



# Artificially applied late-terminal drought stress in the field differentially affects *Ramularia* leaf spot disease in winter barley

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## Abstract

*Ramularia* leaf spot disease (RLS) is one of the most dominating fungal diseases in barley. The disease typically appears late in the season after flowering and results in a rapid loss of photosynthetic leaf area. A recent decline in fungicide efficacy and a lack of RLS-resistant cultivars hamper effective control. Global warming will provoke increasing droughts which influence host plant physiology and probably affect outbreak and severity of RLS. Relatively little is known about genetic resistance to RLS in winter barley and about the influence of various weather conditions and climate change on RLS pathogenesis. Hence, we evaluated severity of RLS on 15 winter barley genotypes under persistent late-terminal drought stress or controlled irrigation, respectively, in a field rainout shelter. Over three consecutive years, we observed reproducible differences in quantitative RLS field resistance of the used cultivars and variable suppression of RLS under drought. Our results support a function of drought in suppression of RLS in winter barley, but also reveal strong year effects even under semi-controlled rainout shelter conditions. Data may be relevant for genotype selection in breeding programmes for RLS resistance and for farmers in the frame of integrated disease management under a changing climate.

## Graphical abstract



**Keywords** *Ramularia collo-cygni* · Physiological leaf spot · Winter barley · Climate adaptation · Drought stress · Phenotyping · Rainout shelter

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## Introduction

In the last four decades, *Ramularia* leaf spot disease (RLS) became an emerging barley disease and RLS is to date one of the most dominating foliar diseases in many barley growing areas worldwide (Havis et al. 2015). RLS disease causes losses in grain yield averaging between 5 and 10%, but

occasionally losses reach up to 75% (Pinnschmidt and Jørgensen 2009; McGrann and Havis 2017). The late and rapid appearance of symptoms in the field, relatively fast adaptation of causal *Ramularia collo-cygni* to fungicides and the lack of fully resistant barley genotypes may have promoted the current dominance of this fungal disease. Furthermore, comparatively little knowledge about epidemiology and effects of environmental conditions on RLS disease impede the development of effective and integrated control measurements, which are still not sufficiently adapted to RLS disease (Dussart et al. 2020). Hence, control of RLS strongly relies on fungicide treatments although *R. collo-cygni* independently achieved multiple resistances towards several fungicides in many different field populations (Matusinsky et al. 2010; Rehfus et al. 2019; Assinger et al. 2021, 2022; Kiiker et al. 2021; Mäe and Kiiker 2022; Erreguerena et al. 2022). As a consequence of this, resistance breeding becomes more important to effectively manage the disease in an integrated approach that should also consider environmental impacts on plant resistance and epidemiology of RLS.

RLS disease is characterized by a relatively long phase of endophytic and symptomless growth of *R. collo-cygni* in the host plant. Hence, the relation between time of infection event and symptom development is hardly predictable under field conditions. Infection and disease spreading usually occur via airborne spore inoculum (Havis et al. 2015, 2023) that mediates a bridge between spring and winter barley (Frei et al. 2007). Additionally, infected seeds, alternative hosts or colonized crop debris are possible sources of inoculum (Oxley et al. 2002; Walters et al. 2008; Matusinsky et al. 2011; Havis et al. 2013, 2015; Brown et al. 2014). In general, symptoms rapidly appear within a few days at post-heading stage when barley is in transition towards its reproductive stage. Environmental conditions are further suspected to trigger disease outbreaks and severity. At late growth stages, reddish to dark brown spots surrounded by a yellow halo appear in a speckled pattern on the entire leaf. The spots are typically small and confined by leaf veins which give the spots a rectangular shape. The fungus further releases photodynamic and toxigenic rubellins into the apoplast, which provoke formation of spots usually on light exposed sides of infected leaves (Heiser et al. 2003; Miethbauer et al. 2003, 2006). This illustrates the strong interaction of the disease with environmental factors and host plant physiology (Schützendübel et al. 2008; McGrann et al. 2014; Havis et al. 2015). Small RLS symptoms at early stages of the disease often resemble physiological leaf spots (PLS) and are likewise associated with abiotic stress and physiological imbalances. This makes accurate disease assessment and decision on fungicide applications difficult for farmers. Both RLS and PLS decrease green leaf area. As a consequence, the entire leaf becomes gradually senescent starting from the leaf tip to the base increasingly

restricting photosynthesis, production and transport of sugars and energy, which further limits starch storage in grains (Tetlow and Emes 2017). Loss of green leaf area results in yield loss and, when it comes to malting barley, quality losses by low thousand grain weight and limited germination capacity (Havis et al. 2015). Following symptom formation, conidiophores start to break through cell walls and stomata on symptomatic, necrotic or senescent leaf tissue resulting in a massive release of spores, which often coincides with moist periods or single rain events, respectively. A recent study by (Havis et al. 2023) revealed crop surface wetness as major environmental parameter strongly associated with spore release.

Environmental conditions affect both host plants and pathogens resulting in variable RLS incidence and severity across various seasons. For this reason, under a changing climate, host–pathogen interactions become more tilted and further unpredictable due to the high influence of increasingly extreme weather conditions such as heavy rainfalls or long-lasting heat and drought periods. Extreme environmental conditions directly affect the host resulting in stress (Chakraborty et al. 2000; Anderson et al. 2004; Juroszek and von Tiedemann 2012; West et al. 2012). From a physiological perspective, abiotically stressed plants are in an imbalanced state which might affect host resistance and provoke increased disease severity (Liu and Liu 2016; Zhang and Sonnewald 2017; Choudhary and Senthil-Kumar 2022). In parallel, abiotic stress situations can create conditions outside the optimum of the pathogen and this can have a negative impact on vital inoculum present on site, pathogen dispersal and infection capacity in the field. Thus, reduced infections and lower disease severity may take place despite abiotic stress is also negatively affecting pathogen defence of the host plant (Hoheneder et al. 2021; Sewelam et al. 2021). Consequently, understanding effects of abiotic factors on pathogen epidemiology and on genotype-dependent disease resistance is indispensable for breeding climate-adapted and stress-resistant crops. In this context, the epidemiology of RLS disease is directly linked to the environment. The high dominance of RLS in recent years and in many barley growing areas under increasingly unfavourable climatic conditions for crops put the focus on future breeding of robust and RLS-resistant cultivars.

Analogous to our previous study in spring barley (Hoheneder et al. 2021), we wanted to assess differences between individual winter barley cultivars in quantitative RLS field resistance under well-watered and drought conditions in the field. Additionally, we aimed to study general effects of late-terminal drought stress on the expression and severity of different RLS disease parameter in diverse winter barley genotypes and whether such potential effects would be influenced by conditions in individual seasons. For this purpose, we used a field rainout shelter to expose a panel of

15 diverse winter barley cultivars to long-lasting dry conditions from spike emergence onwards.

## Material and methods

### Field trials under controlled and continuous drought stress

The incidence of RLS disease on a diverse collection of 15 winter barley genotypes (table S-1) was assessed under controlled irrigation and continuous drought stress in the field. Therefore, we used a moveable rainout shelter (transparent polyvinyl roof with open fronts) at the Bavarian State Institute of Agriculture (LfL) in Freising (Southern Germany; exact location: 48.411872, 11.722039) as previously described in the studies from Wenzel et al. (2015) and Hoheneder et al. (2021). Briefly, plants were sown in four fully randomized blocks containing one field plot (size: 3.5 m<sup>2</sup>) of each barley cultivar. The rainout shelter remained completely open between sowing date in autumn (mid- to end of September) and early winter (December) to maintain sufficient natural precipitation for germination and seedling growth. Automatic opening and closing were applied from beginning of the following year (January) to exclude natural rainfall and to keep soil moisture under control within the rainout shelter. For this purpose, all four blocks were weekly irrigated with 20 mm until the beginning of spike emergence (GS 50) with a sprinkler irrigation system. The amount of irrigation was set according to long-term precipitation data at the field location. From spike emergence on (April), two of four blocks were continuously irrigated, while two other blocks remained without irrigation to gain prolonged dry conditions as expected in near future in Europe (Zebisch et al. 2005; Shah et al. 2022). Time period of applied weekly irrigation, sum of irrigation of the watered plots and sum of excluded natural rainfalls during application of drought conditions in each season are shown in Table 1.

Pesticide treatments were conducted according to the integrated pest management model system for barley in Bavaria

(Maier and Hofmann 1993; Tischner et al. 2006) though without using fungicides in each field season. The plots were harvested after full ripening (growth stage 99) with a single plot combine harvester. Total grain yield (kg) for each plot was weighted after harvest and dried to 14% moisture. Grain yield was further converted into dt/ha according to harvested dry grain weight and respective plot area.

Weather data were recorded by a nearby weather station about 500 m away from the field location. Sum of precipitation, average temperature, relative air humidity, leaf wetness and global radiation were assessed from the agro-meteorology web portal of the Bavarian State Institute of Agriculture (Agrarmeteorologie Bayern 2022). According to our previous study on spring barley (Hoheneder et al. 2021), the assessed data on mean leaf wetness and average temperature are representative for irrigated field conditions. Mean values were calculated for each month during the growing seasons between 2018/2019 and 2020/2021 (Table 1; table S-2).

### Assessment of leaf symptoms or clusters of conidiophores and leaf sampling

RLS symptoms on flag leaf and leaf stage below (F-1) were visually examined at late milk stage to mid-dough stage (growth stage 78–85) according to strongest severity in RLS symptoms during each individual field season. Due to the usually occurring rapid increase of RLS symptoms at the beginning of post-flowering stage onwards, the field plots were controlled every 5 days to determine the maximum progression of leaf symptoms for final assessment of RLS symptoms. Symptoms of net blotch (spot type; *Pyrenophora teres* f. *maculata*) were additionally assessed as the only notable foliar disease beside RLS at the field location and over the three field seasons. Furthermore, physiological leaf spots (PLS) and area of senescent leaf tissue were assessed due to its association with abiotic stress, physiological imbalances and plant maturity. PLS were considered as black spots with a round to irregular shape missing a yellow halo and hence were clearly distinguished from RLS symptoms as described in “Introduction.”

**Table 1** Dates of weekly irrigations in the rainout shelter during the seasons 2019 to 2021

Year	Start of irrigation	Stop of irrigation	n (irrigation events)	Sum of irrigation in irrigated plots (mm)	Sum of natural precipitation excluded from the rainout shelter between April and June (mm)
2019	24/04/2019	12/06/2019	8	160	149.2
2020	22/04/2020	10/06/2020	8	160	85.7
2021	28/04/2021	23/06/2021	9	180	244.7

Sum of irrigation in the rainout shelter and sum of natural precipitation at the field location which was excluded from the rainout shelter are shown in mm during the period of weekly applied irrigation events from spike emergence on until plant maturity. Precipitation was recorded by a weather station about 500 m away from the field location, and data were assessed from the agro-meteorology web portal of the Bavarian State Institute of Agriculture (Agrarmeteorologie Bayern 2022)

Leaf area with clusters of conidiophores was visually quantified in the laboratory with a binocular microscope (magnification: 40) in combination with a flat angled light from the side to increase visibility of usually bright conidiophores of *R. collo-cygni*. Therefore, the bottom side of 20 randomly selected individual leaves per cultivar was screened to determine mean leaf area with visible clusters of conidiophores in per cent according to variable leaf sizes (see also Hoheneder et al. (2021)).

### Isolation of genomic DNA from leaf material and quantification of *R. collo-cygni* DNA

Isolation of genomic DNA was conducted from 20 leaflets per cultivar according to the protocol of Fraaije et al. (1999) with minor modifications as described by Hofer et al. (2016) and after previous freeze-drying overnight. Total *R. collo-cygni* DNA was quantified according to the real-time quantitative polymerase chain reaction (qPCR) protocol as previously published by Taylor et al. (2010). Quantitative PCR was performed with a MX3005P Multiplex Quantitative PCR System (Stratagene, CA, USA). Data were analysed with MxPRO qPCR software (Stratagene, CA, USA).

### Calculation of disease severity ranking

In order to compare genotypes and to balance year effects on RLS severity, disease rankings were calculated from different RLS disease parameters assessed in the field and laboratory (mean ranking for (i) RLS, (ii) leaf area with clusters of conidiophores and (iii) DNA contents) as previously described by Hoheneder et al. (2021). Therefore, a mean rank for each disease parameter was calculated for each genotype and year per field environment (minimum rank: 1; maximum rank 15). Finally, the individual ranks were averaged over the three considered seasons to obtain an overall disease rank for each genotype. Low ranks were equal to low values of disease parameters and indicate resistance; high ranks were equal to high values and indicate susceptibility. Shared ranks were associated with same single values.

### Analysis of variance

RLS symptoms, leaf area carrying conidiophores and fungal DNA contents were subjected to analysis of variance (two-way ANOVA) for the genotype, environment and genotype x environment interaction affecting assessed disease parameters. For this purpose, field plots under irrigated or drought conditions, respectively, were defined as environments. Data from each season and environment were used as replication.

### Yield stability analysis

Stability of the genotypes in yield was determined with an additive main effect and multiplicative interaction (AMMI) model according to Purchase et al. (2000) to analyse stability and performance of each genotype in different field environments. The model fits additive effects for genotypes and environments and multiplicative interactions between genotypes and environments (Crossa et al. 1990). Stability of yield was ranked for each genotype based on calculated AMMI stability values (ASV). The yield stability index (YSI) was calculated by summing the ranking of the overall mean yield and the ranking for ASV of each genotype under irrigated and drought conditions as previously described in our study on spring barley (Hoheneder et al. 2021). The lower the YSI, the more stable is a genotype according to stability and quantity of yield according to seasons and treatments.

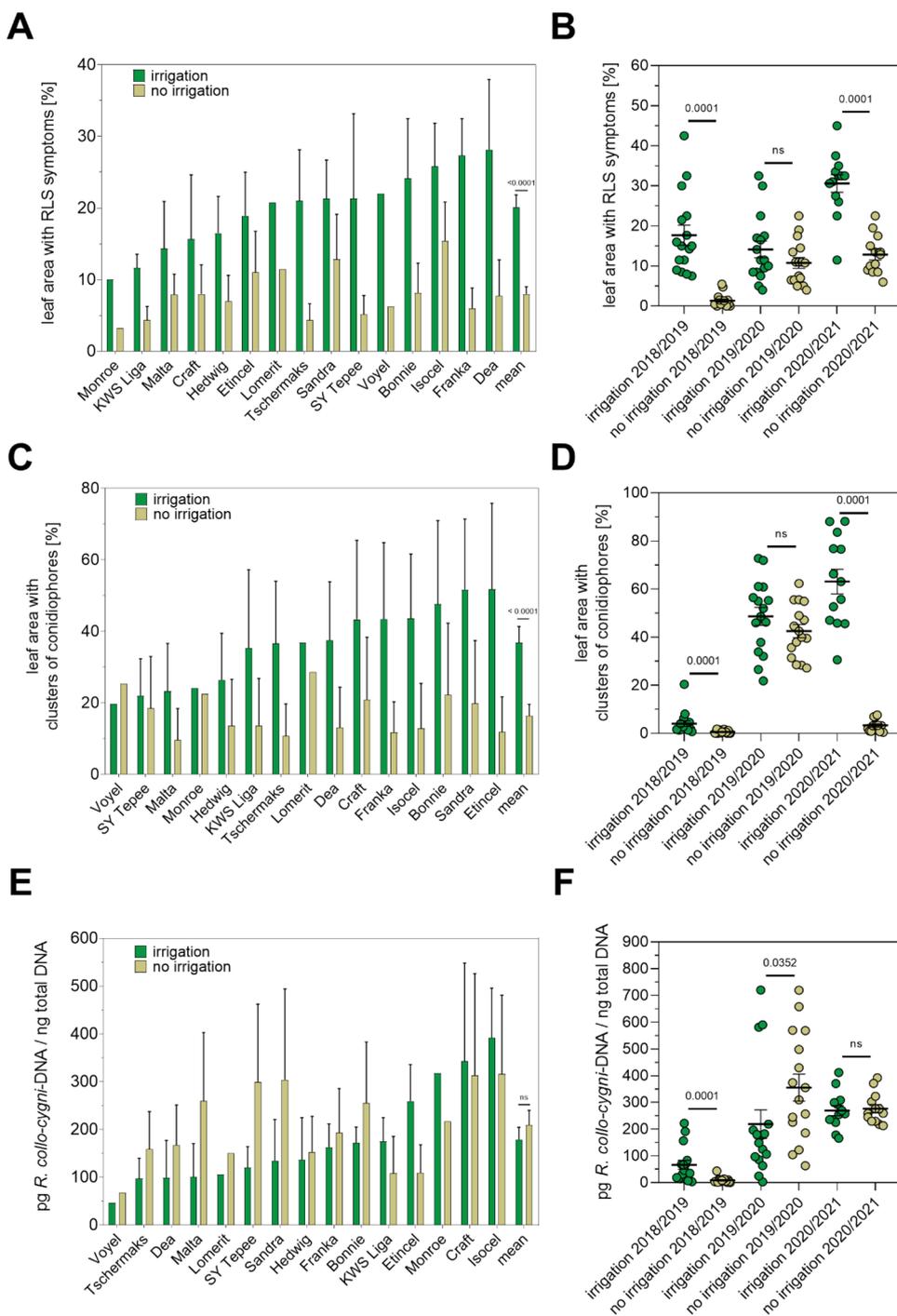
## Results

### RLS severity under irrigated and continuous drought conditions

In the present study, we assessed RLS disease severity of an assortment of diverse winter barley cultivars. The evaluated genotypes represent a set of modern to old European two and six rowed winter barley cultivars. The assortment was preselected to evaluate various winter barley genotypes for their general performance and disease resistance under continuous drought conditions in the field (see table S-1, and material and methods). Different diagnostic tools were used in the field and laboratory. We visually rated RLS symptomatic leaf area on both flag leaves and flag-1 leaves. We further collected leaf samples from the field to determine leaf area with clusters of conidiophores of *R. collo-cygni* under a stereo microscope in the laboratory to directly assess fungal reproduction. Subsequently, genomic DNA was extracted from collected leaves and the amount of fungal biomass was determined via quantitative PCR.

In three consecutive field seasons (2018/2019–2020/2021), we compared RLS severity in a field rainout shelter under either controlled irrigation or long-lasting drought conditions from spike emergence onwards. RLS was generally the dominating foliar disease under both conditions over the three consecutive field seasons (Fig. S-1). Under irrigation, we found a strong cultivar-dependent differentiation in the incidence of RLS severity on upper leaves. Drought conditions generally decreased leaf area with RLS (total mean; irrigation: 20.10%, drought stress: 7.99%; Fig. 1a) and further leaf area with visible sporulating fungus (total mean; irrigation: 38.86%, drought

**Fig. 1** Levels of *Ramularia* leaf spot disease according to assessed disease parameters under drought stress and irrigation in the rainout shelter experiment between 2019 and 2021 (four randomized blocks consisting one plot per cultivar). The bar graph indicates calculated mean values of 3 years. Error bars indicate the standard error of the mean. Data for cultivars Lomerit, Monroe and Voyel represent mean of seasons 2018/2019 and 2019/20. **A** represents percentage of leaf area with *Ramularia* leaf spots per genotype and **B** shows a variation of RLS symptoms per field environment and individual season. **C** indicates percentage of leaf area with clusters of conidiophores and **D** shows the respective data for each individual season. **E** shows detected values of *Ramularia collo-cygni*-DNA in pg Rcc DNA/ng total DNA per genotype, and **F** shows respective data according to each individual field season. Statistical analysis: Comparison of total means of assessed disease parameters under irrigated and non-irrigated conditions over all genotypes was performed with an unpaired Mann–Whitney test. *p* value indicates significance of the slope from zero; alpha = 0.05



stress: 16.36%, Fig. 1b), but not DNA contents in upper leaves (total mean; irrigation: 178.92 pg/ng total DNA, drought stress: 209.03 pg/ng total DNA). Average fungal DNA contents even increased in ten out of 15 barley genotypes growing under drought conditions compared to irrigated plants, but this effect was not evident in every season (Fig. 1e, f).

Under irrigation, mean leaf area with RLS ranged from 10.00% (cultivar Monroe) to 28.17% (cultivar Dea), whereas

mean RLS symptoms on drought stressed plants remained between 3.25% (cultivar Monroe) and 15.42% (cultivar Isocel). The cultivar Monroe hence showed least mean area with RLS symptoms under both conditions, followed by KWS Liga, Malta and Craft (irrigation: 11.67%, 14.33% and 15.67%; drought stress: 4.33%, 7.92% and 8.00%). Most mean RLS symptoms were found for the cultivars Dea, Franka, Isocel and Bonnie under irrigated conditions (28.17%, 27.33%, 25.83% and 24.17%, respectively),

whereas Isocel showed most symptoms under drought (mean: 15.42% mean) (Fig. 1a).

Despite an overall decrease of disease symptoms under drought, suppression of RLS was not generally evident regarding fungal DNA contents and for all genotypes: among all tested genotypes, least leaf area showing clusters of conidiophores (mean 19.74%) and DNA contents (mean 46.66 pg/ng total DNA) were determined for the cultivar Voyer under irrigation (Fig. 1c, e), whereas this genotype showed moderate leaf symptoms with RLS symptoms (22.00%) (Fig. 1a). Drought conditions strongly decreased RLS symptoms on Voyer (Fig. 1a), but leaf area with conidiophores and DNA contents slightly increased under drought (Fig. 1c, e). Similarly, Malta showed relatively little symptomatic leaf area (14.33%) and leaf area carrying clusters of conidiophores (23.22%) under irrigation and further a decrease for those two disease parameters under drought conditions. In contrast, drought conditions resulted in increased DNA contents (irrigation: 100.92 pg/ng total DNA; drought conditions: 259.65 pg/ng total DNA), indicating a stronger colonization within leaves of cultivar Malta under drought stress. For the cultivar Hedwig, which showed relatively little disease over all determined parameters, we found a suppression of RLS symptoms and leaf area carrying conidiophores under drought conditions, but no decrease of DNA contents (Fig. 1a, c, e).

In addition, in order to test a possible relationship between plant ripening and different RLS disease parameter due to a possibly accelerated maturity under drought, a Pearson's correlation analysis was calculated over all tested genotypes (table S-3). Preselection of the genotype assortment for uniform agronomic traits, including plant ripening type, resulted in minor differences in recorded growth stages between genotypes in general and between irrigated and drought stressed plants. Hence, no significant relations were found between growth stages and RLS disease parameters (table S-3).

A year-wise comparison of RLS disease parameters revealed strong year effects in RLS severity under irrigation or drought conditions, respectively. In seasons 2018/2019 and 2020/2021, mean symptomatic leaf area with RLS was suppressed by drought conditions compared to the irrigated plants across the entire genotype assortment (Fig. 1b). In contrast, in season 2019/2020, mean RLS symptoms were only marginally reduced on plants growing under drought (total mean; irrigation: 14.13%; drought conditions: 10.77%; Fig. 1b). According to individual field seasons, mean leaf area with clusters of conidiophores reflects observed effects of drought conditions on RLS symptoms. Mean leaf area with conidiophores was strongly suppressed in seasons 2018/2019 and 2020/2021 under drought conditions, but not in field season 2019/2020, where no significant difference was found between irrigated and drought stressed plants. In

addition, lowest means of leaf area carrying conidiophores were found in season 2018/2019 under both conditions (total mean; irrigation: 4.70%; drought conditions: 0.62%) (Fig. 1d). Despite suppression of RLS symptoms and leaf area with conidiophores in two of three seasons, we could not observe similar effects on fungal DNA contents in upper leaves in every respective field season (Fig. 1f). In season 2018/2019, mean *R. collo-cygni* DNA contents were significantly suppressed under drought conditions, although general colonization of the leaves remained relatively low (total mean, irrigation: 66.62 pg/ng total DNA; drought conditions: 8.52 pg/ng total DNA). An opposite effect on fungal colonization was observed in the following season 2019/20, indicating that under drought stress, conditions were favourable for the fungus to grow within leaves without strong induction of leaf symptoms or increased production of conidiophores compared to irrigated barley genotypes (Fig. 1b, d, f). By trend, similar results were found for fungal DNA contents in season 2020/2021 suggesting a minor effect of drought on leaf colonization in this specific season. As mentioned, leaf symptoms and area with clusters with conidiophores significantly ( $p < 0.0001$ ) decreased under drought conditions, suggesting little relations between visible symptoms or sporulation and fungal colonization in this respective season (Fig. 1b, d, f).

In season 2018/2019, we exclusively observed a strong suppression ( $p < 0.0001$ ) of all assessed RLS disease parameters under drought conditions independent from the individual cultivar (Fig. 1b, d, f). Noteworthy, mean temperatures late in the growth season post-flowering of barley (June 2019) revealed warmest conditions (19.5 °C), lowest leaf wetness (40.0%) and highest global radiation (2049 W/m<sup>2</sup>) compared with the following seasons as recorded by a nearby weather station (table S-2). Additionally, warm mean temperature, lowest leaf wetness and highest global radiation were measured over the entire season 2018/2019, suggesting that such seasonal weather conditions were unfavourable for RLS disease in general and moreover in combination with drought (Fig. 1b, d, f; table S-2).

Furthermore, PLS symptoms were assessed in parallel with RLS symptoms. Total mean of leaf area with PLS symptoms was suppressed under drought conditions for each genotype and over the three individual field seasons (Fig. S-2a, b). No relation between RLS and PLS was found under irrigation. A significant relation between both types of leaf symptoms was found under drought (Fig S-2c).

### Ranking of winter barley genotypes according to RLS disease parameters

In order to compare basal quantitative RLS resistance of cultivars under the applied environmental conditions, we calculated ranks according to yearly assessed disease

parameters and averaged ranks over 3 years as previously described by Hoheneder et al. (2021). Ranks for each disease parameter (minimum 1, maximum 15) were further averaged to a total mean rank (3 years × three RLS disease parameters;  $n = 9$ ) to equalize year effects on RLS severity under both field conditions in the rainout shelter. Ranking revealed a differentiation of cultivar resistance under the two given field conditions (Fig. 2). The cultivar Malta (mean rank: 2.67) and Hedwig (mean rank: 4.67) occupied the first and second rank and can thus be rated as quantitatively resistant to RLS under irrigated field conditions (Fig. 2a). Cultivar ranking changed when we compared irrigated and drought-treated genotypes. The cultivars Tschermaks (mean rank: 3.89) and KWS Liga (mean rank: 4.22) ranked first and second under drought conditions, which indicates high RLS resistance of these genotypes under drought (Fig. 2b). The genotypes Isocel, Etincel and Franka (total mean ranks: 11.78, 10.11 and 9.33) showed highest ranks under irrigation which suggests high susceptibility towards RLS. Similarly, Sandra and Isocel were rated as susceptible under drought conditions (mean ranks: 10.22 and 9.00). Cultivar Lomerit was most susceptible under drought (mean rank under drought = 12.83) although it was only moderately susceptible under irrigation (mean rank = 9.17). Similarly, SY Tepee was moderately susceptible under drought, but ranked with third lowest infection scores under irrigation. Interestingly, some cultivars ranked high with regard to fungal DNA, but moderate or low according to RLS symptoms or fungal sporulation (KWS Liga, Craft, Monroe) (Fig. 2a).

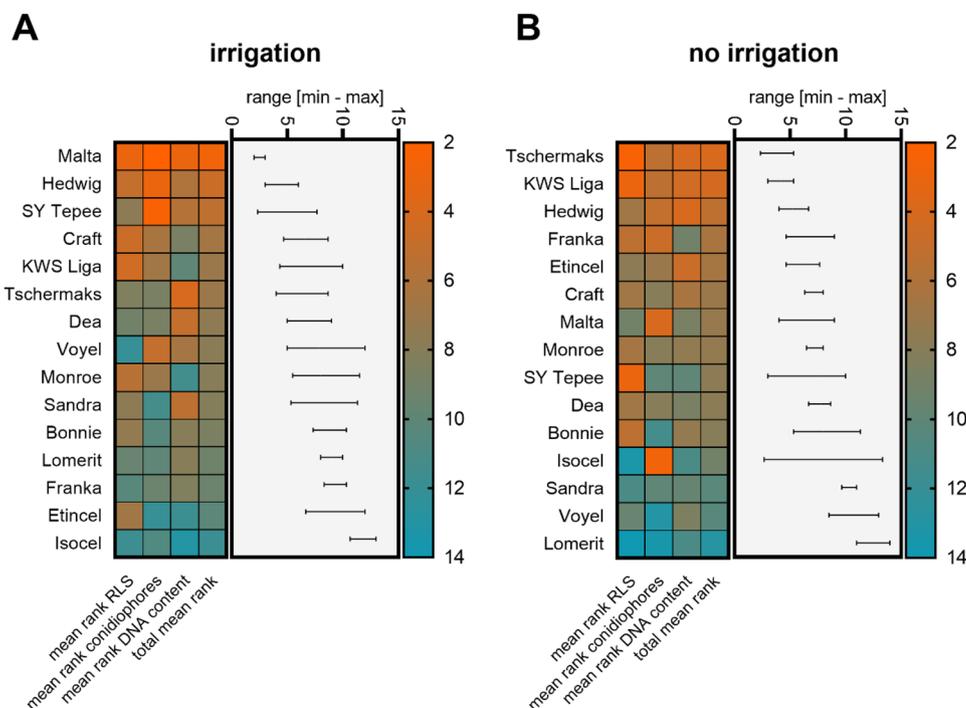
### Correlation between assessed RLS disease parameters

To assess relations between different RLS disease parameter, we calculated Pearson’s correlations between each disease parameter under irrigated and drought conditions over 3 years and 15 cultivars (Fig. 3). By trend, higher rates of RLS symptoms were associated with increased leaf area covered with clusters of conidiophores under irrigation ( $p = 0.0642$ ) (Fig. 3a). Interestingly, RLS symptoms were not correlated with fungal DNA contents (Fig. 3b), indicating that RLS symptoms did not quantitatively reflect fungal colonization of leaf tissue under irrigation. Under irrigation, we found, however, a positive correlation ( $p < 0.0001$ ) between leaf area with conidiophores and *R. collo-cygni* DNA contents among the tested genotypes (Fig. 3c). Under drought conditions, *R. collo-cygni*-DNA contents, RLS symptoms and leaf area with clusters of conidiophores, respectively, were each positively correlated with each other (Fig. 3d–f). The results indicate a closer relation between disease parameters under drought conditions than under irrigation (Fig. 3).

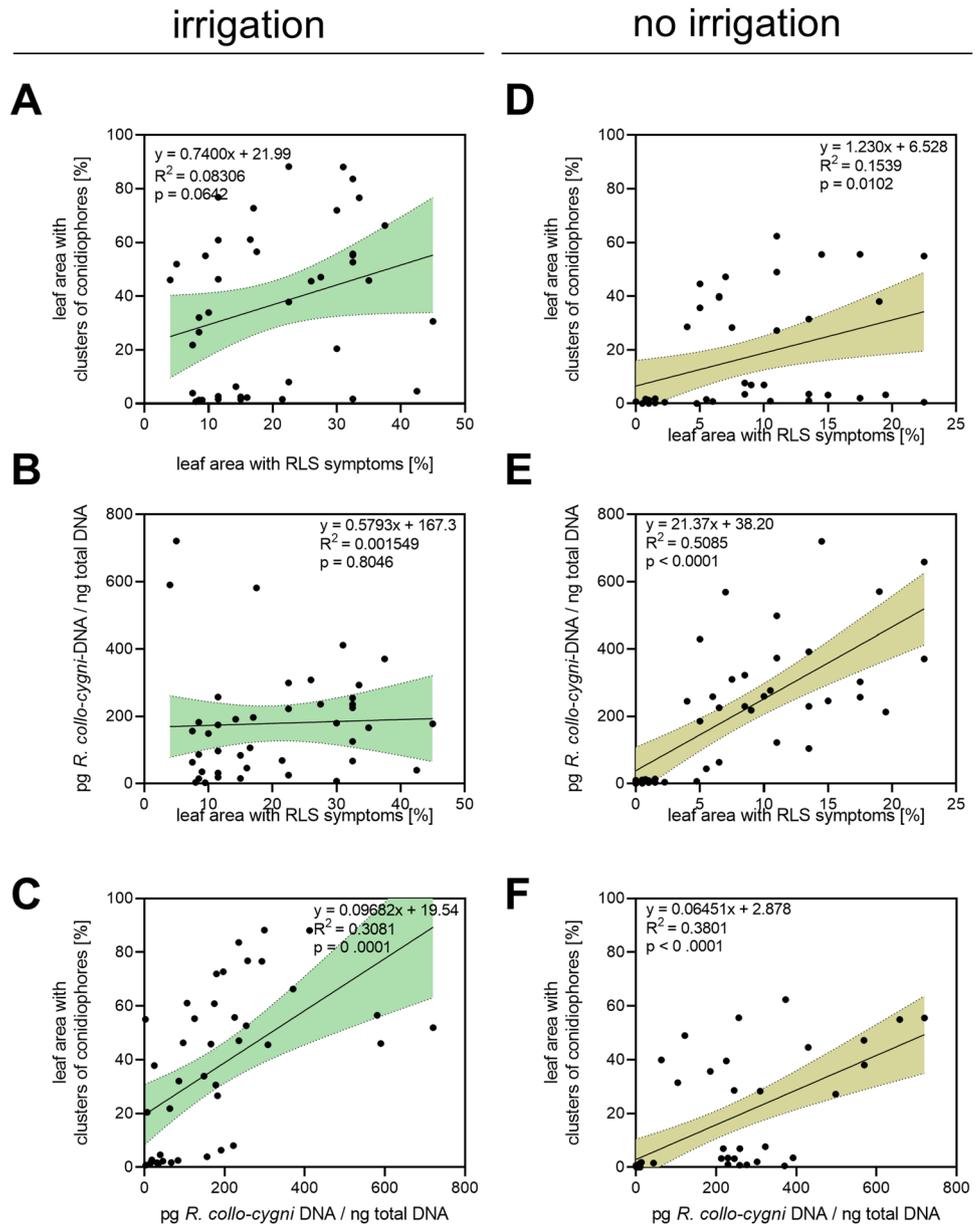
### Analysis of variance of genotype × environment interactions

In order to determine significance of the genotype, environment and the genotype × environment interaction on variation in disease parameters, a two-factor ANOVA was conducted. The analysis of variance showed significant variations among the two environments (drought versus irrigated)

**Fig. 2** Disease severity ranking according to assessed disease parameters in the irrigated control (A) and under drought stress (B) in the rainout shelter experiment between season 2018/2019 and 2020/2021. The heat maps show mean ranks over the three consecutive years for each disease parameter (3 years × three parameters;  $n = 9$  ranks per genotype) and respective range of minimum and maximum values. The genotypes are sorted in an ascending order by total mean rank whereby low ranks indicate quantitative resistance and high ranks indicate higher susceptibility to RLS. Individual ranks are in a range of minimum 1 and maximum 15. If two or more genotypes share the same value, the same rank was associated to those lines



**Fig. 3** Relations between assessed *Ramularia* leaf spot disease parameters in the rainout shelter between season 2018/2019 and 2020/2021. Data points represent year-wise means of 15 winter barley genotypes under irrigated (A–C) and drought (D–F) conditions. Area with RLS symptoms and clusters of *Ramularia* conidiophores are presented as percentage of leaf area, respectively. Visual assessment of upper leaves was conducted at growth stage 78–85. Statistical analysis: linear regression; *p* value indicates significance of the slope from zero;  $\alpha=0.05$ . Colour-shaded areas represent 95% confidence bands of the best-fit line



for the mean of RLS symptoms ( $p < 0.0001$ ) and leaf area carrying clusters of conidiophores ( $p < 0.0045$ ). Neither the genotype nor the environment nor genotype  $\times$  environment

interaction had a significant effect on fungal DNA contents (Table 2). We performed a similar analysis of variance using data of spring barley under irrigation and drought stress

**Table 2** Two-factor analysis of variance for RLS symptoms, fungal sporulation and DNA contents of 15 winter barley genotypes grown in two field environments (irrigation, drought stress) in seasons 2018/2019 to 2020/2021

	DF	RLS			sporulation			DNA content		
		SS	MS	<i>p</i> value	SS	MS	<i>p</i> value	SS	MS	<i>p</i> value
G $\times$ E	14	532.7	38.05	0.9758	2998	214.1	0.9969	197,870	14,134	0.9797
G	14	1064	76.03	0.7103	2758	197	0.998	465,124	33,223	0.6103
E	1	2948	2948	<0.0001	7562	7562	0.0045	15,256	15,256	0.5339

DF, degree of freedom; SS, sum of squares; MS, mean square; G  $\times$  E = genotype by environment; G, genotype; E, environment

(Hoheneder et al. 2021) and revealed significant effect of the environment on disease parameters (sum of RLS and PLS, leaf area with conidiophores and fungal DNA content). The genotype and genotype x environment interaction had also a significant effect on leaf area showing clusters of conidiophores in spring barley (table S-4).

### Evaluation of genotype-dependent stability for yield

To assess stability of genotypes concerning to yield across irrigated and drought conditions, we used an additive main effect and multiplicative interaction (AMMI) model (Purchase et al. 2000) to calculate a yield stability index (YSI; (Hoheneder et al. 2021)) based on grain yield data determined between season 2018/2019 and 2020/2021. The YSI indicates a more stable performance for yield of a certain genotype under the given conditions, the lower the index is. The cultivars Isocel and Lomerit (both YSI 6) showed lowest yield stability indices and hence produced most stable yields under the two considered field environments. Lowest yield stability was found for the genotypes SY Tepee (YSI 27) and Malta (YSI 22). Furthermore, the cultivar with highest yield stability (Isocel) also produced high yields under both field conditions. Lowest yields were produced by the relatively old cultivar Tschermaks (release: 1921; suppl. table S-1) under both field conditions (irrigation: 38.3 dt/ha; drought conditions: 34.8 dt/ha). The least stable genotypes according to yield showed also low yields in both environments (suppl. Fig. S-3).

### Discussion

The multilateral interactions between crop plants, pathogens and the environment might strongly affect performance of individual genotypes under field conditions. In such complex field environments, accurate assessment of disease resistance is often hindered due to multivariable effects of weather conditions on coincidence of pathogen life cycle and infection events with phenological stages of the host (Miedaner and Juroszek 2021). Consequently, accurate phenotyping of disease resistance under diverse stress situations is challenging for plant breeders, but critical to select robust genotypes. Therefore, genotype selection needs to similarly address both biotic and abiotic stress resistance which, at best, should be stable over multiple environments. Analogous to our previous study in spring barley (Hoheneder et al. 2021), the present study focused on assessment of RLS severity on different winter barley genotypes under irrigated and late-terminal drought conditions in the field as it will increasingly occur in near future in Central Europe (Shah et al. 2022; Zebisch et al. 2005). Therefore, we used

a field rainout shelter to obtain data on RLS disease parameter and cultivar performance under irrigation and drought conditions in the field. Data revealed a partial suppressive effect of long-lasting drought conditions on RLS disease. Genotype-dependent differentiation between RLS severity was possible but less distinct in winter barley compared to spring barley (Hoheneder et al. 2021). Furthermore, permanent drought stress did not as strongly suppress RLS as we observed in spring barley (Hoheneder et al. 2021) and only partially affected fungal colonization in the host. The high variability in disease development among cultivars and years under irrigation and drought conditions, respectively, suggests that the epidemiology of RLS in winter barley is influenced by parameters that we could not control under our experimental setup such as temperature, air humidity and global radiation.

Over all tested winter barley cultivars, mean RLS symptoms and leaf area showing clusters of conidiophores of *R. collo-cygni* (Fig. 1a, c) were suppressed under drought conditions when compared to irrigated plots. However, this was not evident for average *R. collo-cygni* DNA contents in upper leaves (Fig. 1e) or when looking into individual years (Fig. 1f). We even recorded increased mean fungal DNA contents under drought in 10 out of 15 barley cultivars, indicating that under the given conditions, fungal colonization was probably little affected or even promoted by drought stress conditions. In this context, quantification of fungal DNA contents produced different results over the three consecutive seasons (Fig. 1e, f). One might thus consider that visual symptom monitoring is not fully sufficient for rating RLS resistance, but should be combined at least with fungal DNA quantification or even more disease parameters. However, it remains an open question whether symptomless endophytic *R. collo-cygni* generally harms plant performance or rather reflects a risk of possible later symptomatic disease outbreak and spore dispersal under distinct environmental conditions. To date, we can only speculate on reasons for partly increased loads of fungal DNA in non-irrigated plants, but data emphasize the high potential of *R. collo-cygni* to quickly colonize barley even under unfavourable environmental conditions. Concomitantly, signals triggering the endophyte to switch for a necrotrophic lifestyle probably failed to appear under permanent drought stress conditions. This may have led to high levels of fungal DNA within the host without causing strong symptoms. Therefore, the epidemiological consequences of potential tolerance to RLS are little understood, but should be considered in future RLS disease monitoring and research on RLS management (Dussart et al. 2020).

A comparison of cultivars revealed a differentiation in RLS severity under both field conditions and highlights significant effects of the environment on disease progression (Fig. 1; Table 2). Under irrigation, the genotypes Monroe,

KWS Liga and Craft showed little RLS symptoms although the leaves were highly colonized by the fungus (Fig. 1a, c, e). This demonstrates that, despite a high fungal colonization of the leaves, the genotypes did not express a rapid loss of photosynthetic active leaf area via development of leaf spots until early dough ripening onwards, when symptoms strongly and rapidly appeared in other cultivars. This reveals a possible suppression of disease outbreak by individual genotypes. Furthermore, the genotype Craft showed a relatively stable yield performance under the given conditions (Fig. S-3), despite a high fungal colonization. However, in our data, we did not observe a general positive or negative association of yield and RLS resistance (table S-5). Although our data set may be too small to draw general conclusions from this, it may be worth considering a possible trade-off between yield and RLS resistance in future phenotyping experiments.

The cultivar Malta showed relatively little RLS symptoms under irrigation and a reduction under drought, although DNA contents were strongly increased under drought (Fig. 1a, e). This suggests that drought supported fungal development in the cultivar without increased visible damage of leaf tissue. In contrast, cultivar Dea showed high RLS symptoms under irrigation, but little DNA contents were found. Drought suppressed RLS symptoms and increased DNA contents compared to irrigated Dea plants. Such drought-dependent effects on RLS disease were not visible for all examined cultivars, which raises the question how individual genotypes differently regulate *R. collo-cygni* defence at a genetic level in particular when combined with abiotic stress. Additionally, drought stress in combination with pathogen infection was found to strongly manipulate defence responses of several hosts resulting in either increased or decreased disease severity relative to well-watered infected plants (Liu and Liu 2016; Sinha et al. 2016; Hossain et al. 2019; Sewelam et al. 2021; Irulappan et al. 2022; Hoheneder et al. 2023). It is possible that drought conditions have a direct effect on disease resistance by induction of stress responses, modulated defence gene expression and altered plant metabolite and phytohormone levels, which could coincide with enhanced pathogen defence and lower disease susceptibility. Furthermore, there is evidence that factors contributing towards drought tolerance are highly interconnected with resistance to fungal diseases, e.g. Fusarium crown rot (Su et al. 2021) or RLS (McGrann et al. 2014). This suggests that the outcome of combined abiotic and biotic stress strongly depends on timing, pathogen biology or strength and duration of abiotic stress (Choudhary and Senthil-Kumar 2022; Sinha et al. 2016).

Considering year effects on different disease parameters, we observed a comprehensive drought-dependent suppression of RLS disease in season 2018/2019. This was observed for all determined parameters and over the entire genotype

assortment, while such a decline was only detectable for single disease parameters in other seasons (Fig. 1b, d, f). This demonstrates that environmental factors differentially occurring in each field season (table S-2) strongly influenced RLS severity in winter barley even in the semi-controlled rainout shelter environment. According to recorded weather data, relatively warm and dry weather conditions in 2018/2019 might have provoked lowest RLS severity under irrigation and probably additively functioned in suppressing RLS disease under permanent late-terminal drought (Fig. 1; table S-2). Hence, higher temperatures late in the season might have also suppressed RLS more consistently in spring barley under persistent drought stress (Hoheneder et al. 2021). Noteworthy, such a decline in RLS severity on winter barley was not similarly consistent in the seasons 2019/2020 and 2020/21, when conditions were slightly cooler and moister (table S-2). Such variable weather effects indicate high sensitivity of *R. collo-cygni* to environmental conditions which could result in both altered promotion and suppression of the disease. Permanently unfavourable conditions (e.g. long-lasting drought) might result in low disease severity. However, single rain events could quickly change conditions, which would be sufficient for significant disease outbreaks.

Analysis of variance revealed significant effects of the environment on RLS symptoms and leaf area carrying conidiophores, indicating strong differences in field conditions between irrigated and drought stressed plots. However, fungal DNA contents were neither significantly affected by the genotype nor the environment (Table 2). Using data of disease parameters of our previous study on spring barley (Hoheneder et al. 2021) for an analysis of variance, the drought *versus* irrigated environment had significant effects on all assessed RLS disease parameters including fungal DNA contents. Interestingly, leaf area carrying conidiophores was further influenced by the genotype and genotype x environment interaction (table S-4). This reflects the strong suppressive effect of drought on production of conidiophores and a high differentiation of this parameter between spring barley genotypes under irrigation. Together, our studies suggest that RLS pathogenesis is highly dependent on weather conditions, which complicates genotype selection for breeding. This challenge seems to be even stronger in winter barley, where we observed less strong differentiation between cultivars and higher fungal DNA loads, when compared to spring barley (Hoheneder et al. 2021).

In view of field conditions occurring during growth of winter barley, our data suggest that seasonal year effects strongly influence RLS disease parameters. Interestingly, the differences between the most susceptible and the most resistant genotypes were stronger in irrigated spring than in winter barley over all disease parameters. So far, data of both studies suggest that the suppression of the long-lasting drought conditions on RLS severity had been more consistent in

spring barley (Fig. 1; (Hoheneder et al. 2021)). Under conditions in Germany, spring and winter barley flower with a time offset of about 3 weeks (spring barley: end of May; winter barley mid-May; Fig. S-4), where rapid disease outbreaks are usually observed in each barley type. Potentially, later in summer, warm and sunny weather conditions more strongly support potential long-lasting drought periods in spring barley. However, directly comparing disease resistance of spring and winter barley is difficult, when crops have not been grown in parallel under similar growth conditions (Dreiseitl 2011). This could be tested in future field experiments growing vernalized winter and spring barley together ruling out differences in growth conditions. This would help better understanding whether general characteristics of spring or winter types interact differently with epidemiology of RLS.

Understanding basal disease resistance as a function of the interaction between environmental factors with host plant physiology is crucial for the selection of genotypes that show resistance to multiple stresses. Drought stress greatly changes plant hormone homeostasis, photosynthesis and resource allocation (Munns and Millar 2023), which could affect the host's ability to express its genotype-dependent resistance potential. For single genotypes under drought, we documented a shift in their resistance to fungal infection in terms of fungal DNA concentrations in leaf tissues. This may indicate that harsh environmental condition influence the genotype-dependent ability of barley cultivars to restrict development of *R. collo-cygni*. However, this was not always associated with increased RLS symptoms that more generally were reduced under drought. Stress-induced damage of leaf tissue probably makes secretion of cell wall degrading enzymes, rubellins and other secondary metabolites, relevant for destruction of host tissue and associated with RLS symptom development (Dussart et al. 2018; Sjökvist et al. 2019), more dispensable for the fungus, and hence, it might have partially profited and more easily spread within the host under drought. However, under the applied drought conditions, accelerated leaf senescence followed by loss of green leaf area probably suppressed leaf symptoms. Noteworthy, we could not find relations between growth stage and assessed disease parameter (table S-3), despite drought conditions lead to minor differences in growth stages of individual genotypes growing under the two field environments. Conditions without rainfalls, increased canopy temperature, lower air humidity, less leaf moisture and less formation of dew during the night are clearly suppressive for *R. collo-cygni* progression in the field (Formayer et al. 2002; Mařík et al. 2011; Brown et al. 2014; Havis et al. 2015; Hoheneder et al. 2021) and further affect ripening processes and senescence. During the process of leaf senescence, remobilization of nutrients towards reproductive spike tissue and formation of reactive oxygen species (Munné-Bosch and Alegre

2004) probably lead to an abscission of resources and external stress for the fungus, which might provoke conidiophore formation for its own reproduction and long distance spreading. Strong and visible RLS disease outbreaks usually occurs post-flowering (Havis et al. 2015). We speculate that conditions between spike emergence and full grain maturity had strongest effects on endophytic fungal growth and disease severity. Under the applied drought conditions, plant ripening, leaf ageing and further senescence probably occurred more rapidly. Comparing weather data during post-flowering phases of spring and winter barley, mean temperature could be a significant environmental factor inducing heat stress of the host with further impacts on plant ontogenesis, hormone homeostasis and RLS disease progression (Röhrig and Dussart 2022). In this context, further studies on physiological interactions between the host and the fungus during different developmental stages and environmental stress conditions could point out distinct responses and key regulators of the barley—*R. collo-cygni* pathosystem, which is strongly modulated by abiotic factors (Schützendübel et al. 2008).

## Conclusions

Ramularia leaf spot became a major disease in many barley growing areas. There is increasing evidence, that environmental factors and their effects on plant physiology influence RLS outbreaks and severity. The present study on winter barley revealed a moderate differentiation in RLS severity between genotypes. Data show that permanent drought conditions partially suppress RLS disease in winter barley and with high phenotypic complexity over three seasons. This might be determined by epidemiology of RLS, which appears to be affected by variations in season-dependent environmental conditions that are not controlled in a field rainout shelter. The potential connection between disease severity and plant physiology needs more focus in research to better understand resistance and tolerance of barley to RLS in complex environments and to support breeding success. Nevertheless, we identified a few winter barley genotypes with a reproducible RLS resistance and stable yield under controlled drought and irrigated conditions in the field. Hence, those genotypes might be worth considering as parents for future breeding programmes and for recommendation to farmers for field cultivation.

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## Declarations

**Conflict of interest** The authors declare that they have no conflict of interest. Ralph Hückelhoven is an associate editor of the Journal of Plant Diseases and Protection, but was not involved in the editorial processing and decisions for this paper.

**Ethical approval** This article does not contain any studies with human or animal subjects.

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