible cultivar. In our experiments, this method had a lower inoculation success rate compared with the Novec 7100 suspension, and a large variation was found between experiments. Overall, the application of spores suspended in Novec 7100 with a pipette to fixed leaves gave the widest range of opportunities, and the method did not require any special equipment, materials, or training. Furthermore, all steps in the experimental setup could be performed under controlled conditions in the greenhouse.

The point inoculation method was investigated in relation to both qualitative traits (e.g., IT) and quantitative traits (aggressiveness or partial resistance), the latter based on latent period and lesion growth. The setup with fixation of the leaves facilitated image-based analyses of disease development, which is becoming increasingly important to understand host–pathogen interactions (Garcia and Barbedo 2013). Image analysis can be used to increase the throughput of accurate assessment of disease severity or other relevant parameters to characterize host resistance or pathogen aggressiveness. The quantitative point inoculation method ensured that the diseased areas were comparable between treatments and leaves, and the fixation of leaves made it possible to take multiple photos using a constant focal length at different time points.

New protocols for spray and point inoculation of *P. striiformis* on wheat were developed for virulence phenotyping and assessment of pathogen aggressiveness. These methods may also have relevance in plant breeding, where efficient phenotyping of disease resistance is an important part of the development of crop cultivars with improved disease resistance. The point inoculation method may allow a large number of breeding lines to be screened by multiple races at low cost under well-defined experimental conditions. Multiple races of the same species or different species may even be applied on the same leaf, tiller, or plant. The new inoculation procedures may also prove beneficial for molecular and histological studies of the interaction between pathogen and host.

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