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Biological control of yellow rust of wheat (*Puccinia striiformis*) with Serenade®ASO (*Bacillus subtilis* strain QST713)

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ABSTRACT

Yellow rust (*Puccinia striiformis* f. sp. *tritici*) is an important disease in wheat causing significant yield reductions, if not effectively controlled. The biofungicide *Bacillus subtilis* strain QST 713 suspension concentrate (Serenade®ASO) was investigated for its potential for yellow rust control in winter wheat field trials. Serenade®ASO reduced severity of yellow rust significantly, providing up to 60% control at BBCH growth stage 65–69, under moderate disease pressure. Under high disease pressure reductions were more variable and provided less than 30% control. An increase in the number of applications of biofungicide from two to four per season tended to improve disease control, although differences were not always significant. With a few exceptions no clear dose-response was seen between using 1, 2, 4, 6 or 8 l/ha applied 4 times at 8–10-day intervals. Yield responses were positive, but responses to biofungicide were only significant in a few cases, and in all cases the level of control and yield responses were significantly lower compared with using prothioconazole as chemical control. An outdoor pot trial using artificial inoculation tested preventive and curative application of Serenade®ASO at three dose rates. This trial confirmed the lack of a clear dose response but showed that timing had a major impact on control, with the best control obtained at the day of inoculation. This study revealed that Serenade®ASO cannot stand alone in the control of yellow rust. More research is needed to develop integrated disease management strategies which also include biofungicides.

1. Introduction

Wheat is a dominant cereal crop worldwide and very important as a staple food resource. Multiple diseases can attack the crop and the disease yellow rust caused by *Puccinia striiformis f. sp. tritici* is seen as one of the major threats to wheat production. *P. striiformis* is a biotroph pathogen on wheat commonly found in cooler and wetter regions including Asia, North America, Australia and Europe. It can cause yield reductions between 5 and 50% depending on the year, region and developmental stage of wheat at which the attack occurs (Singh et al., 2015). Following significant epidemics major economic losses have been measured in Europe, Australia and the US (Beddow et al., 2015; Murray and Brennan, 2009).

Yellow rusts have been a major focus for research and breeding due to the ability of the fungi to overcome race-specific resistance genes within a few years, causing major changes in pattern of epidemics and subsequent yield losses. Traditionally, yellow rust has been less prevalent than other wheat diseases in many countries due to efficient resistance in commonly grown cultivars. However, the dynamics of the *P. striiformis* pathogen to generate new races has caused sudden epidemics in cultivars previously regarded as resistant, and particularly since 2010 detection and fast spread of new aggressive races have caused severe losses (Hovmøller et al., 2015; Wellings et al., 2012). This development has increased the pressure on producing new resistant cultivars in order to manage yellow rust, but it has also put pressure on other control measures such as improved cropping practice and use of fungicides. In case of outbreaks of yellow rust, fungicide treatments are usually recommended as soon as the disease is detected in the field in order to prevent a severe epidemic (Jørgensen et al., 2014). Several fungicides belonging to the chemical groups of triazoles, strobilurins and SDHI's are known to be effective against yellow rust. If treatment is applied at the very early stages of attack, reduced dose rates can be applied and provide good control of yellow rust, whereas delayed treatments have proved to be less cost effective (Jørgensen and Nielsen, 1994).

Fortunately, no evidence of fungicide resistance in *P. striiformis*

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has been found so far, but the risk of development of resistance can not be ruled out due to intense use of fungicides over decades (Oliver, 2014). Chemical control relies on few modes of action, which may increase selection pressure and eventually lead to resistance development. This calls for investigating alternative control measures including biological control products, which are also generally seen as more environmentally sound solutions.

Bacillus subtilis is a rhizobacterium that can form endospores and can produce several different antibiotics (Stein, 2005). These are primarily formed during endospore formation in low concentrations, and there has been some uncertainty as to whether disease control is directly linked to the action of antibiotics (Kilian et al., 2000; Leifert et al., 1995). The mode of action of *Bacillus* species is described by Frac and Jezierska-Tys (2010) as microbial disrupters of pathogen cell membranes. Some of the effect may be indirect as some findings suggest that *B. subtilis* also has the ability to induce plant mediated resistance in the host plant (Ongena et al., 2007).

Serenade®ASO is a broad-spectrum biofungicide that contains *Bacillus subtilis* strain QST 713 and has been approved for use in the European Union (Reg. (EC) No 839/2008). Its worldwide utilisation covers all kinds of fungal diseases in diverse crops (Fischer et al., 2013). The fungicide was registered in Europe targeting mainly *Botrytis cinerea* on outdoor grown lettuce and strawberries, aubergine/eggplant, tomato and paprika in greenhouses and *Erysiphe heracle* and *Alternaria dauci* on carrot. The effectiveness of the related endophytic *Bacillus subtilis* strain E1R-j for the control of yellow rust has previously been reported (Li et al., 2013); however, no studies have been conducted on yellow rust control by the *Bacillus subtilis* strain QST 713. The objective of this study was to i) evaluate the efficacy and consistency of *B. subtilis* QST 713 against yellow rust under field conditions, ii) investigate the dose-response relationship both under field and semi-field conditions and iii) identify the importance of timing and intensity when applying *B. subtilis* QST 713 for control of yellow rust.

2. Materials and methods

2.1. Field trials

A total of four field trials were conducted in winter wheat at Flakkebjerg research station (55.3253 N 11.3913 W) on a fine clay loam soil in the growing season 2013/14 and 2014/15. Disease developed naturally during both seasons and severity was regarded as moderate in 2014 and severe in 2015. The weather in both seasons started with mild winters giving good possibilities for inoculum of yellow rust to survive the winter. In 2014 the disease developed from late April and gave rise to moderate levels of disease. In 2015 yellow rust was established already in the autumn and further development started already in February. These early attacks led to very high and significant infections in susceptible crops in 2015. An overview of the weather conditions can be found in the supplementary material. The four independent trials were conducted using the susceptible cultivars Ambition and Baltimor in 2014 and Ambition and Substance in 2015. The experimental set-up was a completely randomised block design with four replicates, a plot size of 22.5 m^2 (9 m length and 2.5 m width) and with 25 cm space between the plots. Plots were sown with a plot sowing machine at 2–4 cm depth aiming at 400 seeds per m^2. Apart from fungal treatment crop management was conducted according to common crop practice. Chemical control was performed with prothioconazole 250 g/l, (Proline EC250, Bayer CropScience) and the biofungicide used was *B. subtilis* QST 713 10^{12} colony forming units per l (CFU/l), (Serenade®ASO, Bayer CropScience). The products were applied with a self propelled sprayer (Speedy 2500) operating at a speed of 4.5 km/h, and a boom height of 40 cm. The boom was fitted with Teejet 9504 nozzles, operating at a pressure of 2.4 bar and delivering a volume rate of 150 l/ha. Fungicide applications included six treatments plus an untreated control and four treatments plus untreated control in 2014 and 2015, respectively (Table 1). The number of included doses was reduced in 2015 due to the limited dose-response seen in 2014. Treatments and assessments followed a similar schedule in both years (Table 2). Disease assessment was carried out visually as percentage of yellow rust coverage of green leaves evaluated at specific leaf layers at intervals of ten days, starting at the first application and finishing at senescence, following European plant protection standards (EPPO/OEPP (2012) PP 1/26(4)). For the statistical analyses of disease severity, three representative time points (BBCH (Lancashire et al. (1991)) 39, 51 and 69 in 2014 and BBCH 33, 49 and 65 in 2015) were chosen to illustrate the performances of the treatments (Table 2).

The trials were harvested using a plot combine harvester. Yield responses in t/ha were adjusted to 15% moisture.

2.2. Semi-field trial

The pot trial was conducted in a covered outdoor area in 8 l pots (semi-field) in 2015. Each pot was watered individually with an automatic drip irrigation system and temperature conditions were similar to the ones described for the field trials. Twenty seeds per pot of the spring wheat variety Trappe, known for its susceptibility to *P. striiformis*, were sown in each pot. A spring wheat cultivar was chosen as it does not need a vernalisation period. *P. striiformis*

Table 1
Description of treatments used in the four field trials. Replicate trials in 2014 and 2015 followed the same protocol.

Year	Treatment	Product[a]	Application rate (l/ha)	Application time points[b]
2014	1	Untreated		
2014	2	Chemical control	0.8	AD
2014	3	Biofungicide	1	AD and ABCD
2014	4	Biofungicide	2	AD and ABCD
2014	5	Biofungicide	4	AD and ABCD
2014	6	Biofungicide	6	AD and ABCD
2014	7	Biofungicide	8	AD and ABCD
2015	1	Untreated		
2015	2	Chemical control	0.8	AD
2015	3	Biofungicide	4	ABCD
2015	4	Biofungicide	6	ABCD
2015	5	Biofungicide	8	ABCD

[a] Chemical control: 250 g/l prothioconazole (Proline EC250, Bayer CropScience); Biofungicide: 10^{12} CFU/l, *B. subtilis* QST 713 (Serenade®ASO, Bayer CropScience).
[b] Dates of application time points (A to D) are given in Table 2.

Table 2
Description of the dates of application (A, B, C, D), dates of assessments of yellow rust severity (1, 2, 3) and the cultivars used in the four field experiments.

BBCH[a]		Application dates				Assessment dates		
Trial	Variety	A 31–32	B 32–33	C 33–37	D 39–53	1 33–39	2 49–51	3 65–69
2014, Exp. 1	Ambition	24.04.	02.05.	13.05.	28.05.	22.05.	27.05.	17.06.
2014, Exp. 2	Baltimore	24.04.	02.05.	13.05.	28.05.	22.05.	26.05.	17.06.
2015, Exp. 1	Ambition	27.04.	06.05.	12.05.	26.05.	07.05.	07.06.	23.06.
2015, Exp. 2	Substance	27.04.	06.05.	12.05.	26.05.	07.05.	07.06.	23.06.

[a] Growth stages in BBCH scale Lancashire et al. (1991).

spores of isolate (Flak9/11) were multiplied on the cultivar Anja and harvested by shaking the plants inside a cellophane bag. Subsequently, *P. striiformis* spores were dried, transferred to cryo vials and stored at −80 °C until experimental use. At wheat growth stage 33 the plants were inoculated with 1.5 mg *P. striiformis* spores per pot and dissolved in Novec 7100. Spores were applied with an airbrush spray gun (standard class; Revell GmbH) in the early evening. Plants were moistened and covered with plastic for the following two nights, ensuring 100% relative humidity.

The plants were treated either preventively (2 days before inoculation) or curatively (0, 1, 2, 4 and 8 days after inoculation) with 3 concentrations of biofungicide (2, 4 and 8 l/ha) and compared to the chemical treatment (tebuconazole, 125 g/ha). A treatment with the adjuvant Silwet Gold (0.1%) and a mixture of 4 l/ha biofungicide + 0.1% Silwet Gold was only applied at two timings (0 and 2 days after inoculation) (Table 3).

Disease assessment was carried out visually, counting number of infected leaves at an interval of two days and the severity was assessed according to EPPO standards, starting at the first observation of disease 13 days after inoculation. The set-up was a complete randomised design with four replicates.

2.2.1. Statistical analysis

Statistical analysis was performed using R version 3.1.3 (R Core Team (2016)). Yellow rust AUDPC was calculated for leaf 2 from BBCH 37 to 69. All disease assessment data were log transformed prior to ANOVA to meet the assumption of normal distribution. Differences between groups were calculated with Tukey's test with a confidence level of 0.95.

3. Results

3.1. Field trials

3.1.1. Yellow rust control levels

Yellow rust disease pressure varied between the two years with severe attacks in 2015 and only moderate attacks in 2014 (Fig. 1). Treatments with the triazole fungicide prothioconazole generally resulted in significantly better and less variable control of yellow rust compared with control by biofungicide (Tables 4 and 5). In contrast to chemical treatment, yellow rust control by biofungicide varied considerably between sites and years with up to 60% control in 2014 (BBCH 69) compared with less than 30% control in 2015 (BBCH 65) (Tables 4 and 5).

Biofungicide applications of minimum 4 l/ha significantly reduced disease development in both experiments in 2014 compared to untreated controls (Fig. 1). In 2015 significant reductions of disease development were only recorded for one treatment. When AUDPC data of all four experiments were analysed together biofungicide treatment did not lead to any significant reduction in disease development (not shown). In all trials, grain yields were significantly increased, following two treatments with prothioconazole (11–42%), compared with the untreated control (Table 6). Biofungicide treatments increased yields by 1–7%, but total yield was not significantly different from untreated in any of the four trials (Table 6).

3.1.2. Dose-response relationship

Application rates below 4 l/ha did not result in significant reductions of disease progress in any of the trials in 2014 compared with the untreated control (Fig. 1). Treatments with 4, 6 and 8 l/ha reduced disease progress significantly in 2014 compared with the untreated control, but disease severity in all biofungicide treatments were higher compared with chemical control. Under more severe disease pressure in 2015, biofungicide treatments did not reduce disease progress with the exception of the treatment with 6 l/ha Exp.1 (Fig. 1). In summary, a minimum of 4 l/ha biofungicide had to be applied to obtain a reduction in disease progress, but no dose-response relationship was observed for higher application rates.

3.1.3. Application frequency

No significant reduction in disease progress was found between the control and one or two applications of biofungicide in 2014 (dark colours in Fig. 2). However, observations at BBCH 51 showed that an increase in treatment frequency from one to three applications resulted in a significant reduction in disease severity in 2014, Exp. 2. In 2014, Exp. 1 the differences were not statistically

Table 3
Description of application rates and application time points of the semi-field trial.

Treatment	Product[a]	Application rate (l/ha)	Application time (days before or after inoculation)
1	Biofungicide	2	−2, 0, 1, 2, 4, 8
2	Biofungicide	4	−2, 0, 1, 2, 4, 8
3	Biofungicide	8	−2, 0, 1, 2, 4, 8
4	Chemical control	0.5	−2, 0, 1, 2, 4, 8
5	Biofungicide + Adjuvant	4 + 0.1%	0, 2
6	Adjuvant	0.1%	0, 2
c	Control (non inoculated)		
ci	Control (inoculated)		

[a] Chemical control: 250 g/l tebuconazole (Folicur 250 EW, Bayer CropScience); Biofungicide: 10^{12} CFU/l *B. subtilis* QST 713 (Serenade®ASO, Bayer CropScience); Adjuvant: organosilicone surfactant (Silwet Gold, Bayer CropScience).

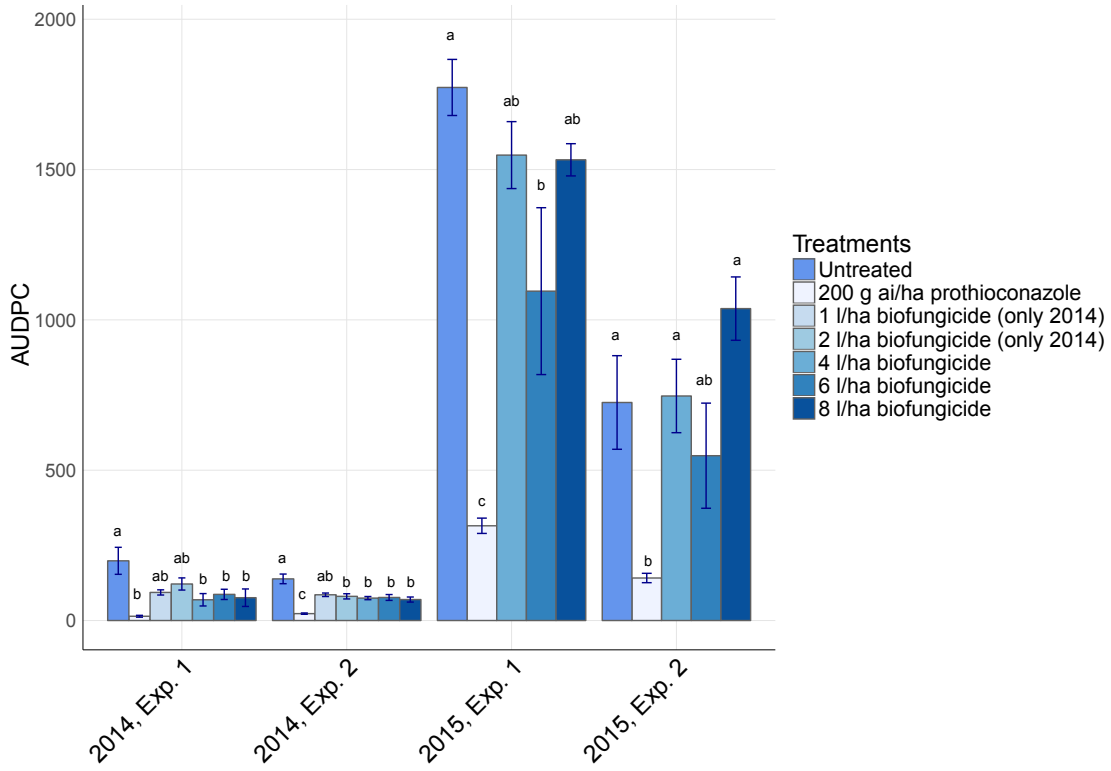

Fig. 1. Disease control for the four experiments in 2014 and 2015 using area under disease progress curve (AUDPC) from BBCH growth stage 37–69 at leaf 2. Treatments with 1 and 2 l/ha biofungicide are only included in trials conducted in 2014. Error bars represent standard errors around each mean and different letters represent significant differences between treatments (P = 0.05). Log transformation was applied prior to statistical analysis. Chemical control: 250 g/l prothioconazole (Proline EC250, Bayer CropScience); Biofungicide: 10^{12} CFU/l, *B. subtilis* QST 713 (Serenade®ASO, Bayer CropScience).

Table 4
Percent control of yellow rust in the growing season 2014, at selected growth stages in BBCH scale. Each value is given together with its standard error except for the untreated control for which yellow rust coverage of green leaves (%) is given in brackets. Different letters represent significant differences between treatments (Tukey, confidence level of 0.95). Log transformation was applied prior to statistical analysis.

Trial	2014, Exp. 1	2014, Exp. 2	2014, Exp. 1	2014, Exp. 2	2014 Exp. 1	2014 Exp. 2
BBCH	39 (leaf 2)	39 (leaf 2)	51 (leaf 2)	51 (leaf 2)	69 (leaf 2)	69 (leaf 1)
Untreated	0 (2.08) a	0 (2.00) a	0 (5.12) a	0 (9.5) a	0 (14.25) a	0 (12.25) a
Chemical control 0.8 l/ha	86.75 ± 4.12 b	70.00 ± 10.41 b	97.07 ± 0.98 b	84.21 ± 5.26 b	98.6 ± NA b	93.88 ± 2.04 b
Biofungicide 1 l/ha	48.19 ± 15.66 ab	37.50 ± 16.14 ab	70.73 ± 5.63 b	60.53 ± 7.89 c	56.14 ± 8.77 c	42.86 ± 14.53 c
Biofungicide 2 l/ha	51.81 ± 17.04 ab	52.50 ± 18.98 a	48.78 ± 18.41 ab	63.16 ± 5.26 c	38.60 ± 10.48 c	46.94 ± 7.07 ac
Biofungicide 4 l/ha	55.02 ± 25.85 ab	72.50 ± 22.50 a	82.44 ± 8.01 b	68.42 ± 12.15 c	61.40 ± 7.3 c	42.86 ± 15.98 c
Biofungicide 6 l/ha	30.92 ± 37.98 ab	21.25 ± 29.47 ab	77.56 ± 16.59 b	57.89 ± 6.08 c	57.89 ± 14.61 c	38.78 ± 20.41 ac
Biofungicide 8 l/ha	38.96 ± 41.86 ab	37.50 ± 37.5 ab	73.66 ± 13.58 b	64.91 ± 9.28 c	57.89 ± 14.61 c	56.46 ± 11.86 bc

Chemical control: 250 g/l prothioconazole (Proline EC250, Bayer CropScience).
Biofungicide: 10^{12} CFU/l, *B. subtilis* QST 713 (Serenade®ASO, Bayer CropScience).

Table 5
Percent control of yellow rust in the growing season 2015, at selected growth stages in BBCH scale. Each value is given together with its standard error except for the untreated control of which yellow rust coverage of green leaves (%) is given in brackets. Different letters represent significant differences between treatments (Tukey, confidence level of 0.95). Log transformation was applied prior to statistical analysis.

Trial	2015, Exp. 1	2015, Exp. 2	2015, Exp. 1	2015, Exp. 2	2015, Exp. 1	2015, Exp. 2
BBCH	33 (leaf 3)	33 (leaf 3)	49 (leaf 2)	49 (leaf 2)	65 (leaf 1)	65 (leaf 1)
Untreated	0 (2.00) a	0 (0.28) a	0 (35.00) a	0 (41.75) ab	0 (60.00) a	0 (76.25) ab
Chemical control 0.8 l/ha	97.50 ± 2.50 b	100 ± NA b	78.10 ± 4.15 b	86.43 ± 5.59 c	97.50 ± 0.83 b	94.59 ± 1.78 c
Biofungicide 4 l/ha	58.33 ± 30.05 bc	−627.27 ± 331.95 ab	23.81 ± 12.60 ac	−22.75 ± 19.78 ab	2.78 ± 12.11 a	14.75 ± 15.84 ab
Biofungicide 6 l/ha	23.75 ± 31.32 ac	−263.64 ± 363.64 ab	42.86 ± 21.82 c	20.36 ± 38.29 bc	27.08 ± 21.35 a	16.39 ± 11.16 bc
Biofungicide 8 l/ha	65.00 ± 32.53 bc	−506.06 ± 320.70 ab	35.71 ± 7.14 c	−79.64 ± 18.29 a	29.17 ± 14.63 a	−0.55 ± 5.78 a

Chemical control: 250 g/l prothioconazole (Proline EC 250, Bayer CropScience).
Biofungicide: 10^{12} CFU/l, *B. subtilis* QST 713 (Serenade®ASO, Bayer CropScience).

Table 6
Harvested yield (t/ha) of plots treated with fungicide and biofungicide products, followed by the relative yield in brackets. Different letters represent significant differences within each experiment (Tukey, confidence level of 0.95).

Treatment	2014, Exp. 1	2014, Exp. 2	2015, Exp. 1	2015, Exp. 2	Mean[a]
Untreated	9.92 (100.00) a	7.72 (100.00) a	8.32 (100.00) a	6.57 (100.00) a	8.13 (100.00) a
Chemical control	11.02 (111.09) b	9.96 (128.97) b	10.83 (130.15) b	9.37 (142.56) b	10.3 (128.19) b
Biofungicide 1 l/ha	10.04 (101.18) a	8.11 (104.99) a	NA	NA	NA
Biofungicide 2 l/ha	9.75 (98.29) a	8.17 (105.86) a	NA	NA	NA
Biofungicide 4 l/ha	10.59 (106.75) ab	7.86 (101.88) a	8.57 (102.97) a	6.83 (104.03) a	8.47 (103.90) a
Biofungicide 6 l/ha	10.11 (101.94) a	8.02 (103.92) a	8.50 (102.10) a	6.89 (104.91) a	8.38 (103.21) a
Biofungicide 8 l/ha	10.64 (107.31) ab	8.00 (103.66) a	8.36 (100.51) a	6.62 (100.84) a	8.41 (103.07) a
Mean ± standard error	10.29 ± 1.05	8.26 ± 1.59	8.92 ± 2.38	7.26 ± 2.67	8.78 ± 1.46

Chemical control: 250 g/l prothioconazole (Proline EC250, Bayer CropScience).
Biofungicide: 10^{12} CFU/l, *B. subtilis* QST 713 (Serenade®ASO, Bayer CropScience).
[a] Mean of all four experiments in two growing seasons.

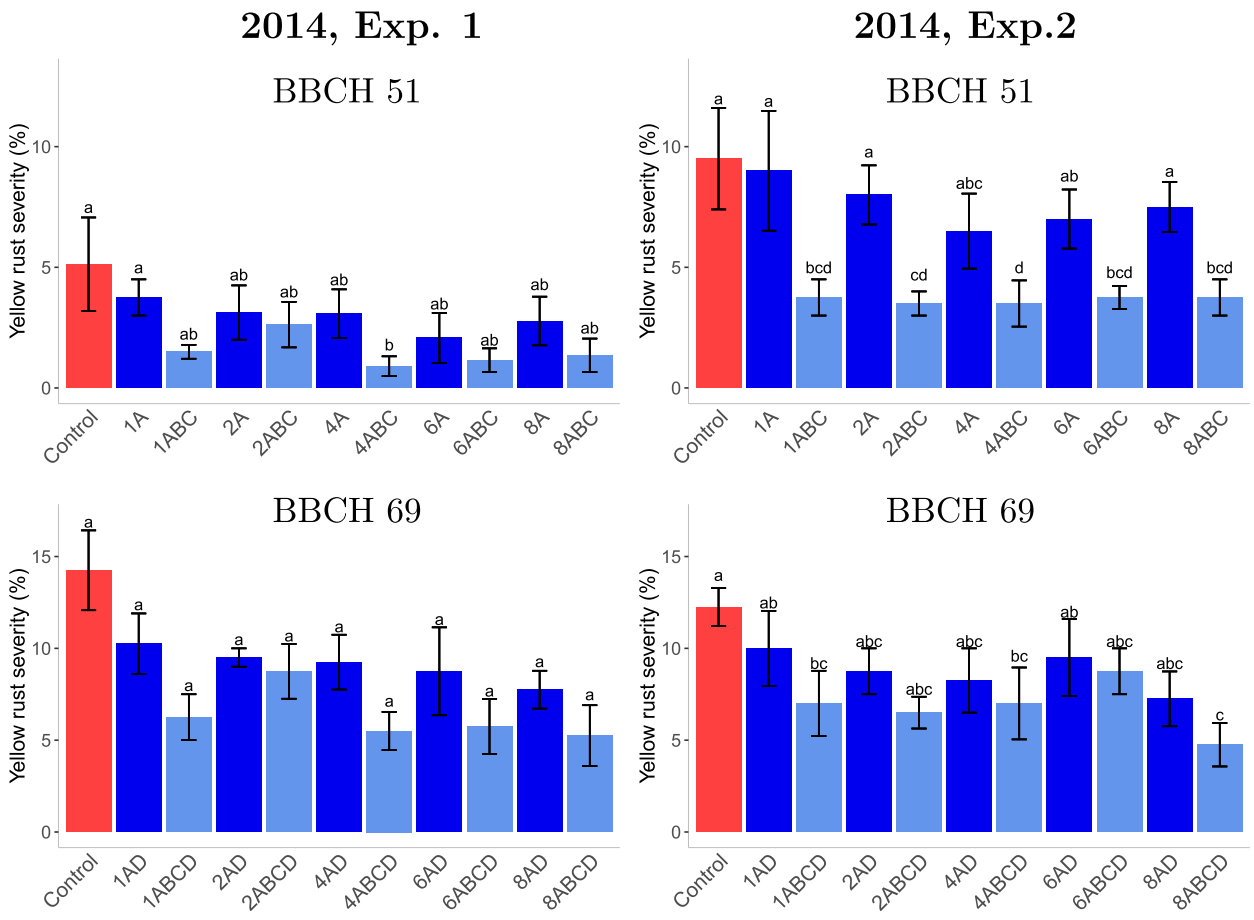

Fig. 2. Severity (%) of yellow rust on winter wheat during the 2014 growing season, observed at growth stages 51 (top) and 69 (bottom). X axis labelling: numbers indicate the amount of biofungicide applied (l/ha); capital letters from A to D indicate application time point (Table 2). Error bars represent standard errors and different letters represent significant differences between treatments (P = 0.05). Log transformation was applied prior to statistical analysis.

significant, but the trends were identical. Likewise, the observations after 4 treatments assessed at BBCH 69 showed a clear trend to superior control of yellow rust compared with two applications (lower part of Fig. 2), although differences were not significant.

3.2. Semi-field trial

Yellow rust inoculation was successful with a moderate disease severity assessed on an average of 10 leaves per pot and a 3% severity on infected leaves. The untreated inoculated plants developed significantly more yellow rust than any of the treatments. The non-inoculated control confirmed the absence of yellow rust infections potentially caused by airborne inoculum (Fig. 3).

All treatments resulted in a significant reduction of both number of leaves infected and % severity on infected leaves (Fig. 3). The treatment with tebuconazole resulted in the lowest severity compared with the treatment with biofungicide alone and in combination with the adjuvant Silwet Gold. There was neither correlation between application rate of biofungicide and disease severity nor the addition of adjuvant resulted in a significant improvement. Compared with the untreated control, the treatment

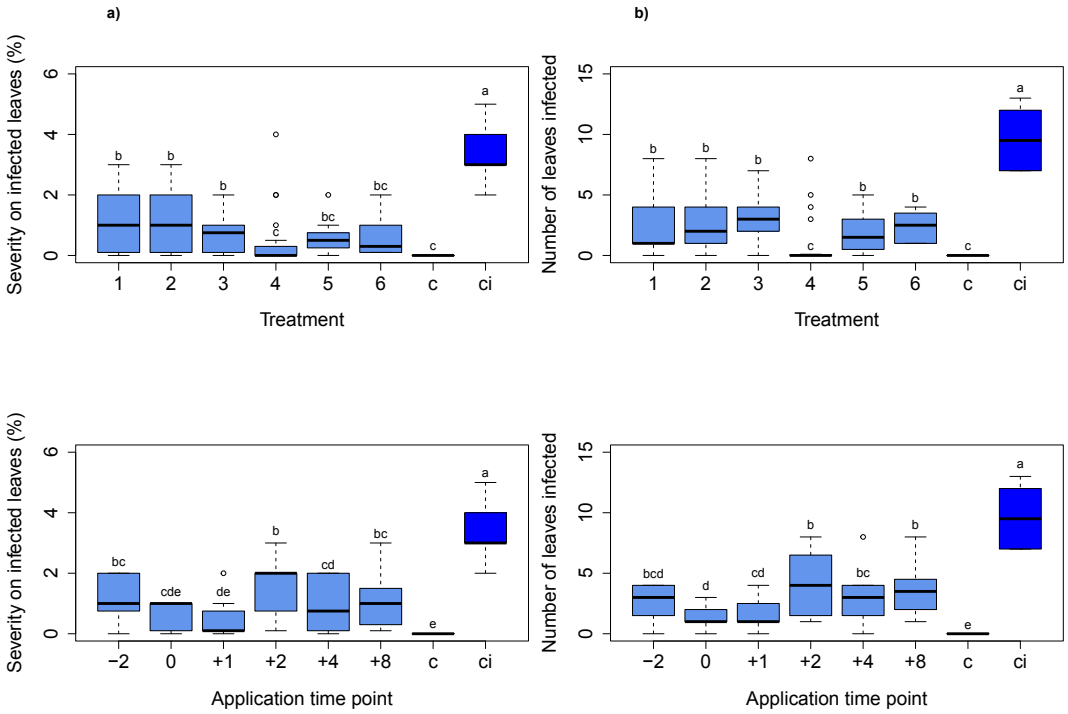

Fig. 3. Disease severity of yellow rust on spring wheat as a) % coverage of infected leaves and b) number of leaves infected per pot. Top figures reflect treatments 1–6 and bottom figure shows control from different application time points(-2 to + 8 days after inoculation), see Table 3; c, control; ci, inoculated control. In each box, the central mark represents the median, the edges of the box are the 25 th and 75 th percentiles and the whiskers mark the minimum or maximum of the data. Outliers are represented by individual dots.

with adjuvant alone also significantly reduced the severity of yellow rust The investigation of the optimum application time for biofungicide resulted in a clear and significant reduction in numbers of infected leaves when treatments were applied at the day of inoculation and one day after inoculation. Assessments of disease severity showed the same trend although not all the observed differences were significant (Fig. 3).

4. Discussion

Bacillus subtilis is one of the most studied biological control agents (BCA). The pronounced increase in the interest in biological control has led to a search for potential candidates for BCA to replace or supplement synthetic pesticides, with the overall aim of moving towards more sustainable agriculture (Wei et al., 2016). This study investigated the efficacy of the biofungicide Serenade®ASO formulation for control of yellow rust in wheat. Preventive and curative treatments with *Bacillus subtilis* QST713 under semi-field condition revealed that timing is very important for optimal control. The day of inoculation or one day after inoculation provided the best control. Applying 2 days before gave slightly lower control, although the effect was not significantly different from applying the day or the day after inoculation. Delaying treatments to 2, 4 and 6 days after inoculation reduced the level of control significantly. This implies that *B. subtilis* as a biofungicide is mainly acting preventively and is only curative at the very early stages of disease development. These findings are in line with the outcome of a study by Li et al. (2013) where applications 0 and 24 h before inoculation significantly reduced disease severity by approximately 50%. The importance of timing using *B. subtilis* products has also been highlighted by Rytter et al. (1989), who showed that *B. subtilis* had to be applied before or at the time of inoculation to achieve significant disease reductions. Furthermore, specific studies have shown that disease progress after penetration of the crop was not influenced by *B. subtilis* cells or their produced substances (Li et al., 2013; Rytter et al., 1989). Our findings therefore confirmed that timing is essential for obtaining significant control.

In the field trials presented in this study *B. subtilis* QST713 provided varying reductions of the yellow rust disease. At the best, 60% control was obtained under low to moderate levels of severity as seen in 2014. At more severe epidemics, as seen in the season 2015, the effects were more variable and generally below 30% control. No information from other studies using *B. subtilis* QST713 for control of yellow rust is available for comparison. Nevertheless, our results are in agreement with the findings from another study using *B. subtilis* strain E1R-j for control of yellow rust. This study recorded 51% and 43% control in two years of field experiments (Li et al., 2013). Other studies with the *B. subtilis* QST713 formulation have tested the efficacy on powdery mildew (*Podosphaera xanthii*) on cucumber and bacterial spot of tomato (*Xanthomonas* spp.). These trials also showed varying control levels of a size similar to the levels of control in our experiments. Powdery mildew on cucumber was not significantly inhibited in a greenhouse experiment, while bacterial spot of tomato was significantly reduced by 21–43% in two out of three field experiments (Abbasi and Weselowski, 2015; Cerkauskas and Ferguson, 2014).

In the present study, no significant dose-response relationship was observed. This might be attributed to the capacity of *B. subtilis* strains to cause an induced resistance plant mediated reaction (Choudhary and Johri, 2009; Fischer et al., 2013). If this is the mode of action of biofungicide, this response would most likely be triggered by a certain amount of *B. subtilis* present and would not necessarily be enhanced by higher concentrations. This view is, however, in contrast to other studies, which have shown that *B. subtilis* also acts by preventing spore germination, germ tube elongation and germ tube penetration (Liu et al., 2010; Rytter et al., 1989; Romero et al., 2007). Specifically, Li et al. (2013) have shown that strains of *B. subtilis* can cause uredospore and germtube

rupture leading to dysfunction of fungal structures.

A pronounced variation in yellow rust control was seen in the field experiments between replicates and across growing seasons, which is in line with other studies where the use of *B. subtilis* against different pathogens usually led to higher variability compared with synthetic fungicides (Wei et al., 2016). It is likely that factors such as relative humidity, temperature and direct sunshine are abiotic factors influencing *B. subtilis* survival on the leaf surface. Li et al. (2013) suggested that *B. subtilis* products might be acting more effectively if conditions for colonisation on the wheat leaves could be improved. This would prolong bacterial lifetime and also give more time for secreting antibiotic substances.

In addition to this, insufficient rainfastness of the Serenade®ASO formulation, tested in this study, might contribute to variable yellow rust control. Crane and Bergstrom (2014) investigated the spacial distribution of *Bacillus amyloliquefaciens*, used as a BCA, on the surface of wheat leaves. They identified rainfastness as a limiting factor for the control of *Fusarium graminearum* under field conditions. A recent study in strawberries investigated the phyllosphere on leaves from plants treated with *B. subtilis* QST713 using next generation sequencing. The study showed that open air crops have lower population persistence than indoor crops (Wei et al., 2016). A loss of 50% biofungicide was seen 8 days after application in outdoor crops. Within the same time interval a much lower loss was recorded on greenhouse crops. It is believed that the fast reduction in the *B. subtilis* population on outdoor crops may explain the variable and relatively low efficacy under field conditions. Extensive loss of *B. subtilis* from the leaf surface, paired with bacterial death, would also explain the absence of a dose-response relationship and suggest that frequent applications of *B. subtilis* are required to ensure a high efficacy. The present study supports this, as 4 applications generally provided better control, compared to 2 applications of biofungicide. Addition of the adjuvant Silwet Gold to the Serenade®ASO formulation in the semi-field trial did not lead to a significant improvement of yellow rust control, even though this adjuvant used alone reduced yellow rust severity significantly compared with the control. This effect of Silwet Gold is a common phenomenon and was also observed in field experiments in 2015 (unpublished results).

As mentioned, rainfall events are a factor expected to cause differences between indoor and outdoor conditions. Rainfall can wash off bacterial cells but could also promote activity by giving dislodging and dispersing of conidia on the leaf surface, increasing the possibility of the biofungicide to spread to new growth. Indoor production did in contrast to outdoor production not show any spread to new growth (Wei et al., 2016). In 2015, 4.4 mm of rain was registered following application C (BBCH 33). At the following observation at BBCH 49, yellow rust control was very variable and relatively low compared with the corresponding observation at BBCH 51 in 2014 with only 0.4 mm of rain after application C (Table 4 and 5). Whether the low control levels in 2015 are solely correlated with more severe disease pressure compared with 2014 or can partly be explained by inferior rainfastness can not be verified, but rain might have played a role in the lower control levels achieved in 2015.

On the basis of this study, the use of *Bacillus subtilis* QST713 for yellow rust control in the field can only be recommended under low disease pressure or in organic fields where no alternatives are available. Although a slight but not significant yield increase was measured from treatments with *Bacillus subtilis* QST713, it is at present too risky to rely on this product for control of yellow rust under severe disease pressure. In comparison with *Bacillus subtilis* QST713, traditional chemistry using two applications provided very stable control and yield responses.

It is proposed to investigate mixtures of *B. subtilis* with other fungicides or adjuvants in order to aim at improving the stability and rainfastness and to achieve less variable control under field conditions. The tolerance of different *B. subtilis* strains to a range of pesticides has already been investigated in laboratory tests, but the study did not include the most widely used fungicides for rust control in cereals (Furuya et al., 2011). Successful use of *B. subtilis* is considered to require frequent treatments (Wei et al., 2016). As also seen in this study where an improved level of control was observed when increasing the number of treatments from 2 to 4 during crop elongation and heading. Whether this strategy will be adopted by the farming community is doubtful, as it will increase the time and costs spent on disease control. Even so, treatments with biofungicide can potentially become part of an integrated pest management strategy by replacing some of the synthetic fungicide treatments and assist in delaying resistance evolution to synthetic fungicides and thereby contribute to a more sustainable disease control.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.cropro.2016.11.009.

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