Point inoculation method for measuring adult plant response of wheat to stripe rust infection

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Key words: *Puccinia striiformis* f. sp. *tritici*, adult plant resistance screening, *Triticum aestivum*, point inoculation

Abstract

Depending on the pathogenicity of the stripe rust fungus *Puccinia striiformis* f. sp. *tritici*, the nature of resistance in the wheat host plant, and the environment, a broad range of disease phenotypes can be expressed. Therefore, the phenotyping of partial adult plant stripe rust resistance, requires reliable and repeatable procedures, especially under controlled conditions. In this paper, the development of a flag leaf point inoculation method, which resulted in a 100% initial infection rate, is presented. Flag leaf inoculations were achieved by placing 6 mm antibiotic test paper discs, dipped into a urediniospore and water suspension and covered with water-proof plastic tape, on the adaxial side of leaves. Results from independent trials allowed for the statistical comparison of stripe rust lesion expansion rate in wheat entries that differ in

resistance. The technique is inexpensive, reliable and applicable to routine screening for adult plant response type, quantitative comparison of stripe rust progress, environmental influences and pathogenicity of different isolates.

The short-lived effectiveness of many major genes for all stage resistance to rusts in wheat (*Triticum aestivum* L.) has focused attention on more durable resistance types. When several minor genes or QTL for adult plant resistance (APR) to the wheat rusts are combined, lower disease levels occur, protection against a broader range of races follows, and expectations of durability increase (Singh et al. 2015; Klarquist et al. 2016; Dong et al. 2017; Pretorius et al. 2017; Mundt 2018). When deployed singly, many APR genes do not confer adequate resistance under high disease pressure; however, the combination of four or five minor genes can approach immunity or a high level of resistance (Singh et al. 2014). Chen (2005) mentioned that high-temperature APR to wheat stripe rust caused by *Puccinia striiformis* Westend. f. sp. *tritici* Erikss. is race-nonspecific and durable. Despite the appeal of APR, Michelmore et al. (2013) and Ellis et al. (2014) warned that our knowledge of this resistance type is incomplete and that certain components may indeed be race-specific and non-durable. The emphasis on APR to rust diseases, whether from a mechanistic or breeding perspective, requires accurate and reliable phenotyping. While successful rust assessment is carried out in field nurseries across the world, certain germplasm collections may require more detailed and quantitative assessments of infection type and disease development over time. Reliable inoculation protocols in controlled conditions are also of great importance to enable and speed up the cloning of APR genes.

Several studies have investigated inoculation procedures for cereal rust assessment under controlled conditions. Andres and Wilcoxson (1984) developed a device for precise deposition

of urediniospores on individual leaves of cereal plants, a system successfully used for quantifying components of resistance in wheat to the leaf rust pathogen *P. triticina* Erikss. (Kloppers and Pretorius 1997). Singh et al. (1996) described a convenient adhesive tape method for evaluating leaf rust response in flag leaves. In their approach, urediniospores were applied to sections of clear adhesive tape which were then fixed to the upper surface of flag leaves. A further advantage of this technique was that more than one pathotype could be applied to the same leaf. Settling towers have also been successfully used for quantitative deposition of urediniospores on wheat seedlings or adult plants (Eyal et al. 1968; Mortensen et al. 1979). A challenge in using a settling tower is the uniform application of spores on plant parts such as erect stems or twisting flag leaves that are not fully or similarly fully exposed in a horizontal position.

Spray inoculations are also commonly used. Browder (1971) described an efficient inoculation system where a suspension of urediniospores in light mineral oil, contained in gelatin capsules, is atomized onto foliage by custom-made inoculators. Hickey et al. (2012) and Riaz et al. (2016) applied spore-oil suspensions of respectively, *P. striiformis* and *P. triticina*, by means of an air brush. Following procedures described by Pretorius et al. (2000 and 2007) for flag leaf infection by *P. striiformis* and *P. triticina*, Bender et al. (2016) found that a suspension of urediniospores of the stem rust pathogen *P. graminis* Pers. f. sp. *tritici* Erikss. & Henning in water was most suitable for inoculating adult wheat plants in greenhouse experiments.

Broers and Lopez-Atilano (1994) placed agar blocks pre-inoculated with *P. striiformis* urediniospores on flag and flag-1 leaves of the highly susceptible cultivar Morocco to achieve uniform infections in quantitative studies of resistance components. However, the success rate of infections depended on the number of spores applied to the agar blocks. Milus et al. (2009) attained 72% successful infections by inoculating wheat flag leaves with *P. striiformis*

76 urediniospores suspended in an agar solution. More recently, Sørensen et al. (2016) compared
77 different point inoculation methods for *P. striiformis*. In their experiments, treatments with
78 urediniospores suspended in the engineered fluid Novec 7100, or mixed with *Lycopodium*
79 spores in a dry inoculum product, gave a 100% infection rate. Although consistent infection
80 was achieved for quantitative measurements, this method was developed for seedlings and
81 required the horizontal fixing of leaves on a base plate.

82 Taking into account the advantages and disadvantages of the different methods, the
83 objective of our study was to develop an uncomplicated yet reliable point inoculation method
84 for assessing stripe rust response in wheat flag leaves. Primary requirements were a robust,
85 easy to use technique, consistent infection, and assessment of infection type and / or rate of
86 disease development.

87

88 **Materials and Methods**

89 **Plant materials and growing conditions.** The development of a stripe rust point
90 inoculation method was investigated in two independent greenhouse trials. In the first trial the
91 cultivars Avocet 'S' (susceptible control), Kariega (resistant control [South African spring
92 wheat]) (Smit et al. 2010)) as well as two experimental lines, MP152 + *QYr.sgi-4A.1* and
93 Avocet S + *QYr.sgi-4A.1* (hereafter refer to as MP152_4A and AvS_4A, respectively) were
94 included. Both MP152_4A and AvS_4A were derived from a cross between Kariega and
95 Avocet 'S' and express moderate levels of stripe rust resistance (slow rusting) under field
96 conditions. Plants of the respective entries were grown in steam-sterilized soil in 2 L pots, three
97 plants per pot, at 18-22˚C in a greenhouse. After emergence plants were watered twice a day
98 with reverse osmosis water and fertilized twice per week with Multifeed-Classic water-soluble
99 fertilizer (Effekto® NPK 19:8:16 (43)), 2.5mg/L water and 200ml/pot, for the duration of the
100 trial.

In a second trial, the technique was validated using seven wheat entries known to vary in their field response to *P. striiformis* race 6E22A+. These include the stripe rust susceptible entries Avocet 'S', Avocet 'R', Jupateco 'S', Krokodil and JIC871, the moderately resistant entry Jupateco 'R' and the cultivar Trident (*Lr37/Yr17/Sr38* gene complex) which expresses stripe rust APR under field conditions. Avocet 'S' and Avocet 'R' are near-isogenic selections for the absence or presence of *YrA* from the Australian wheat cultivar Avocet (Wellings et al. 1988) whereas Jupateco 'S' and Jupateco 'R' were reselected for the absence or presence of the *Lr34/Yr18/Sr57* gene (Singh 1992). Krokodil is a South African spring wheat cultivar (Smit et al. 2010) and JIC871, coded as entry W6241 in Prins et al. (2016), is a highly susceptible line from Ethiopian origin obtained from the Genome Resource Unit, Norwich Research Park, UK.

Inoculation experiments. In both trials, plants were inoculated with *P. striiformis* race 6E22A+ when the majority were at mid heading to mid flowering (Zadoks growth stages 55-65) (Zadoks 1961). Inoculum consisted of 100 mg freshly collected urediniospores in 100 ml of reverse osmosis water without any added surfactant. Using a pair of forceps, a 6 mm antibiotic Munktell (www.munktell.com) test paper disc was dipped into the spore layer on top of the suspension and placed in the middle of the adaxial surface, equidistant to the base and tip, of each flag leaf. Each disc was immediately covered with a strip of white-colored, PVC electrical insulation tape (0.2 mm by 18 mm) placed across the width of the leaf. To facilitate easy removal, the adhesive tape was cut long enough to allow for a 1cm extension on either side of the leaf. When covering discs special care was taken to not put undue pressure onto the disc area itself in an effort to retain maximum moisture and contain the point of inoculation. Plants were kept at 10°C in the dark in a cold room for 24 h (under prevailing conditions

without additional humidifying) before placement in a greenhouse at 14-18°C. The tape and discs were carefully removed 24 h later.

In the first trial, four pots per entry, with five inoculated flag leaves per pot, were used in each trial replicate. To evaluate the effect of post-inoculation temperature on the expression of resistance two treatments were applied. Two pots of each entry were kept in separate greenhouse cubicles at a mean low (14.4-17.8°C) and mean high (19.1-23.7°C) night/day temperature regime respectively, measured at 30 min intervals with an Onset® data logger (model HOBO® 4-channel analog logger). Prevailing day length applied with no additional lighting used in the greenhouse cubicles. Pots were randomized on the respective greenhouse benches and the trial was replicated in an independent experiment applying the same methodology.

In the second trial, which was replicated, each trial replicate was presented by two pots per entry, with four inoculated flag leaves per pot kept in greenhouse cubicles with mean night/day temperature range of 14.55°C and 18.54°C, respectively.

Stripe rust assessment. In the first trial, the maximum length of visible lesion progression was measured at three-day intervals from seven dpi to 22 dpi using a Mitutoyo digital calliper. In the second trial the initial lesion size was measured at seven dpi and 12 dpi and thereafter every five days until the final measurements at 27 dpi.

Data analysis. Data collected from the four pots per treatment in the first trial, representing two trial replicates with 10 data points per trial replicate, were pooled as the data did not differ significantly ($P \leq 0.05$) between the two trial replicates. Non-linear regression was used to determine the relationship between variables using NCSS: Statistical System (Hintze, 2007). The Power model ($Y=Ax^b$) was used to describe the relationship between time (days after

150 inoculation) and lesion length (mm). Lesion length data obtained 22 dpi were analysed for
151 variance (ANOVA) including all variables. Mean separation was conducted using Fisher's
152 unprotected test to determine the least significant difference (LSD) at the 5% significance level.
153 Similarly, in the second trial data obtained from the four pots per entry, representing two trial
154 replicates with eight data points for each entry per trial replicate, were pooled as the data did
155 not differ significantly ($P \leq 0.05$) between the two trials. Non-linear regression analysis as
156 described for trial one was applied using the Logistic model ($Y=A/(1+B(\exp(-Cx)))$) to
157 describe the relationship between time (days after inoculation) and lesion length (mm).
158
159 **Results**
160 The point inoculation technique developed for studying stripe rust infection on flag leaves
161 was highly reliable with a 100% infection rate achieved over trials, entries and treatments. The
162 appearance of the first symptoms of infection, which was visible as chlorosis 7 days after point
163 inoculation on a flag leaf of Avocet 'S', is shown in Fig. 1. In the first trial involving two
164 temperature treatments, the Power model (Fig. 2) was selected as it accommodated variation
165 in the nature of response curves across entries and treatments. The progression in lesion length
166 responses observed in MP152_4A, AvS_4A and Avocet 'S' was similar at the low temperature
167 incubation (Fig. 2A). However, ANOVA analysis (Table 1) for data points 22 dpi shows that
168 MP152_4A produces significantly shorter lesion lengths (58.64 mm, $P<0.05$) when compared
169 with AvS_4A (67.29 mm) and Avocet 'S' (65.93 mm). In the high temperature treatment (Fig.
170 2B) the responses observed for both MP_152 and AvS_4A were similar and differed
171 significantly ($P<0.05$) from that of Avocet 'S', which was supported by ANOVA analyses on
172 the 22 dpi data points (Table 1). For the resistant cultivar Kariega a slight increase in lesion
173 length only occurred between 7 and 10 dpi for both treatments and the lesion length remained
174 constant at 6.59 and 6.32 mm for the low and high incubation temperature treatments,

175 respectively from day 13 to day 22 post inoculation. Lesion development on flag leaves of
176 Avocet 'S' was delayed by the high temperature regime (40.82 mm) when compared with the
177 low temperature regime (65.93 mm), but remained significantly longer in expansion when
178 compared with that of the other entries, which did not exceed 13.6 mm in this environment.
179 Comparative phenotypes for Kariega, Avocet 'S' and AvS_4A at the mean low (A) and mean
180 high (B) temperature treatments is shown in Fig. 3.

181 In the second trial, the Logistic model (Fig. 4) best described the sigmoidal curve
182 responses. However, no significant response was found for the cultivar Trident which lesion
183 lengths remained constant at 6.52 mm from 7 dpi to 27 dpi. The responses observed in Avocet
184 'S', Avocet 'R', Jupateco 'S', Krokodil and JIC871 clustered together, whereas the Jupateco
185 'R' response was independent of a group. A box plot representing lesion length data of the
186 respective entries 27 dpi confirms the grouping of the entries (Fig. 5). Comparative phenotypes
187 for Trident, Jupateco 'R', Krokodil, Avocet 'S' and JIC871 27 dpi are shown in Fig. 6.

188

189 **Discussion**

190 A flag leaf point inoculation technique, allowing for the reliable measuring and
191 comparison of stripe rust lesion expansion under controlled conditions, was developed. One of
192 the most noteworthy advantages of this technique is that flag leaves similar in age and
193 appearance can be selected which adds to accuracy and precision of phenotyping. Knowing the
194 exact point of inoculation permits the reliable measurement of lesion expansion in wheat leaves
195 where systemic colonization over time occurs. In wheats displaying a strong hypersensitive
196 response without substantial expansion of a sporulating leaf area, final lesion size can be
197 described, measured and statistically compared. Disc application did not result in inoculum
198 run-off away from the initial point of application as was experienced in optimization trials with
199 liquid suspensions (Boshoff WHP, unpublished data). The paper discs are inexpensive and their

application is uncomplicated and quick. Furthermore, the technique does not require a dew chamber cycle often limiting the number of pots, entries and treatments manageable on a given day. The waterproof tape retained sufficient moisture in the discs over a 48 h incubation period, was easy to apply and remove, and did not show any signs of phytotoxicity.

The technique in its current form did not consider standardization of the exact spore load per disc. The number of spores that successfully infects the plant within the 6 mm disc area may thus vary. Measures to optimize or standardize spore load per disc could be considered in future studies to allow for comparison of entries differing in components of resistance such as initial infection rate. Necrosis observed at the point of inoculation for some of the infection points did not result in any significant differences in lesion expansion and can be attributed to plant cell death as lesions aged. Measuring lesion size at regular intervals on many flag leaves was tedious. However, depending on the objective of an experiment, the final lesion dimensions may be enough to discriminate amongst trial entries. Should more detailed comparisons be required over a time course, e.g. the characterization of a slow rusting response, more regular ratings will be necessary. Here, the application of automated phenotyping technology should be useful.

Previous point inoculation methods applied to achieve uniform infections with *P. striiformis* have been less successful when considering the success rate of infections achieved (Broers and Lopez-Atilano 1994; Milus et al. 2009). The use of agar blocs or agar solutions may also result in colonization and contamination by other micro-organisms at the point of inoculation. The technique described by Sørensen et al. (2016) was efficient and resulted in a 100% infection rate of *P. striiformis* on wheat seedlings. Resources required for fixing a large number of leaves on a horizontal plate will limit the number of entries that can be handled and the technique is considered less practical when working with adult plants due to variation in plant height and leaf position. As the current point inoculation technique allows for the

retention of moisture at the inoculation point plants can be incubated under conditions of low humidity without affecting infection negatively. This facilitates the handling of a larger number of plants per inoculation cycle as dew cabinets often are restricted in capacity.

The significant shorter mean stripe rust lesion development measured at the higher temperature regime for Avocet 'S' clearly shows the sensitivity of *P. striiformis* race 6E22A+ to higher ambient temperatures. Previous efforts to detect any resistance response in AvS_4A under controlled conditions failed. These experiments were carried out using the spray inoculation method under a lower temperature regime (15-18°C night/day temperature regimes) in the greenhouse. The results obtained with the current point inoculation method confirmed previous results obtained with spray inoculation indicating that resistance conferred by the *QYr.sgi-4A.1* QTL against stripe rust is not expressed at prevailing cooler temperatures. The strong response observed at the higher temperature regime for MP152_4A and AvS_4A indicates that resistance conferred by this QTL is temperature sensitive. Trials to induce earlier expression of the *QYr.sgi-4A.1* resistance in Avocet_4A and MP152_4A by incubating post inoculated seedlings at three set temperature regimes (20-25°C, 18-21°C and 15-18°C, respectively) were unsuccessful (Boshoff WHP, unpublished data). Resistance conferred by the *QYr.sgi-4A.1* QTL therefor appears to be influenced by both growth stage and temperature, which is typical for high-temperature adult plant stripe rust resistance reviewed by Chen (2013). This has implications for phenotypic expression of resistance under field conditions as temperature varies within and between seasons.

Results obtained with the point inoculation technique indicated the successful characterization of different stripe rust response types in adult wheat plants. Numerous applications for detailed phenotyping are possible, e.g. routine screening, environmental influences, the effects of different *P. striiformis* races on selected germ plasm, and even the possibility of field studies provided point inoculation occurs before natural infection.

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Table 1. Two-way interaction in lesion length between four wheat entries incubated at mean low temperature (14.4-17.8°C) (A) and mean high temperature (19.1-23.7°C) (B) incubation treatments 22 days post point inoculation with *Puccinia striiformis* f. sp. *tritici* race 6E22A+.

	Temperature Treatment (°C)		
Cultivar	**A**	**B**	**Mean**
Kariega	6.59 a	6.32 a	6.46 a
MP152-4A	58.64 b	13.57 b	36.11 b
AvS-4A	67.29 c	13.45 b	40.37 c
Avocet 'S'	65.93 c	40.82 c	53.37 d
Mean	49.61 b	18.54 a	34.08

LSD: Cultivar x treatment = 2.68; Cultivar = 4.24; Treatment= 3.00

*Mean values followed by the same letter in the respective columns as well as for the treatment means do not differ significantly according to Fishers LSD ($P<0.05$).

Fig. 1. Flag leaf of cultivar Avocet 'S' (A) showing clear signs of infection visible through chlorosis seven days post point inoculation with urediniospores of *Puccinia striiformis* f. sp. *tritici* race 6E22A+. Expansion of stripe rust lesion length is clearly visible 14 days post inoculation from the upper leaf surface (B).

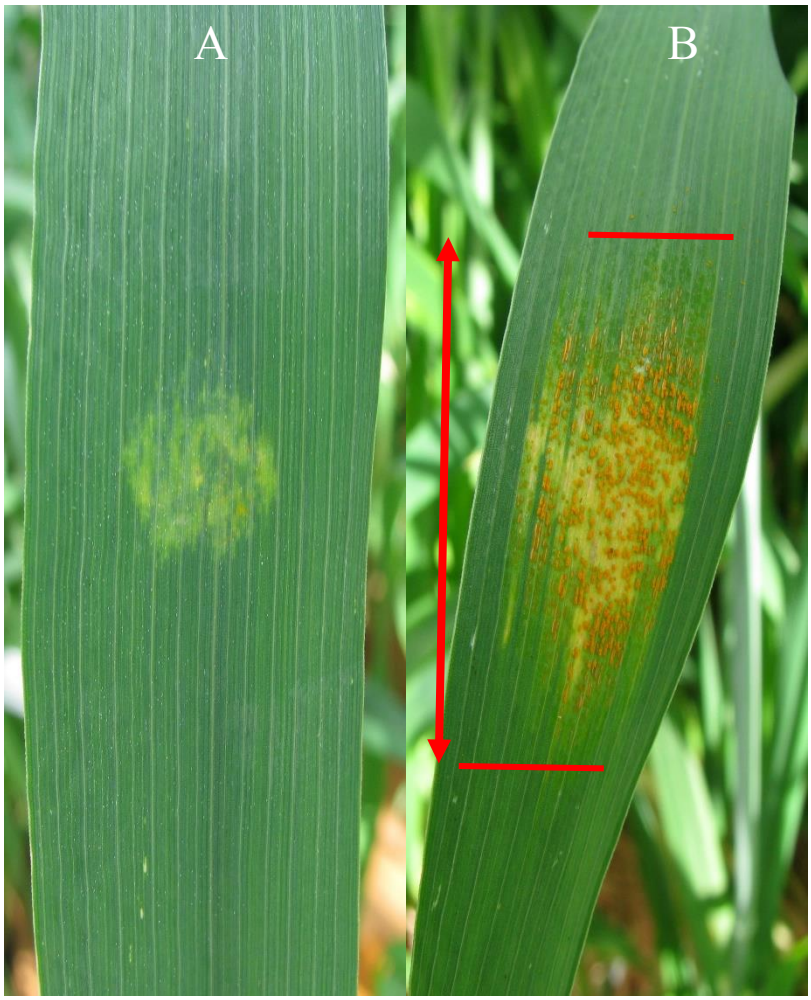
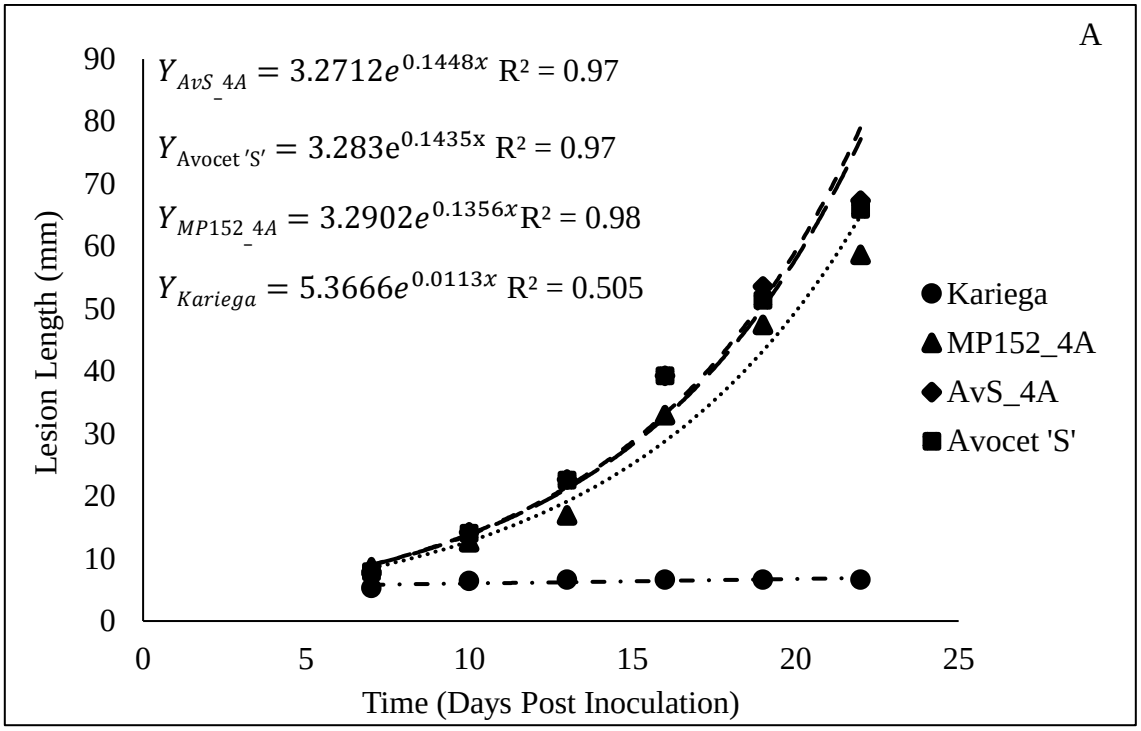


Fig. 2. Lesion length responses (mm) of four wheat entries at mean low temperature (14.4-17.8°C) (A) and mean high temperature (19.1-23.7°C) (B) incubation treatments over a 22 day period post point inoculation with *Puccinia striiformis* f. sp. *tritici* race 6E22A+.



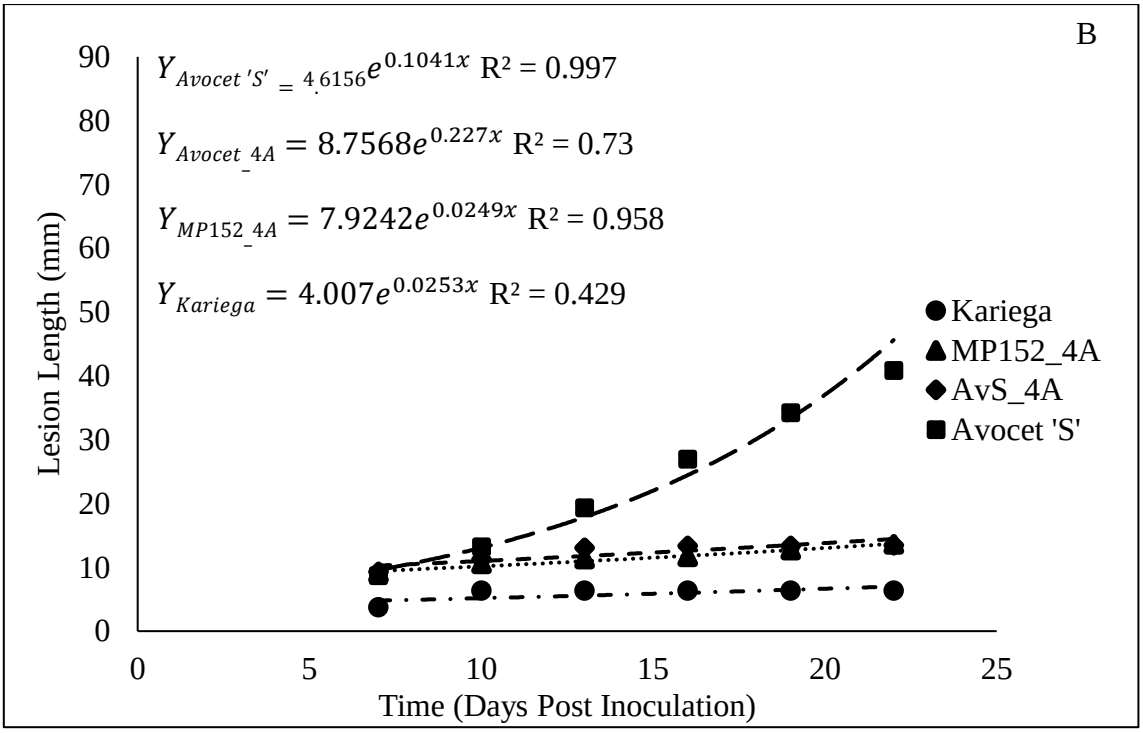
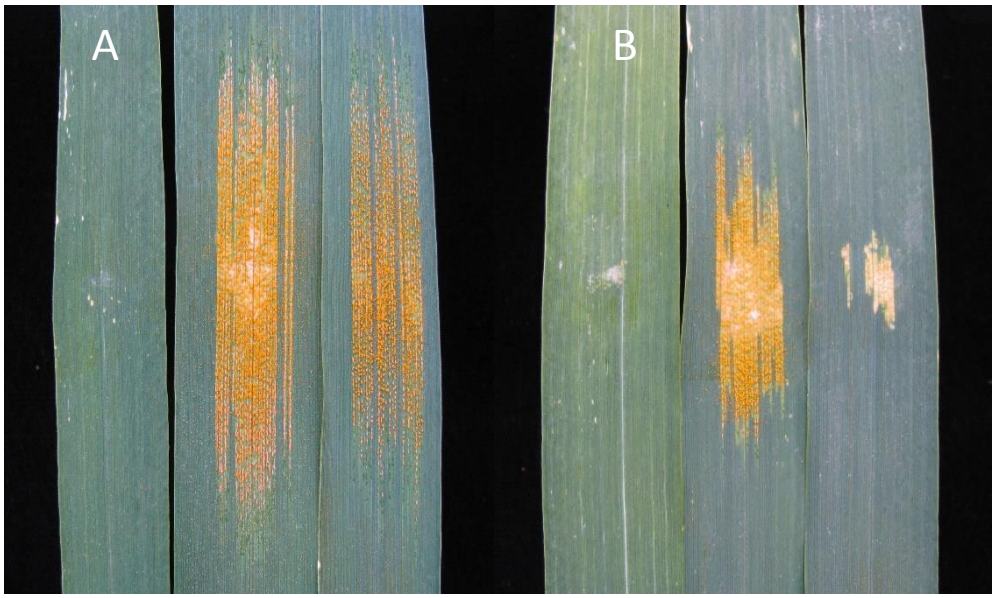


Fig. 3A and B. Flag leaves from left to right showing representative stripe rust infection types and lesion lengths for the wheat entries Kariega, Avocet 'S' and AvS_4A 22 days post point inoculation with *Puccinia striiformis* f. sp. *tritici* race 6E22A+. The mean post inoculation night/day temperatures were at a mean low (**A** 14.4-17.8°C) and mean high (**B** 19.1-23.7°C), respectively.



382 **Fig. 4.** Lesion length responses (mm) of seven wheat entries over a 27 day period post point inoculation with *Puccinia striiformis* f. sp. *tritici* race

383 6E22A+.

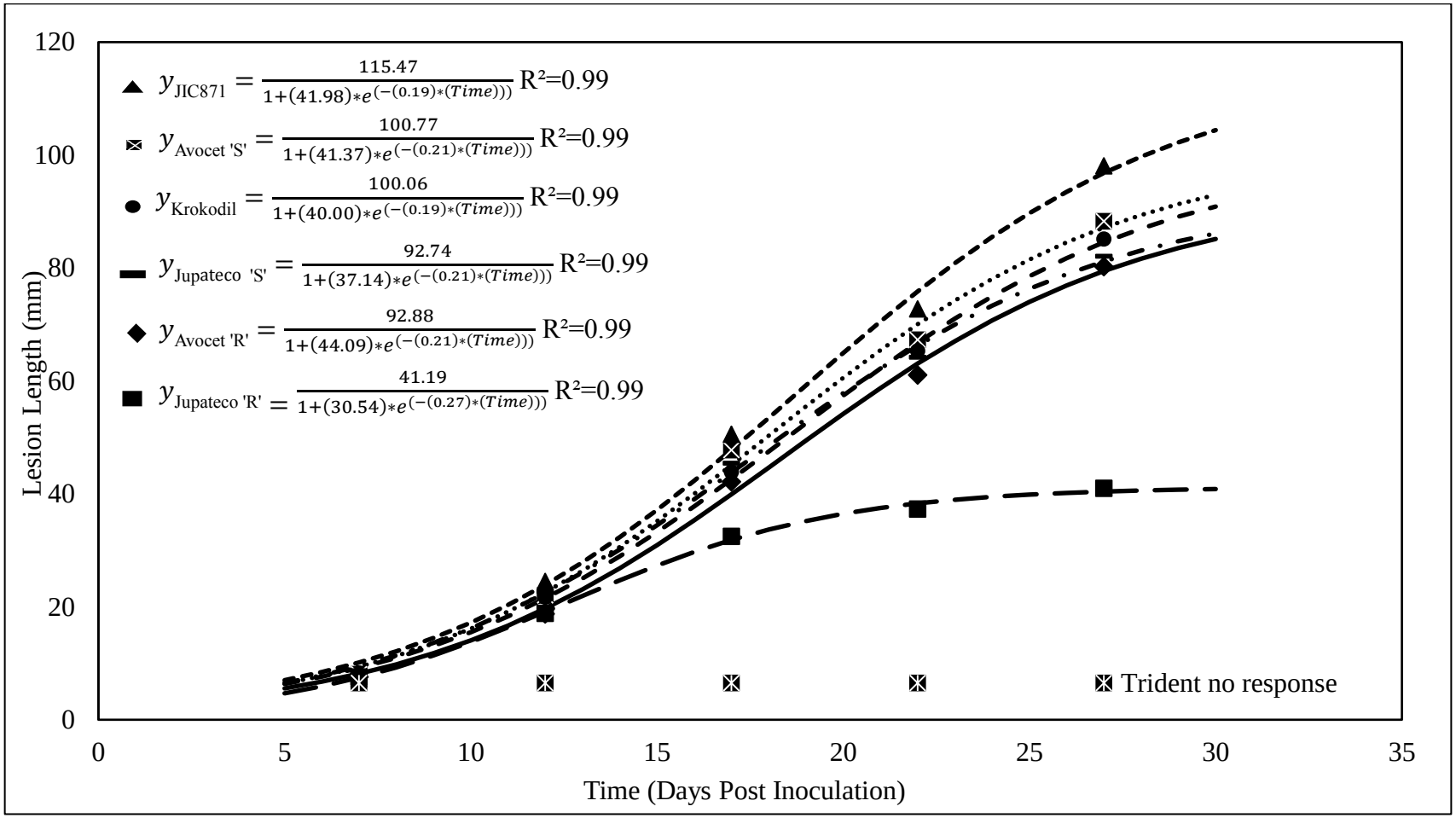


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Fig. 5. Box plot representing lesion length of seven wheat entries 27 days post point inoculation with *Puccinia striiformis* f. sp. *tritici* race 6E22A+. Means are marked with X and error bars indicate data falling outside the upper and lower quartiles.

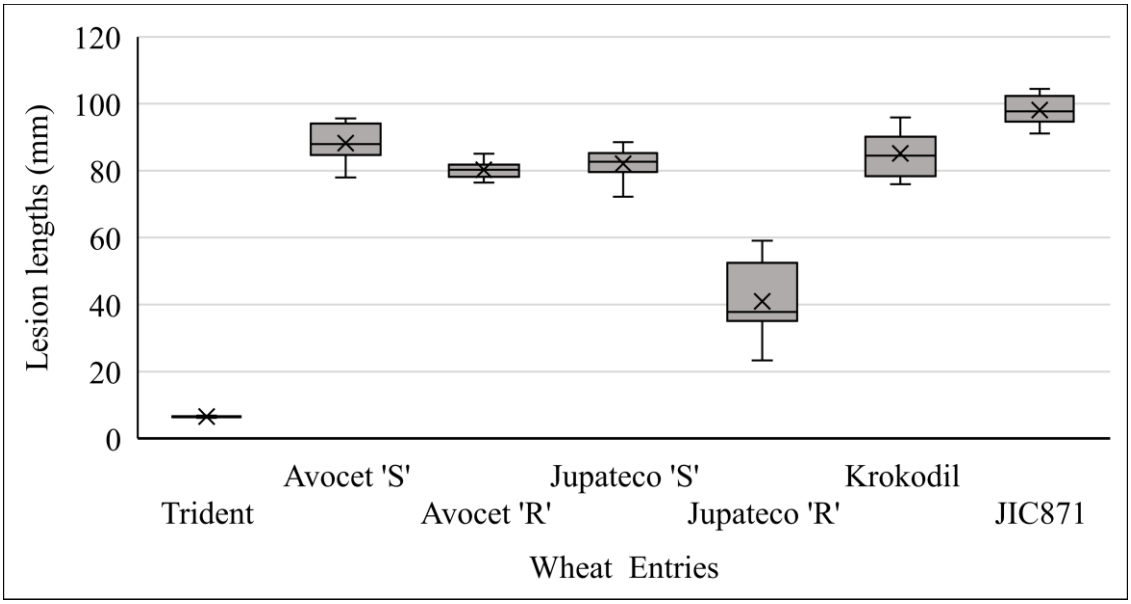

Fig. 6. Flag leaves from left to right showing representative stripe rust infection types and lesion lengths for the wheat entries Trident, Jupateco 'R', Krokodil, Avocet 'S' and JIC871 27 days post point inoculation with *Puccinia striiformis* f. sp. *tritici* race 6E22A+. The mean post inoculation night/day temperatures maintained were 14.55 and 18.54°C, respectively.