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ORIGINAL ARTICLE

Fungicide strategies and resistance of *Ramularia collo-cygni* to demethylation and succinate dehydrogenase inhibitors in Austrian winter barley (*Hordeum vulgare*)

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Abstract

Ramularia collo-cygni B. Sutton and J.M. Waller is a major disease in Austrian barley-growing regions. To date, fungicide application is the most effective method to manage the disease; however, fungicide resistance to demethylation and succinate dehydrogenase inhibitors has developed over the last few years. In the growing seasons 2016/2017 and 2017/2018, field trials were carried out to analyze the efficiency of fungicide strategies based on different fungicide classes. Disease development, growth parameters and monitoring of *CYP51* and *sdh* mutations were determined. Fungicide treatments resulted in higher disease control, green leaf area and grain yield. In Austrian *R. collo-cygni* field populations, the frequency of the mutations *CYP51*-I325T and *CYP51*-I328L was low to moderate. Frequency of mutations *sdhC*-H146R and *sdhC*-H153R was low. Frequencies of *CYP51*-I325T and -I328L were similar and increased following DMI application. Frequency of *sdhC*-H146R was higher compared to *sdhC*-H153R. The SDHI benzovindiflupyr showed a higher selection rate for *sdh* mutations compared to bixafen. These *sdh* mutations were not selected if chlorothalonil was used as mixing partner, leading to a stable composition of *sdh* resistance alleles over the last two years. Chlorothalonil was proven to be an effective tool for anti-resistance strategies. Currently, SDHIs and DMIs are the backbone of Ramularia leaf spot control in Austria; however, the level of resistance is likely to increase in absence of suitable anti-resistance strategies and following the ban of chlorothalonil.

Introduction

In recent years, Ramularia leaf spot (RLS) has become a major barley disease across Europe. The causal agent, *Ramularia collo-cygni* B. Sutton and J.M. Waller, is already well established in Austria and poses a high risk to winter barley (*Hordeum vulgare* L.), the third most important cereal crop in Austria (BMNT 2019). The ascomycete *R. collo-cygni*, first described as *Ophiocladium hordei* (Cavara 1893), is a global disease with growing importance in numerous regions (Havis et al. 2015). In Austria, it has been detected for the first time in the late 1980s (Huss et al. 1987). West et al. (2012) assumed an increasing risk of RLS following climate changes, as confirmed further by McGrann and Brown (2017), who found that barley plants exposed to abiotic stress such as drought and warm temperatures, showed further increased RLS disease levels. Havis et al. (2015) also mentioned the importance of the worldwide spread and the relevance of a strong relationship between environmental factors such as leaf surface wetness and RLS epidemiology. Overall, the *R. collo-cygni* population shows a low genetic diversity and can only be divided in two main genomic groups (Stam et al. 2019). This is not in line with other pathogens like *Puccinia striiformis* or *Fusarium graminearum*, which show differences in geographical lineages (O'Donnell et al. 2000). The combination of long-distance wind dispersal and additionally seed transmission could be the reason

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for a broad spread around the globe and the lack of geographical clusters (Stam et al. 2019). This may also lead in a broad dispersal of DMI and SDHI-resistant populations and hence poses a challenge for further fungicide control of the pathogen. Therefore, a future increase and evolution of RLS must be considered for all grain growing regions.

Symptoms of RLS typically appear late in the growing season, usually after heading (Walters et al. 2008) or even later after flowering (Salamati and Reitan 2006). During the advanced development the dark brown spots surrounded by a yellowish halo on the leaves can merge and finally the infected parts die. Leaf sheaths and awns can also get infected (Huss and Sachs 1998). Ramularia leaf spot can easily be confused with physiological spotting. In some cases, net blotch caused by *Pyrenophora teres* or necrosis caused by *Blumeria graminis* f.sp. *hordei* can also look very similar. To determine the presence of RLS infection, the use of the "5Rs" (rectangular shape, reddish-brown coloration, restricted by the veins, right through the leaf and ringed with yellow margin of chlorosis) is essential to correctly classify the presence of the disease in the field (Havis and Brown, 2019).

Harvey (2002) reported seed infection with an infection rate of approximately 10%. This implies that seedborne transmission in addition to spore dispersal within barley plants is a major source of inoculum for RLS (Kaczmarek et al. 2017; Stabentheiner et al. 2009). The pathogen has been reported on rye, oats, wheat and several grass species as well (Huss 2004; Huss et al. 2005; Kaczmarek et al. 2017). This leads to a continuous source of RLS during the whole growing season and can dramatically impact crop rotations (Cromey et al. 2004; Frei 2004; Huss 2004; Huss 2008).

In normal years, yield losses caused by *R. collo-cygni* are in the range of 15–25% with a potential yield impact of 0.4–0.9 tons per hectare (Cromey et al. 2004; Greif 2004; Harvey 2002; Havis and Evans 2016; Huss et al. 1992; Oxley and Burnett 2010; Oxley and Havis 2004; Oxley et al. 2010). Havis et al. (2015) also reported yield reduction of up to 70% in years when the disease was found at high levels and in highly susceptible varieties. In the recent years, RLS occurred in many Austrian barley growing areas, often accompanied by a partial presence of net blotch (*Pyrenophora teres*) and barley rust (*Puccinia hordei*). Under such circumstances, yield losses ranged between 20 and 45% (Syngenta, unpublished data). Hein (2019) reported that there is no barley variety that had usable full resistance to *R. collo-cygni* in the field. Currently, 47 winter barley varieties are listed in the Austrian variety list (https://bsl.baes.gv.at/kulturen/getreide/wintergerste/) with only 5 of them having a low susceptibility (AGES 2020). Nevertheless, growing partial resistant varieties is an essential tool to reduce *R. collo-cygni* incidence in the field.

In conventional Austrian barley fields, one or two fungicide applications are performed to control winter barley diseases. A broad range of fungicides are registered mainly belonging to quinone outside inhibitors (QoIs), demethylation inhibitors (DMIs), succinate dehydrogenase inhibitors (SDHIs) and multisite inhibitor chlorothalonil (CTN). The single point mutation G143A confers a complete resistance to all QoI fungicides (FRAC 2010; Leadbeater 2012; Walters et al. 2012). Resistance to QoI fungicides was first reported by the Fungicide Resistance Action Committee (FRAC) in 2010 although resistance was first detected in 2002 leaf samples from the Rothamsted Hoosfield Archive (Fountaine et al. 2010). DMIs such as prothioconazole, introduced into the market in 2005, and SDHIs such as boscalid, introduced in 2002, have provided effective control of RLS (Lucas 2016). The first shift in *R. collo-cygni* sensitivity to DMI was reported in 2016 in Austria. Low frequencies of amino acid alterations *CYP51*-I325T, I328L and Y403C/Y405H, which were showing a significant loss of sensitivity, were found in Austrian samples (FRAC 2018a). A European study, carried out between 2009 and 2017, documented 12 *CYP51* alterations (*CYP51*-V136A, Y137F, A311S, I381T, I384L, D458G, Y459C, Y459N, G460D, G460V, Y461N and Y461H; based on *Z. tritici* CYP51 sequence) and 15 different *CYP51* haplotypes (Rehfus et al. 2019). Furthermore, they pointed out that the *C1*- and *C3*-haplotype based on *CYP51*-I381T + I384L + Y459C (*C1*) or Y461H (*C3*) were found in high frequencies and resulted in reduced field efficacy of DMI fungicides. The *R. collo-cygni* sequence I381T is homolog to I325T, I384L to I328L and Y403C/Y405H are homolog to Y459C/Y461H (S. Torriani, 2020; personal communication).

Numerous point mutations in the coding region of subunits SDH-B, SDH-C and SDH-D of the succinate dehydrogenase (SDH) enzyme were found in various fungal plant pathogens (Avenot and Michailides 2010). In European *R. collo-cygni* samples, first resistance to SDHIs was found in 2015 (Germany, Ireland, Slovenia); the first evidence for Austria was documented for 2017. European monitoring data reveal the mutations *sdhC*-G91R, *sdh*C-G171D *sdhC*-H146R/L, *sdhC*-H153R all are strongly involved in the reduced field efficacy of SDHIs as well as the mutations *sdhC*-N87S, *shd*B-T267I and *sdh*B-N224T associated with a lower reduction in sensitivity (FRAC 2020). Further mutations in European *R. collo-cygni* population (e.g., *sdhC*-R152M, B-R264P, B-H266Y/R/L) have also been documented by Rehfus et al. (2019).

Since the first detection of these mutations, a reduction in efficacy of both DMI's and SDHI's for the control of RLS was observed. Treatments against RLS were until May 2020

mostly limited to a main foliar fungicide application which also included chlorothalonil after booting stage.

To date, our knowledge on the effect of different fungicide strategies to manage resistant populations of *R. collo-cygni* in barley is still limited. The low to moderate frequencies of mutations *CYP51*-I325T and *CYP51*-I328L and low frequencies of mutations *sdhC*-H146R and H153R detected in Austrian *R. collo-cygni* populations (FRAC 2018a, b) were the starting point of this study. The objective of this study was to analyze different fungicide strategies using different active compounds (DMI solo and in mixture with SDHI, chlorothalonil and QoI), application rates and application timings to explore the population dynamics on *R. collo-cygni*. The impact on RLS disease development, leaf senescence and yield was monitored, as well as the frequency of *CYP51* and *sdh* resistance alleles. These data provide valuable insight to understand fungicide resistance evolution in *R. collo-cygni* and help to develop anti-resistance strategies in this increasingly important plant disease.

Materials and methods

Field trial design and fungicide application

Field plot experiments were performed in Austria's major winter barley-growing areas Lower Austria (Czech-Slovak border; site 1-KO, site 2-TU, site 3-P), Upper Austria (German-Czech border; site 4-WL) and Styria (Slovenian border; site 5-WZ) during 2016/2017 and 2017/2018 (Table 1). Soils in all trial sites were silt to silty clay loam, except for site 5-WZ that was loamy sand. Average annual rainfall, temperature and duration of sunshine were recorded and is shown in Supplementary Table 1. The treatments (Trt) included ten fungicide treatments and an untreated "fungicide-free" control. Tables 1 and 2 provide an overview of the trial sites, listing all treatments/products with their active ingredients. Each trial was arranged as a randomized complete block design with four replicates (in total 44 plots per trial site). The plot size ranged between sites and was between 15 and 30 m^2.

Table 1 Experimental sites, variety information, growth stages (GS), application dates and harvest dates in 2017 and 2018

Year	State District	Trial location (site; coordinates)	Variety and RLS-susceptibility	GS application 1 date	GS application 2 date	GS application 3 date	Harvest date
2017	LA (Lower Austria) KO (Korneuburg)	Kleinwilfersdorf (site 1-KO; 48.39889 16.27473)	Valentina high	30-31 12-04-2017	39-49 06-05-2017	57-59 22-05-2017	28-06-2017
	LA (Lower Austria) TU (Tulln)	Preuwitz (site 2-TU; 48.34058 15.81478)	Sandra high	30-31 12-04-2017	39-49 06-05-2017	52-59 17-05-2017	29-06-2017
	LA (Lower Austria) P (Sankt Pölten)	Witzendorf (site 3-P; 48.20144 15.58497)	Sandra high	31-32 24-04-2017	39-51 16-05-2017	57-59 25-05-2017	05-07-2017
	UA (Upper Austria) WL (Wels Land)	Bad Wimsbach (site 4-WL; 48.07581 13.88532)	KWS Meridian moderate	30-31 03-04-2017	49-51 16-05-2017	59 26-05-2017	07-07-2017
	ST (Styria) WZ (Weiz)	Karberg (site 5-WZ; 47.17661 15.61206)	Semper moderate	32 02-05-2017	39 10-05-2017	59 29-05-2017	No harvest
2018	LA (Lower Austria) KO (Korneuburg)	Weinsteig (site 1-KO; 48.44448 16.40789)	Sandra high	31 14-04-2018	43-49 03-05-2018	57-59 10-05-2018	21-06-2018
	LA (Lower Austria) TU (Tulln)	Preuwitz (site 2-TU; 48.35689 15.82166)	Sandra high	32 17-04-2018	39-49 01-05-2018	57-59 09-05-2018	26-06-2018
	LA (Lower Austria) P (Sankt Pölten)	Witzendorf (site 3-P; 48.206157 15.59590)	Valentina high	31 17-04-2018	39-49 02-05-2018	53-59 09-05-2018	21-06-2018
	UA (Upper Austria) WL (Wels Land)	Bad Wimsbach (site 4-WL; 48.06947 13.89422)	Valentina high	32 18-04-2018	39-49 01-05-2018	51-55 09-05-2018	02-07-2018
	ST (Styria) WZ (Weiz)	Oberdorf (site 5-WZ; 47.17850 15.60863)	Christelle moderate	31-32 23-04-2018	39 02-05-2018	59 16-05-2018	No harvest

Table 2 Treatments and fungicides used in field trials

Treatment (Trt)	Application 1* (GS 30-32)	Dose L ha⁻¹	Application 2 (GS 39-51)	Dose L ha⁻¹	Application 3 (GS 51-59)	Dose L ha⁻¹
Trt 1	Untreated control					
Trt 2	Gladio	0.6	Proline	0.6		
Trt 3	Gladio	0.6	Aviator Xpro	1.0		
Trt 4	Gladio	0.6	Elatus Era	1.0		
Trt 5	Gladio	0.6	Proline + Balear	0.6 + 0.833		
Trt 6	Gladio	0.6	Aviator Xpro + Balear	1.0 + 0.833		
Trt 7	Gladio	0.6	Elatus Era + Balear	1.0 + 0.833		
Trt 8	Gladio	0.6	Elatus Era + Amistar Opti	0.8 + 1.2		
Trt 9	Gladio	0.6	Elatus Era + Amistar Opti	1.0 + 1.5		
Trt 10	Gladio	0.6	Elatus Era	1.0	Amistar Opti	1.5
Trt 11	Gladio	0.6	Elatus Era	1.0	Elatus Era + Amistar Opti	1.0 + 1.5

Product name	Company	Active ingredients (a.i.)
Gladio® 625 EC	Syngenta Agro GmbH	375 g/l fenpropidin; 125 g/l propiconazole; 125 g/l tebuconazole
Proline® 250 EC	Bayer Austria GmbH	250 g/l prothioconazole
Aviator® Xpro 225 EC	Bayer Austria GmbH	150 g/l prothioconazole; 75 g/l bixafen
Elatus™ Era 225 EC	Syngenta Agro GmbH	150 g/l prothioconazole; 75 g/l benzovindiflupyr
Balear® 720 SC	Cheminova Austria GmbH	720 g/l chlorothalonil
Amistar Opti® 480 SC	Syngenta Agro GmbH	400 g/l chlorothalonil; 80 g/l azoxystrobin

*To exclude the impact of powdery mildew and net blotch. Application 1 was not set up for primary RLS control

The two trial seasons were dryer and warmer compared to the long-term average between 1961 and 1990 (Supplementary Table 1; ZAMG 2000). Over all trial sites, spring 2018 was warmer, and as a result a shorter vegetation period with an earlier harvest time was achieved. The fungicide application was oriented to the growth stage (GS), and the first application was done around GS 31, the second application around GS 44, and the third application around GS 57/58.

Fungicides were applied using a plot sprayer, equipped with flat fan nozzles at 3 bar pressure and 300 L spray volume per hectare. Other maintenance applications and fertilization were applied uniformly over the whole trial. Additionally, a period of at least 5 days before or after fungicide application was carried out to avoid stress or potential phytotoxic effects.

Disease, green leaf area and yield assessments

Foliar disease severity (DS %) was evaluated three to five times depending on disease pressure and disease development following the last spray application. Diseases were visually assessed as percent diseased leaf area per leaf level at four points across the length of the plot according to EPPO guideline PP 1/26 (Anon. 2012). Ramularia leaf spot was assessed on the upper two leaf levels. For statistical analyses, RLS assessments were pooled and the area under the disease progress curve (AUDPC) (Shaner and Finney 1977) was calculated from the scores for each plot. Using the following calculation

$$AUDPC = \sum_{i=1}^{n} \left[\frac{Y_{i+n1} + Y_i}{2} \right] \times [X_{i+1} - X_i]$$

where Y_i = RLS severity (per unit) at the ith observation, X_i = time (days) at the ith observation, n = total number of observations, and $Y_0 = X_0 = 0$.

The green leaf area (GLA) was assessed as mean percentage of green area on the two upper leaf levels at four to seven times between GS 59 and 89. The area under the green leaf area curve (AUGLAC; Cromey et al. 2004) was calculated for each plot from the GLA measurements using the same formula as for AUDPC where Y_i = GLA in % at the ith observation, X_i = time (days) at the ith observation, n = total number of observations, and $Y_0 = X_0 = 0$.

Eight trials were harvested and data were transformed to yield (dt ha^{-1}) at 86% dry matter for each plot. At trial site 5-WZ, heavy rain showers before harvest resulted in lodging and made harvest very difficult and at the end measurements impossible.

Analyses of amino acid alterations associated with demethylation inhibitor and succinate dehydrogenase inhibitor resistance

To enable the quantification of the DMI (I325T, I328L) and SDHI (H146R and H153R) resistance alleles, a q-PCR method was developed. Total DNA was extracted from at least 30 leaves, randomly collected from the four replicates of each treatment and at five time points during the growing season. The first three collection times were connected to the fungicide application date. The fourth and fifth samplings were done around 2 weeks after last application (around GS 77) and 4 weeks after last application (around GS 87), depending on the treatment (Table 1). Leaves were dried at room temperature and stored at −18 °C until further use. The preparation of the biological material used in the q-PCR method was similar as described in Wullschleger et al. (2015) to quantify G143A mutation in *Z. tritici*. Briefly, leaf disks of 2 mm diameter were punched out preferentially from typical RLS symptoms.

The total reaction volume for all q-PCR´s was 10 μl; this contained 2 μl template DNA, plus 8 μl Mastermix and primers, [5 μL FastStart Universal SYBR Green Master, 0.3 μL forward and 0.3 μL reverse primers at a final concentration of 300 nM and 2.4 μL water]. Primers for q-PCR assay were produced by Microsynth—The Swiss DNA Company and listed in Table 3. The forward primers were allele-specific (amplification refractory mutation system = ARMS) (*S. Torriani* and *S. Accardo*, 2020; written communication).

Q-PCR assays for the mutation *CYP51*-I325T were carried out in a 384-well plates on the BIO-RAD "CFX384" (Bio-Rad Laboratories, Hercules, CA, USA), under following conditions: After an initial heating step for 10 min at 95 °C, 40 cycles at 95 °C for 15 s, 59 °C for 30 s and 72 °C for 7 s were applied, followed by a final amplification step at 95 °C for 6 min (59 °C to 95 °C, increment by 0.5 °C for 5 s). For the mutation *CYP51*-I328L, the same instrument with following conditions was used: After an initial heating step for 10 min at 95 °C, 40 cycles at 95 °C for 15 s, 63 °C for 20 s and 72 °C for 15 s were applied, followed by a final amplification step at 95 °C for 6 min 10 s (58 °C to 95 °C, increment by 0.5 °C for 5 s). For the mutation *sdhC*-H146R and *sdhC*-H153R, the q-PCR assays were carried out in 384-well plates on the Applied Biosystems "ABI 7900 HT Sequence Detection System" (Applied Biosystems, Foster City, USA), according to the following program: Initial heating at 95 °C for 10 min, followed by 40 cycles of 15 s at 95 °C, 30 s at 56 °C, 30 s at 72 °C and final elongation step at 15 s at 95 °C, 15 s at 55 °C, 15 s at 95 °C. The specificity of the amplification products is monitored by melting curve analysis, which is required in SYBR Green qPCR assays. Primers

Table 3 Primers used for qPCR-assays

Mutation	Forward (FW) or reverse (RV)	Wild type (WT) or mutated type (MT)	Primer
sdhC-H146R	FW	WT	5'-GCACTGCCATTCACTTTGCA-3'
	FW	MT	5'-GCACTGCCATTCACTTTGCG-3'
	RV	WT & MT	5'-CACAACAGTCCATCCCGACT-3'
sdhC-H153R	FW	WT	5'-TCTTTCAACGGTGTGAGACA-3'
	FW	MT	5'-TCTTTCAACGGTGTGAGACG-3'
	RV	WT & MT	5'-CACAACAGTCCATCCCGACT-3'
CYP51-I325T	FW	WT	5'-TCCGGATCCACGCGCCGAT-3'
	FW	MT	5'-TCCGGATCCACGCGCCGAC-3'
	RV	WT & MT	5'-TGCGGTTCCCACAAGAGGCA-3'
CYP51-I328L	FW	WT	5'-ACGCGCCCAYCCACTCCA-3'
	FW	MT	5'-ACGCGCCCAYCCACTCCC-3'
	RV	WT & MT	5'-TGCGGTTCCCACAAGAGGCA-3'

S. Torriani and *S. Accardo*, 2020; written communication

were cross-tested against other barley diseases and barley DNA to ensure the correct amplification of *R. collo-cygni* (S. Torriani and S. Accardo, 2020; written communication).

A validation experiment was run using plasmid DNA with a part buildup of the different primers. A dilution series were used to determine the window of specificity, in order to show how distinct the discrimination of the wild-type allele in the mutant allele PCR is and vice versa. The frequencies of the resistant allele (% mut) were calculated by the following formula (Brent and Hollomon 2007; rectified after Syngenta CP, Stein, Switzerland).

$$\% \ mut = \frac{1}{2^{\Delta Ct} + 1}$$

Statistical analysis

Data were analyzed using proc mixed procedure of SAS 9.4 (SAS®, SAS Institute Inc., Cary, NC) combined with Hochberg´s GT2 multiple comparison test for unequal variances ($\alpha = 0.05$). For testing fixed effects, the method was specified with DDFM = kenwardroger to approximate the degrees of freedom for means. Fungicide treatment, location and interactions between them were considered as fixed effects, block, year and their interactions as random effects. Due to the different disease occurrence between locations and years, single trials were separately analyzed and presented as means and standard error.

Results

Overall, mean RLS on the upper two leaves in untreated control was moderate to high and reached between 23.4 and 86.3% in both years, except site 1-KO where a very low

disease severity of 7.1% occurred in 2017 (Fig. 1). Hardly any other diseases were detected besides RLS. Net blotch was assessed in 6 trials (site 1-KO in 2017, site 2-TU in 2017, site 3-P and site 4-WL in 2017 and 2018). The mean disease severity in the untreated control was 6.5%, which was reduced to 0.8% by fungicide treatment Trt 2 and to 0.3% and 0.1% by fungicide treatment Trt 4 and Trt 9. Barley brown rust appeared in 2 trials (site 2-TU in 2017 and 2018) with a mean disease severity of 3.0% in untreated control (data not shown).

Yield, Ramularia leaf spot disease severity and green leaf area

The main factors treatment ($p < 0.001$) and location ($p < 0.001$) affected yield; nevertheless, there was no significant interaction between treatment x location and treatment x year (Table 4). Yield was increased by 5.3% with prothioconazole (Trt 2) and 6.5% with prothioconazole + bixafen or benzovindiflupyr (mean Trt 3 and Trt 4). By adding 600 g ha⁻¹, chlorothalonil (Trt 6 and Trt 7) yield was increased by 9.4% compared to the untreated control (Table 4). Significant differences were only observed between the untreated control (Trt 1) and treatments including chlorothalonil (Trt 5 – Trt 11). In untreated plots, yields were 5 to 14 dt ha⁻¹ higher in 2017 compared to 2018, except site 4-WL (Supplementary Table 2). There were no significant differences in yield at site 2-TU and site 4-WL in 2017 and site 1-KO in 2018. Additionally, only significant differences were found between untreated and treated plots at site 1-KO in 2017 and site 4-WL in 2018 (Supplementary Table 2). Similar to RLS severity, the different application timings as well as the different dose rates at application time 2 showed no significant differences in yield.
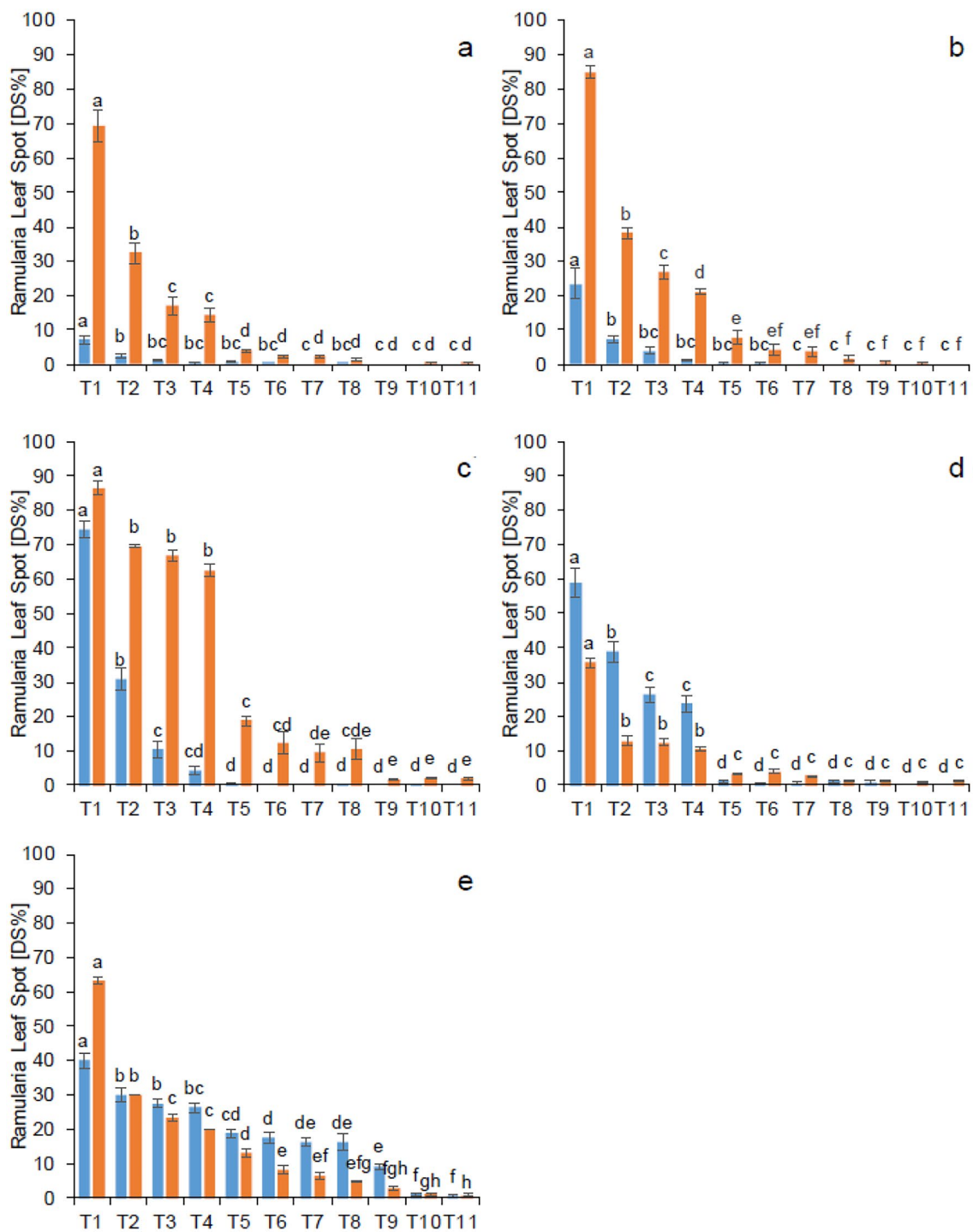
Fig. 1 Effect of different treatments (T) on Ramularia leaf spot disease severity (DS%; ±SE) (a = site 1-KO, b = site 2-TU, c = site 3-P, d = site 4-WL and e = site 5-WZ) assessed between GS 85 and 87 on upper two leafs (blue bars 2017; orange bars 2018). T1 = UTC, T2 = P (E), T3 = P + BI (E), T4 = P + BE (E), T5 = P + C (E), T6 = P + BI+ C (E), T7 = P + BE + C (E), T8 and T9 = P + BE + C + A (E, T8 = lower dose rate compare T9), T10 = P + BE (E) and A + C (L), T11 = P + BE + C + A (L). UTC = untreated control, P = prothioconazole, BI = bixafen, BE = benzovindiflupyr, C = chlorothalonil, A = azoxystrobin, E = early application around GS 39-51, L = late application around GS 51-59. For each site and year, means with the same letter were not significantly different according to GT2-Hochberg test (p = 0.05)

Table 4 Effect of treatment, location and year on averaged grain yield, Ramularia leaf spot (AUDPC) and green leaf area (AUGLAC) on winter barley trials in 2017 and 2018 (Means ±SE). Means within the same category with the same letter were not significantly different according to GT2-Hochberg test (p = 0.05)

Treatment	Yield		Ramularia leaf spot		Green Leaf Area	
(Trt)	dt ha^{-1}	(±SE)	AUDPC	(±SE)	AUGLAC	(±SE)
Trt 1	78.28	1.6[a]	589.95	60.9[a]	2069.52	54.2[a]
Trt 2	82.41	1.7[ab]	283.93	35.3[b]	2336.39	46.4[b]
Trt 3	83.10	1.7[ab]	212.55	28.0[c]	2417.46	49.8[bc]
Trt 4	83.61	1.7[ab]	178.06	25.2[c]	2461.93	50.4[bcd]
Trt 5	84.87	1.8[b]	56.83	9.7[d]	2524.60	48.9[cd]
Trt 6	85.15	1.8[b]	41.67	8.0[d]	2548.29	48.7[cd]
Trt 7	86.08	1.8[b]	33.73	7.0[d]	2594.15	46.9[d]
Trt 8	86.14	1.7[b]	30.73	7.5[d]	2609.47	52.5[d]
Trt 9	87.74	1.8[b]	13.40	3.5[d]	2627.37	53.6[d]
Trt 10	87.12	1.6[b]	7.12	1.5[d]	2621.00	50.8[d]
Trt 11	86.59	1.7[b]	7.39	1.4[d]	2625.28	51.3[d]
	P value	DF	*P* value	DF	*P* value	DF
Treatment (Trt)	< 0.001	10	< 0.001	10	< 0.001	10
Location (L)	< 0.001	3	< 0.001	4	< 0.001	4
Year (Y)	< 0.001	1	0.0063	1	< 0.001	1
L × Trt	1.0000	30	< 0.001	40	0.9993	40
Y × Trt	0.9940	10	0.2814	10	0.6207	10

The fungicide treatments reduced RLS significantly compared to the untreated control ($p < 0.001$; Table 4). There were also differences in locations ($p < 0.001$) and the interaction between treatment and location ($p < 0.001$). Trt 2, which contains prothioconazole in the main application, reduced RLS AUDPC by 52%, and with prothioconazole + bixafen or benzovindiflupyr (Trt 3 and Trt 4) an additional reduction by another 15% was achieved. Fungicide programs containing chlorothalonil reduced RLS by more than 90%, whereby no significant differences between prothioconazole + chlorothalonil (Trt 5), prothioconazole + bixafen or benzovindiflupyr + chlorothalonil (Trt 6 or Trt 7) and prothioconazole + benzovindiflupyr + azoxystrobin + chlorothalonil (Trt 8 – Trt 11) were detected. There were no differences in RLS severity between the different application timings (Trt 9 – sprayed at GS 39-51; Trt 10 – splitting application GS 39-51 and GS 51-59; Trt 11 – sprayed at GS 51-59), except for site 5-WZ, where significant differences between Trt 9 and Trt 10/Trt 11 were observed in 2017 (Fig. 1e).

Over all trials, RLS level was reduced by spraying fungicides (Fig. 1). RLS severity was generally higher in 2018, except for site 4-WL. With 74.4 and 86.3% RLS severity in untreated control plots, site 3-P reached highest RLS severity in both years, followed by site 2-TU with 85% in 2018 and site 4-WL with 58.8% in 2017. In 2018 at site 3-P (Fig. 1c), we observed lowest RLS efficiency for Trt 2, Trt 3 and Trt 4 with 69.7, 66.9 and 62.7% RLS severity. This was significantly lower than Trt 1 with 86.3%. In same trial, by adding of chlorothalonil (Trt 5 – Trt 11) we observed RLS levels under 18.9%. In another location (site 5-WZ in 2017; Fig. 1e), the differences between treatments without chlorothalonil (Trt 2 – Trt 4) and treatments with chlorothalonil (Trt 5 – Trt 11) did not show that high difference in RLS development. Overall, the data show that when more treatments with a greater number of active ingredients (AIs) were included, this resulted in a better control of RLS.

Average area under the green leaf area curve (AUGLAC) of the untreated control across all 10 trials was 2069.52 (Table 4). Similar to AUDPC, the AUGLAC was significantly different for treatments ($p < 0.001$), locations ($p < 0.001$) and years ($p < 0.001$). Treatments, which included prothioconazole (Trt 2) or prothioconazole + bixafen or benzovindiflupyr (Trt 3 or Trt 4), significantly increased AUGLAC by 13% and 18%. By adding of chlorothalonil (Trt 5 – Trt 11), GLA could be increased by more than 23% compared to the untreated control (Trt 1). Treatments applied at different application timings (Trt 9—sprayed at GS 39-51; Trt 10—splitting application GS 39-51 and GS 51-59; Trt 11—sprayed at GS 51-59) as well as different dose rates at application time 2 (Trt 8—80% of spray rate of Trt 9

Table 5 Mean frequency (%) of DMI (I325T and I328L) resistance alleles in untreated control and changes caused by selected fungicide treatments in Austrian trial sites

Mutation		Treatment	Year / Site										Average of all sites and years
			2017					2018					
			Site 1-KO	Site 2-TU	Site 3-P	Site 4-WL	Site 5-WZ	Site 1-KO	Site 2-TU	Site 3-P	Site 4-WL	Site 5-WZ	
CYP51-	I325T	Trt 1	0.0	33.2	20.3	43.7	0.5	10.5	13.2	22.9	37.2	1.6	18.3
		Trt 2	0.0	66.9	99.6	99.3	0.0	62.2	47.6	61.3	89.7	8.3	53.5
		Trt 3	0.0	51.4	94.1	99.1	0.7	79.4	82.6	78.9	92.9	9.0	58.5
		Trt 4	0.0	81.7	99.6	95.2	0.0	69.1	73.6	84.3	97.3	17.9	61.9
	I328L	Trt 1	0.0	29.7	23.4	46.7	0.1	11.8	13.6	24.4	39.2	0.9	19.0
		Trt 2	0.0	74.6	99.6	98.8	0.2	60.6	51.9	64.6	90.6	7.8	54.9
		Trt 3	0.0	50.3	94.3	96.9	0.1	88.1	84.0	82.0	93.2	5.8	59.5
		Trt 4	0.0	85.8	99.8	92.8	0.1	76.0	69.8	86.0	97.5	16.0	62.4

compared to Trt 9—full dose rate) did not show differences in AUGLAC.

Analyses of mutations associated with fungicide resistance

For this study, we evaluated the frequency of *CYP51* and *sdh* resistance alleles. Tables 5 and 6 illustrate the effect of treatments on the frequency of *CYP51* and *sdh* mutations at the first sampling date when a full data set was available (GS 87). Resistant *R. collo-cygni* strains were found in all trial sites and in both years, except site 1-KO in 2017 where only wild-type alleles have been found. Monitoring of *R. collo-cygni* samples showed differences in the frequencies of mutations between treatments and locations but only small differences between years.

The frequency of *CYP51* mutations (Table 5) in untreated plots was higher at site 1-KO and lower at site 2-TU in 2018 compared to 2017 and within the same range for all other sites. I328L occurred at same frequency as I325T and reached the highest level (39.2–46.7%) at site 4-WL; lower frequencies were found at site 1-KO and 5-WZ. In general, if mutations were present in the untreated control, *CYP51* mutations were highly selected after Trt 2, 3 and 4 (Trt 2 = prothioconazole, Trt 3 = prothioconazole + bixafen; Trt 4 = prothioconazole + benzovindiflupyr). In trial sites with more than 1% *CYP51* mutation in the untreated (Trt 1), Trt 2 showed a frequency increase for *CYP51* mutations of two to six fold changes.

Frequencies of *sdh* mutations in *R. collo-cygni* populations (Table 6) were generally on a low level. Frequencies of *sdhC*-H146R were generally higher than *sdhC*-H153R. Site 4-WL was the only location, which reached more than 1.4% of both *sdh* mutations in both years, in untreated control. With 8.1% in the untreated control, the population at site 3-P reached the highest frequency of *sdhC*-H146R in 2017 and with 5.2% also the highest frequency in 2018. In all sites where *sdhC*-H146R was found in Trt 1 (untreated control), except site 2-TU in 2017, mutation H146R was highly selected for by Trt 4. When applying chlorothalonil (Trt 5 - Trt 11) and compered to Trt 1, the frequencies of *sdhC*-H146R were reduced or could be not detected, except for Trt 6–Trt 8 at site 3-P in 2017 and Trt 5 at site 4-WL in 2018. Frequency of mutation H153R was also affected by the application of fungicides and highly selected through Trt 4. Here, the highest frequencies were measured in Trt 4 with 19.6% at site 2-TU in 2017 and 11.6% at site 4-WL in 2018. Averaged over both years and all sites, Trt 4 showed the highest selection compared to the untreated control: *sdhC*-H146R increased from 3.1% to 16.3% and *sdhC*-H153R increased from 1% to 7.9%.

Table 6 Mean frequency (%) of SDHI (H146R and H153R) resistance alleles in untreated control and changes due to fungicide treatments in Austrian trial sites

Mutation		Treatment	Year/site										Average of all sites and years
			2017					2018					
			Site 1-KO	Site 2-TU	Site 3-P	Site 4-WL	Site 5-WZ	Site 1-KO	Site 2-TU	Site 3-P	Site 4-WL	Site 5-WZ	
sdhC-	H146R	Trt 1	0.0	5.2	8.1	3.3	0.0	2.5	2.2	3.2	5.2	1.4	3.1
		Trt 2	0.0	4.6	3.4	21.0	0.0	3.3	2.1	7.5	9.5	0.1	5.2
		Trt 3	0.0	4.0	14.0	37.3	0.0	11.4	7.2	14.2	18.3	0.2	10.7
		Trt 4	0.0	3.0	32.1	39.9	0.0	18.0	17.0	14.1	33.7	4.8	16.3
		Trt 5	n.d.	0.0	2.5	n.d.	0.0	0.0	n.d.	0.0	7.9	0.0	1.5
		Trt 6	n.d.	0.0	12.7	n.d.	0.0	0.0	n.d.	0.0	0.0	n.d.	2.1
		Trt 7	n.d.	n.d.	23.1	n.d.	0.0	0.0	n.d.	0.0	0.0	n.d.	4.6
		Trt 8	n.d.	n.d.	13.7	n.d.	0.0	n.d.	0.0	0.0	0.0	n.d.	2.7
		Trt 9	n.d.	n.d.	0.0	n.d.	0.0	n.d.	n.d.	n.d.	n.d.	n.d.	0.0 or n.d
		Trt 10	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
		Trt 11	n.d.	n.d.	0.0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.0 or n.d.
	H153R	Trt 1	0.0	0.0	0.0	4.7	0.3	3.1	0.1	0.6	1.4	0.1	1
		Trt 2	0.0	13.9	2.0	2.4	0.9	0.0	5.9	2.4	6.4	0.0	3.4
		Trt 3	0.0	9.6	1.9	1.1	0.2	5.7	2.3	2.6	6.2	1.5	3.1
		Trt 4	0.0	19.6	9.5	8.7	0.7	10.0	9.1	7.7	11.6	2.1	7.9
		Trt 5	n.d.	0.0	2.1	n.d.	0.0	n.d.	n.d.	0.9	1.4	0.0	0.7
		Trt 6	n.d.	n.d.	1.1	n.d.	0.0	n.d.	0.0	0.0	3.3	n.d.	0.9
		Trt 7	n.d.	0.0	15.4	n.d.	0.0	n.d.	0.0	n.d.	2.9	n.d.	3.7
		Trt 8	n.d.	n.d.	0.0	n.d.	0.0	n.d.	n.d.	4.1	0.0	n.d.	1
		Trt 9	n.d.	n.d.	0.0	n.d.	0.0	n.d.	0.0	0.0	n.d.	n.d.	0.0 or n.d
		Trt 10	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.0	n.d.	0.0 or n.d
		Trt 11	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

n.d. not detected, no Ramularia-DNA in leaves

When using chlorothalonil (Trt 5–Trt 11), a significant better RLS control was achieved compared to fungicide treatments not containing chlorothalonil (Trt 2–Trt 4) or the untreated control (Trt 1) (Table 4). This was also linked with reduced frequencies of *sdh* mutations (Table 6). In trial site 1-KO, 2-TU and 5-WZ, these treatments reduced the alteration H146R and H153R to 0% (*R. collo-cygni* was found, but only the wild-type population) or the treatments controlled the pathogen well and *R. collo-cygni* was not detected. Chlorothalonil could not reduce alteration H146R and H153R to 0% in Trt 5, Trt 6 and Trt 7 at site 4-WL in 2018 and additionally Trt 5 to Trt 8 at site 3-P in both years.

Discussion

The occurrence of plant diseases and fungicide strategies in cereals has changed over the recent years. DMIs have been registered since the late 1970s and were the core group of fungicides in cereal disease control (Wieczorek et al. 2015). In recent years, SDHIs became the fastest growing fungicide group. In Austria, SDHI applications increased from 26 to 39% of the market in cereals in 2012 (Deter 2013). Recently, low to moderate frequencies of mutations *CYP51*-I325T and I328L and low frequencies of mutations *sdhC*-H146R and H153R were detected in Austrian *R. collo-cygni* populations (FRAC 2018a, b)—this was the starting point of this study.

Our experimental sites were hardly affected by others diseases, as we did a fungicide treatment at GS 30-32 to exclude powdery mildew and net blotch, which can be confused with RLS during field assessments. The application was not set up for the RLS control but still, it cannot be ruled out that there might be an impact in the epidemic development of *R. collo-cygni* (Mulhare et al. 2021). However, in both testing seasons all fungicide treatments increased yield compared to the untreated control, whereby all treatments with chlorothalonil achieved a yield benefit. Significant differences between the fungicide treatments and the RLS control were not clearly reflected in the yield data. This is in line with Stetkiewicz et al. (2019) who reported that fungicide treatments had an impact in disease severity, but this impact could not be directly reflected in a significant impact on yield. In our study, weather conditions shortened the development time for grain fill during the 2017 season, as the yield in the untreated control was lower in 2017 compared to that of 2018, except for site 4-WL. The higher yield in 2018 at site 4-WL can be attributed to the higher RLS severity in 2017. In general, our field trials were exposed to drought stress which lead to a short period between the disease outbreak and leaf senescence. Thus, the fungicide treatments had only a very short period to demonstrate their efficacy.

For prothioconazole (Trt 2), the incidence of RLS was reduced compared to the untreated control and weaker than prothioconazole + bixafen or benzovindiflupyr (Trt 3 and Trt 4). In our study, treatments that included benzovindiflupyr in Trt 4 and Trt 7 showed obviously better yield and RLS control than bixafen in Trt 3 and Trt 6. The performance of benzovindiflupyr might have been better, as this compound is a highly potent SDHI, and secondly, the intrinsic activity is superior to other active ingredients within the same FRAC group (Guicherit et al. 2014). In addition, this greater performance could also be linked to a faster selection for *sdh* mutations within the *R. collo-cygni* population. This would mean that fungicide resistance to the SDHI class of fungicides could develop more quickly.

A correct application, timing and dosage are important factors for optimal disease control. In this study, spray programs with full dose rates of four different active ingredients (Trt 9–Trt 11) achieved better overall RLS control when compared to 80% of full dose rate (Trt 8). There were no significant differences in RLS control between the two application timings, although the splitting and late application increased RLS control marginally compared to an early application. Fungicide applications between GS 37 and GS 55 are considered optimal for disease control (Matusinsky et al. 2010). McCabe (2009) also reported GS 49 or later as optimal fungicide timing for ensuring yield, late season fungicide applications are considered the most effective tool to protect leaves and ears from the fungus (Havis et al. 2014; Heß et al. 2007). However, the application time is still a matter of debate, and due to the ban of chlorothalonil the optimal application timing will become more important.

In general, in Austria a low to moderate DMI and SDHI resistance in *R. collo-cygni* is present. In our 10 selected field trials, we found that changes in *CYP51*- and *sdh*-frequencies occurred in leaf samples collected around GS 87 after fungicide applications. Similar to the monitoring method of the FRAC, we focused our screening on mutations *sdhC*-H146R and H153R as well as *CYP51*-I325T and I328L. These mutations are showing high resistance factors to SDHIs and DMIs, respectively, and are currently among the most frequent in natural populations of *R. collo-cygni* (FRAC 2018a; FRAC 2018b; Rehfus et al. 2019). To our knowledge, this is the first study to evaluate the impact of different fungicide programs on RLS severity and the relative selection of *CYP51* and *sdh* resistance alleles of *R. collo-cygni* populations. In our screening, *CYP51* resistance alleles were found at higher frequencies when compared to *sdh* resistance alleles. The existing trend of higher selection for *CYP51* mutations is reflected by a lower performance in the field with around 52% disease control using prothioconazole. In contrast, the mutation found at *sdhC*-H153R had an average of 1% and at position *sdhC*-H146R 3.1% was observed (in untreated control; over all sites).

This better RLS control in the field using SDHIs results from 64% RLS effectiveness for bixafen and 70% for benzovindiflupyr. Mutation *sdhC*-H146R was generally more frequent than mutation *sdhC*-H153R as reported by Rehfus et al. (2019) at European level. The mutation *sdhC*-H153R is the homolog to mutation *sdhC*-H152R evolved in *Z. tritici* associated to fitness penalties (S. Torriani, 2020; personal communication).

In regions with high RLS distribution such as Upper Austria (site 4-WL), the frequency of *sdhC*-H146R in Trt 4 was found to be between 6 and 12 times higher when compared to the untreated control (Trt 1) and for *sdhC*-H153R mutations from between 2 and 8 times. In all tested trial sites, the frequency for *sdh* mutations was higher after an application with benzovindiflupyr compared to bixafen, with the exception of *sdhC*-H146R at site 2-TU in 2017 and at site 3-P in 2018. The superior RLS control with benzovindiflupyr compared to that of bixafen might be the reason for this stronger selection of *sdhC*-H146R and -H153R. Benzovindiflupyr has a higher intrinsic activity and binds stronger to the wax layer of the plant, where a reservoir of the active ingredient was able to build up. This leads to a continuous and controlled release of the active ingredient in the plant. Additionally, the greater bioavailability of benzovindiflupyr is responsible for an optimal activity and superior performance when compared to other SDHIs (Guicherit et al. 2014). Avenot and Michailides (2010) also reported for *Alternaria alternata* that an SDHI within the same cross-resistance group is more potent than other SDHI's when the intrinsic activity is higher.

Isolates showing significant loss of sensitivity to DMI fungicides harbored mutations in the CYP51 gene, highlighting mutation in the target gene as the major resistance mechanism in *R. collo-cygni* (FRAC 2018a). Several DMI studies in wheat showed that the application of different DMI fungicides were responsible for an increase in frequency of *CYP51* mutations (Heick et al. 2017; Stammler et al. 2008). Gutiérrez-Alonso et al. (2017) reported that high SDHI dose rates can reduce Septoria leaf blotch (caused by *Z. tritici*, with *R. collo-cygni* being the nearest sequenced relative according to Stam et al. 2018). However, lower dose rates may reduce selection for resistance strains. We found that applications without chlorothalonil increase the frequency of *CYP51* and *sdh* mutations and after a several applications with DMIs and SDHIs only this may lead to a rapid decline in RLS control and lower yield benefits.

In recent years, RLS field efficiency through DMI or SDHI decreased from almost 100% in 2014 to around 30% in 2019 (Syngenta, unpublished data). Fungicide resistance management strategies, such as reductions in the number of applications of active ingredients from a single group and mixing with effective fungicide partners, particularly multisite-acting fungicides, are the key to reduce selection of resistant strains (FRAC 2018b). The positive effect of this strategy was demonstrated in this study. With a diverse fungicide treatment (Trt 9 - Trt 11), the amount of *sdhC*-H146R and -H153R mutations were reduced, enabling good RLS control and thus lowering the field infection rate.

Weather and soil conditions, which induce stress in barley plants, were decisive factors for higher RLS severity (Oxley et al. 2008). West et al. (2012) predicted an increase in *R. collo-cygni* populations due to the global climate change, especially through heat stress. Furthermore, non-chemical strategies were also important factors for anti-resistance-strategies and can help to reduce RLS development. It is therefore essential to avoid saving seeds from heavily infected fields and to use not highly susceptible varieties. This can be considered as the primary strategy to reduce seedborne transmission of *R. collo-cygni* on barley (Havis et al. 2014; Kaczmarek et al. 2017). Furthermore, recently published markers associated with RLS (Tsai et al. 2020) will facilitate breeding of more durable resistant barley varieties. However, intensive straw management and weed control to reduce spore distribution, crop rotation and the growth of stress-free barley plants are also important to minimize the impact of the *R. collo-cygni* population size (Stam et al 2019).

Due to legal restrictions, chlorothalonil is no longer available for resistance management measures (BAES 2020). New management strategies—including the guidelines recommended by FRAC—are needed in order to prolong the lifespan of DMIs and SDHIs. Mixtures with different modes of action and the addition of multisite inhibitors are proposed (AHDB 2020). Stracke et al. (2018) reported activity of the multisite inhibitor folpet on RLS. However, more in-depth studies on the multi site inhibitor folpet are needed to understand its effects on RLS and the development of resistant *R. collo cygni* populations.

In summary, this study demonstrates the impact of spray strategies on RLS control and the selection rate of *CYP51* and *sdh* mutations. All fungicide treatments reduced RLS in this study; however, adding the multisite inhibitor chlorothalonil gave a significant lower level which could not be achieved by other treatments. Splitting the application timing between 2 and 3 in Trt 10 including chlorothalonil resulted in the best RLS control and prevented an increase in mutations conferring resistance (*CYP51*-I325T, *CYP51*-I328L, *sdhC*-H146R and *sdhC*-H153R) within the *R. collo-cygni* populations. Without chlorothalonil, these mutations increased in their frequency. Now that this active ingredient is no longer available, new strategies against RLS must be developed, taking into account the avoidance of fungicide resistance.

Declarations

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