




Ergot infection in winter rye hybrids shows differential contribution of male and female genotypes and environment

Anna Kodisch · Peer Wilde · Brigitta Schmiedchen · Franz-Joachim Fromme ·
Bernd Rodemann · Anna Tratwal · Michael Oberforster · Franz Wieser ·
Andrea Schiemann · Lise Nistrup Jørgensen · Thomas Miedaner 

Received: 18 November 2019 / Accepted: 14 March 2020 / Published online: 1 April 2020
© The Author(s) 2020

Abstract Contamination of ergot (*Claviceps purpurea*) in grains continues to be a problem in outcrossing plants like rye, especially in years of favorable infection (cold, rainy) conditions. The problem is not the yield loss, but the contamination of the grains by toxic alkaloids leading to strict critical values within the European Union. This study was conducted to (1) partition the variation of genotype, inoculation treatments and environment for ergot infection of 12 winter rye genotypes, (2) the effect

of varying proportions of a non-adapted restorer gene on ergot, and to (3) reveal within the genotype the relative importance of male pollen fertility and female receptivity on the ergot reaction of single crosses bearing different restorer genes. In total, 12 rye genotypes and two factorial crossing designs with each of five female and four male lines differing in their restorer genes were tested by artificial infection in up to 16 environments in four European countries. High and significant genotypic variation regarding the ergot severity and pollen-fertility restoration were observed. Furthermore significant general combining ability and specific combining ability variances and interactions with environment were obtained. The

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s10681-020-02600-2>) contains supplementary material, which is available to authorized users.

A. Kodisch · T. Miedaner (✉)
State Plant Breeding Institute, University of Hohenheim,
Fruwirthstraße 21, 70599 Stuttgart, Germany
e-mail: miedaner@uni-hohenheim.de

P. Wilde · B. Schmiedchen
KWS LOCHOW GMBH, 29303 Bergen, Germany

F.-J. Fromme
HYBRO Saatzucht GmbH & Co. KG, Kleptow Nr. 53,
17291 Schenkenberg, Germany

B. Rodemann
Federal Research Centre for Cultivated Plants, Institute
for Plant Protection in Field Crops and Grassland, Julius
Kühn-Institute, Messeweg 11/12, 38104 Brunswick,
Germany

A. Tratwal
Institute of Plant Protection – National Research Institute,
Władysława Węgorka 20, 60-318 Poznan, Poland

M. Oberforster
Institute for Sustainable Plant Production, Austrian
Agency for Health and Food Safety (AGES),
Spargelfeldstr. 191, 1220 Vienna, Austria

F. Wieser
Saatzucht LFS Edelhof, Edelhof 1, 3910 Zwettl, Austria

A. Schiemann
Agrotec, Gregersensvej 1, 2630 Taastrup, Denmark

L. N. Jørgensen
Department of Agroecology-Crop Health, Aarhus
University, Forsøgsvej 1, 4200 Slagelse, Denmark

pollen-fertility restoration of the male had by far the highest importance for ergot severity, the female component, however, also revealed a significant effect. In conclusion, selecting for superior restoration ability is the most promising way on the short term, but there are also possibilities to improve the maternal site in future breeding programs.

Keywords *Claviceps purpurea* · Ergot · Inoculation treatments · Pollen restoration · *Secale cereale* · Winter rye

Introduction

Ergot infections caused by *Claviceps purpurea* [Fr.] Tul. have been a nightmare for centuries. The fungus infects only the ovary at or shortly after flowering. Once an ovary is infected, the fungus forms a dark, compact sclerotium where a grain would normally develop (Pažoutová 2002). Typically, the sclerotia are up to 5 cm long and extend out from the glumes. The greatest threat from ergot is not the yield reduction, but the contamination of the grains by alkaloids present in the sclerotia and being toxic to humans and mammals (Hulvová et al. 2013). The fungus is distributed worldwide and has a large host range infecting hundreds of grasses (Wegulo and Carlson 2011). *Claviceps purpurea* is mainly a problem in rye and many cross-pollinating grasses, because the fungus cannot grow through intact glumes, but must be transported to the pistil by wind-borne aerosols or insects. The ergot fungus mimics the pollination of a cereal floret for fertilization (Kirchhoff 1929; Tudzynski et al. 1995; Mielke 2000). After penetration of the pistil, the fungus grows down the style, the ovary wall is completely colonized after some days, but the hyphae do not spread further into the tissue. Generally, resistance to *Claviceps purpurea* develops a few days after fertilization (Tudzynski et al. 1995). Due to this highly specific host–pathogen interaction the availability of high amounts of pollen reduces ergot infection considerably (Miedaner and Geiger 2015). Caused by severe pathological syndromes (Van Dongen and de Groot 1995), the amount of ergot sclerotia and sclerotial fragments in unprocessed cereals in the European Union is restricted to 0.05% by wt. for human consumption (European Union 2015) and to

0.1% by wt. for animal feed (European Communities 2002). Even stricter regulations and maximum levels based on the total alkaloid content are expected to be enacted within the EU.

Winter rye in Europe was grown on about 4.5 million hectares in 2017, mainly in the Russian Federation, Poland, Germany, Belarus, Ukraine, Spain, and Finno-Scandinavia (FAO 2019). Use of rye for dark breads manufactured with sour dough and having high moisture with long shelf life is typical for these countries (except Spain). Alternatively, rye and wheat flours are mixed in different proportions to produce a bread with a lighter texture, color and flavor (Miedaner 2013; Deutsches Brotinstitut e.V. 2019). Today, ergot sclerotia can be retrieved from rye lots to a large extent by grain cleaning machines based on photocells, but this is not standard in all countries and milling companies and it increases the costs and considerably slows down the processing (Miedaner and Geiger 2015). Baking of wheat and rye flour, respectively, reduced ergot alkaloids in bread in differing amounts (Meleard 2016; Bryła et al. 2019), however, no consistent loss was observed during the production of durum pasta (Tittlemier et al. 2019). A recent study in Italy of Debegnach et al. (2019) pointed out that 85% of the wheat- and rye-derived products were contaminated with at least one ergot alkaloid and observed the highest total alkaloid contents in rye and wheat bread. Further, ergot alkaloids were found in healthy grain that developed above and below infected flowers in wheat and barley and for a lesser extent in rye (Gordon et al. 2019). In this context, ergot alkaloids could also be detected in ergot-cleaned grain samples caused by abrasion (Beuerle et al. 2012; Byrd and Slaiding 2017; MacDonald and Anderson 2017). If the farmer delivers rye within the European Union with a higher percentage of ergot than the maximum allowed levels, the harvest is downgraded or in more severe cases rejected outright. Ergot-free rye grain, therefore, has a high economic impact.

Infection of rye by *Claviceps purpurea* is affected by genotypic diversity of the host and the pathogen, environmental variation and their interactions (Miedaner and Geiger 2015). Rye as the major host of *Claviceps* comes in two types of cultivars: open-pollinated cultivars and hybrids. Open-pollinated cultivars are produced by random mating after selection of self-incompatible families. The resulting

cultivars are fully pollen shedding. Commercial hybrids in rye constitute a cross between a single-cross seed parent and a two-line synthetic as a pollen parent ($A \bullet B \times \text{Syn } C, D$; Geiger and Miedaner 2009). The crosses are produced based on cytoplasmic-male sterility (CMS) induced by the Pampa (P) cytoplasm and afford the presence of a restorer gene contributed by the pollinator to result in full pollen shedding in the progenies and, thus, the commercial rye stand. While the European restorer sources for the P cytoplasm are only partial restorers and prone to environmental changes and the seed parents, non-adapted, monogenically inherited restorer genes from Iranian primitive rye and Argentinean landraces provide nearly full restoration (Miedaner et al. 2005). The latter showed, however, a considerable yield penalty even in higher backcross generations (BC_4 to BC_5 , Miedaner et al. 2017). Additionally, the amount of pollen available from hybrid cultivars depends on the ease of restoration and/or physiological resistance mechanisms of the female parent. This genetic female-by-male interaction is called in plant breeding combining ability and divided into general (GCA) and specific (SCA) combining ability (Hallauer et al. 2010). In a diallel or factorial cross design, the genetic variation due to females and males and their interaction as well as the non-genetic variation can be partitioned. Low temperatures and high humidity around the time of meiosis, i.e., some days before pollination, negatively affects pollen production and viability. Wet weather at flowering delays pollination and, thus, greatly increases the period of susceptibility for ergot. Moreover, it promotes the proliferation of the pathogen in the rye stand and increases infection frequency (Miedaner and Geiger 2015). Furthermore, few quantitative trait loci (QTL) were identified recently conferring partial resistance to ergot affecting the size and weight of sclerotia in bread wheat (Gordon et al. 2015). In durum wheat, QTL were associated with different components of the infection process like the hormonal pathway (Gordon et al. 2020). Beside of that, the density of the rye stand (Betz and Mielke 1996), the homogeneity of the cultivar, and the duration of flowering (Thakur and Williams 1980) might play a role in ergot severity. This underlines the necessity to test over several locations and/or years.

The objective of this research was to assess (1) the partitioning of genotypic and environmental variation of 12 winter rye genotypes to ergot reaction, (2) the

effect of varying proportions of a non-adapted restorer gene on ergot, and (3) the relative importance of the fertility restoration ability of male parents (GCA male), the susceptibility of female parents (GCA female), and their interaction (SCA) on the ergot reaction of the hybrid. We expect a high importance of the pollen parent on ergot reaction, but the role of the female parent is unclear. It is also unclear, to which proportion the non-adapted restorer gene must be introgressed to reduce ergot considerably and whether there are specific female \times male interactions that could be used in practical breeding.

Materials and methods

The study consists of two experimental set-ups. The first experiment evaluated the diversity of genotypic and environmental variation of winter rye genotypes for ergot susceptibility for three different inoculation treatments of *Claviceps purpurea* (Fr.: Fr.) Tul. (untreated, German inoculum, country-specific inoculum). This experiment also included four single crosses with varying proportions of a highly effective, non-adapted restorer gene. The second experiment evaluated the relative importance for ergot susceptibility of female parents and the pollen-fertility restoration ability of male parents and their interactions.

Plant material

Experiment 1 (Exp. 1) was conducted with 12 genotypes of winter rye (*Secale cereale* L.) (Table S1). The respective breeder provided seeds of all varieties: eight genotypes by KWS LOCHOW GmbH (KWL, Bergen, Germany), three hybrids by HYBRO Saatzeit GmbH & Co. KG (HYB, Schenkenberg, Germany) and one cultivar by “DANKO” Hodowla Roslin Sp. z o.o. (Koscian, Poland). The KWL genotypes included four single crosses (SC) produced especially for this study by crossing a female non-restorer line with different pollinator lines yielding hybrids with 0, 25, 50 or 100% of the dominant, non-adapted restorer gene from IRAN IX. This is illustrated by the subscript in the name, i.e. SC_{100} is a single cross containing 100% of the plants the non-adapted restorer gene. The other

percentages were produced by mixing near-isogenic lines with/without the respective restorer gene in differing percentages. SC₀ represents a cross of two non-restorer lines, the outcome should be totally male sterile. The hybrid cultivars provided by HYB included 10% open-pollinated rye according to their commercial use.

In experiment 2 (Exp. 2), two sets (set A, set B) of each of 20 factorial crosses, produced by crossing five seed parents (CMS lines) and four pollinator lines were analyzed. The pollinators of each set consisted of two restorer lines, each restorer line with (plus) or without (minus) a non-adapted restorer gene, thus comprising four lines. The crosses in set A were provided by KWL and that in set B by HYB. KWL also provided the CMS single-cross, which was used as border between the plots of the field trials for both experiments.

Field trials

The field tests of Exp. 1 were conducted in 2013 at nine locations from four countries (Table S2) as a split-plot design with the inoculation treatments as the main plots and the genotypes as subplots, both randomized according to a complete randomized block design with two replicates. Exp. 2 was performed in a total of 16 environments (location × year combinations) for set A and eight environments for set B (details see Table S3) as two separate lattice designs that were grown adjacent to each other in two replications in 2014 and 2015. Entries were grown on large-drilled plots of 5.0 to 7.04 m² depending on the location. Each entry plot was surrounded by four plots of a CMS single cross as border in a chessboard-like design (Miedaner et al. 2010b). This design was used to avoid neighboring effects by airborne pollen, to minimize effects of drifting of ergot spores during inoculation, and to avoid direct contact with plants bearing honeydew from the neighboring plot. Sowing was done in the last 2 weeks of September or early in October. The kernel density amounted to 200 kernels/m². Each location applied mineral fertilizers, herbicides, growth regulators and fungicides in a conventional way.

Inoculation and resistance traits

All inocula were produced by the Julius Kühn-Institute, Institute for Plant Protection in Field Crops and Grassland (Braunschweig, Germany) as previously described in detail by Miedaner et al. (2010a). *Claviceps purpurea* was isolated from ergot samples from different locations of each country separately according to Kirchhoff (1929) and conidia for inoculation were produced on wheat-grain medium as described in Mielke (2000) and Engelke (2002). After establishing a starter culture until sporulation by growing them on potato dextrose agar (PDA) for 14 days (dark, room temperature), spores were rinsed from the plates by sterilized water. The spores from one plate were incubated in a 400 ml flask filled with liquid medium (autoclaved), that contain 1% oat meal (Engelke 2002). Incubated sub-cultures (10 days, room temperature, 100 rpm) were used for seeding the wheat-grain medium by soaking (overnight). After a first autoclave (20 Min, 121 °C) step, the samples were filled in a 2-litre polyethylene bag and autoclaved the second time. For incubation (18°C, dark, 4 weeks) the wheat-grain medium containing bags were mixed with the mycelium suspension produced in one flask. Until usage, this was stored at 4°C. For producing spore suspensions for inoculation, the colonized wheat was suspended in tap water (60 min) after removing the bags and the concentrations was adjusted to 3×10^6 spores/ml. To improve the adhesion of the spores onto the heads, some drops of Tween 20 were added to the spore suspension. A machine-driven field sprayer was used to inoculate all field trials in the evening (5:30–9 p.m.) or in the morning (8–10:30 a.m.) to ensure a high enough humidity. Spraying was done with a water volume of about 600 L/ha, starting when the earliest 30% of the plots were fully flowering (BBCH 65, Meier 2001). The inoculation procedure was repeated three to four times at intervals of one to four days to ensure that enough ergot inoculum is available during the whole flowering period in the light of possible variation in flowering among cultivars. In Exp. 1, three different inoculum treatments were used: untreated (without inoculation), German inoculum (isolates collected from sclerotia of infected rye in Germany) and country-specific inoculum (isolates collected from sclerotia of infected rye in the respective country).

Exp. 2 was inoculated with the German inoculum at all locations.

From each plot, 1 m² from the middle of the plot was harvested by hand at dough ripening stage (BBCH 85–89). To avoid the harvest of secondary or tertiary tillers, only the upper third of the rye plants were cut. All heads of one plot were air dried (30 °C) and threshed by a large single-head thresher (Pelz K 35, Saatzuchtbedarf Baumann, 74638 Waldenburg, Germany). After that, the total sample was weighed, all sclerotia fragments were sorted out by hand, weighed again and ergot severity (= % of sclerotia in grain by weight) was calculated as percentage of ergot relative to the grain sample and used as resistance trait. Furthermore, anther scoring (1–9), heading stage (1–9) (1 = very late, 9 = very early) and plant height (cm) were recorded at some locations (Tables S2 and S3). Anther scoring was done to estimate pollen fertility of the genotypes. According to Geiger and Morgenstern (1975), it was assessed visually in the field several times for each plot by scoring the anther characteristics (size, dehiscence) on a scale from 1 to 9. The classes of this scale ranged from male-sterile (score: 1–3), partially male-fertile (score: 4–6) and male-fertile (score: 7–9) plants, differing within the classes by increasing anther size. For a better interpretation, scores were transformed to restorer indices (RI, %). Scores 1–3 were set to RI = 0 and score ≥ 4 were calculated according to the following formula: $RI = 100 \times (\text{Score} - 3) / 6$. The index ranges from 0 (no pollen production) to 100% (fully male-fertile plants) (Geiger et al. 1995).

Statistical analyses

All analyses were based on single-plot data. A statistical outlier test was performed as described in Bernal-Vasquez et al. (2016) for PLABSTAT and detected outliers were handled in the following as missing values. Analyses of variance (ANOVA) were calculated for ergot severity (%) after a square-root transformation, because the residuals were not normally distributed in any environment. ANOVA was computed for each location separately and combined across locations for each trait using standard procedures (Cochran and Cox 1957). In Exp. 1, the effect of ‘genotype’ and ‘inoculation treatment’ was considered as fixed, and ‘replication’ and ‘environment’ as

random. In Exp. 2, the effect of ‘male’ and ‘female’ were considered as fixed, while ‘replication’ and ‘environment’ as random. Repeatability for each environment and entry-mean heritability (h^2) across all environments were estimated from ANOVA as the ratio of genotypic to phenotypic variance considering the number of replicates and environments, respectively (Fehr 1987). Outlier test and ANOVA were conducted with the computer program PLABSTAT (Utz 2011).

The Software R (R Core Team 2018) and R-Studio (Version 3.5.1) (RStudio Team 2016) were used to do graphic visualization, calculating the means and all comparisons of, e.g. type of cultivar and inoculum in Exp. 1, by using a pairwise *t test*. For this, back-transformed data were used. For multiple testing, the Tukey test as implemented in R-Studio was used.

Results

Effect of inoculation treatment and correlation to agronomic traits

In the three German and two Austrian locations, the inoculation treatment resulted on average in significantly higher ergot severity than the non-inoculated variant (Fig. 1). In Denmark, the latter variant had a similar mean than the inoculation with the German inoculum. The country-specific inoculum was in all countries significantly better than the alternative inoculum except in Poland, where all treatments had the same low level.

Eight commercial winter rye cultivars and four single crosses showed considerable differences in their mean ergot severity (%) after inoculation with the German inoculum ranging from 0.13 to 2.07% across environments (Table 1). SC₁₀₀, both under natural and artificial infection, was even better than the population cultivars (Conduct, Dańkowskie Diament). SC₅₀, Visello, and Brasetto were in the same range than the population cultivars after inoculation although their RI was lower. Similarly, restorer index was maximum for both population cultivars (> 80%) while this trait ranged for the hybrids and SC from about 33 to 72% except for SC₀ that represents a male-sterile genotype. Differences in heading stage were low, for plant height again large differences were found ranging from 111 to 151 cm. The 12 entries showed

Fig. 1 Mean ergot severity (%) of 12 winter rye genotypes for each of three inoculation treatments across one to three locations in four countries in 2013 (n = number of locations, Exp. 1). Treatments with the same letter are not significantly different within individual countries (Tukey test, $P < 0.05$)

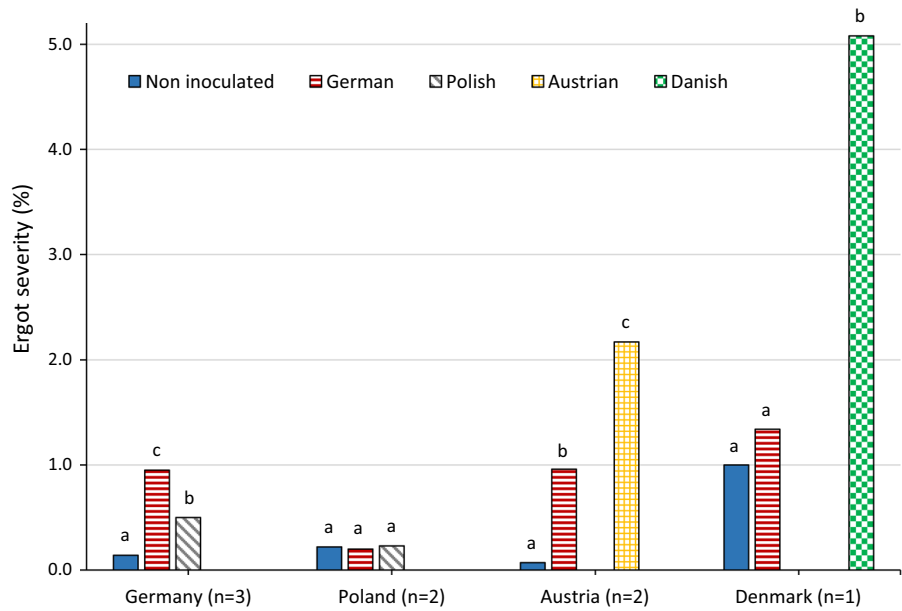


Table 1 Means of eight winter rye cultivars and four single crosses (SC) varying for their percentage of a non-adapted restorer gene (SC_{100} , SC_{50} , SC_{25} , SC_0) for ergot severity (%) after inoculation with German inoculum, restorer index (RI,

%), heading stage (HS, 1–9), and plant height (PH, cm) across eight (ergot severity) and seven (other traits) locations, respectively, and all inoculation treatments (Exp. 1)

Genotypes	Type of cultivar ^a	Ergot severity (%)	RI (%)	HS (1–9)	PH (cm)
SC_{100}	H	0.13	72.29	3.79	139.5
SC_{50}	H	0.40	57.98	4.02	133.6
Dańkowskie Diament	P	0.47	80.90	4.71	145.2
Conduct	P	0.57	85.26	4.69	150.45
Brasetto	H	0.50	45.98	4.33	130.52
Visello	H	0.63	55.98	4.64	131.8
SC_{25}	H	0.78	32.98	3.43	112.52
Palazzo	H	0.81	50.74	3.79	138.57
SU Satellit ^b	H	0.87	47.26	4.98	131.00
SU Allawi ^b	H	0.98	37.26	5.26	134.64
SU Stakkato ^b	H	1.53	39.71	4.83	129.29
SC_0	H	2.07	7.52	3.12	111.38
Mean		0.81	51.16	4.30	132.37
LSD _{5%} ^c		0.21	19.84	1.37	4.42
Heritability ^c		0.90			
CV _G % ^{c,d}		6.90**			

^aP: population cultivar, H: hybrid cultivar

^b10% population rye included

^cFrom square root transformed (sq transf.) data for ergot severity

^dCoefficient of variation of genotype

**Significant at $P < 0.01$

a similar ranking between the non-inoculated and inoculated treatment ($r = 0.94$, $P < 0.001$, Fig. 2a). Even when the male-sterile single cross (SC_0) was removed, the coefficient of correlation was still high ($r = 0.86$, $P < 0.001$). Ergot severity significantly correlated with restorer index ($r = -0.80$, $P < 0.001$) (Fig. 2b) and plant height ($r = -0.61$, $P < 0.05$), whereas no significant correlation was obtained between ergot severity and heading stage.

Effect of different proportions of a non-adapted restorer gene

Ergot severity clearly decreased with increasing proportions of the non-adapted restorer gene in the single cross, for both natural and artificial infection (Table 1). For artificial infection by German inoculum, an amount of the restorer gene of 25% (SC_{25}), 50% (SC_{50}) and 100% (SC_{100}) is followed by a reduction of ergot severity by 62%, 81%, and 94%, respectively, compared to the male-sterile SC_0 . For natural infection, the same proportions of the restorer gene reduced ergot severity by 60% (SC_{25}), 37% (SC_{50}), and 94% (SC_{100}), respectively, compared to SC_0 (data not shown for brevity).

Partitioning of genotypic and environmental variances for ergot infection of 12 genotypes

For ergot severity, the estimates of the variance components were significantly different from zero for all main factors and their interactions (Table 2). The environmental variance was the most important source of variation followed by the genotypic and genotype \times environment interaction variances. As expected, the inoculum treatment variance was significantly different from zero ($P < 0.05$) only for ergot severity. Genotype \times inoculation treatment variance was negligible although significant, while environment \times inoculation treatment variance had the second highest variance component. Entry-mean heritability was 0.88. The other traits also showed important genotypic and genotype \times environment interaction variances except heading stage. Accordingly, entry-mean heritability for this trait was much lower than for restorer index and plant height that reached values > 0.9 .

Relative importance of male and female components on the ergot reaction of single crosses

The restorer lines R2 and R3 showed significantly ($P < 0.01$) different ergot severities and restorer indices for their plus and minus variants (Tables 3, 4). In contrast, for ergot severity and restorer index, no

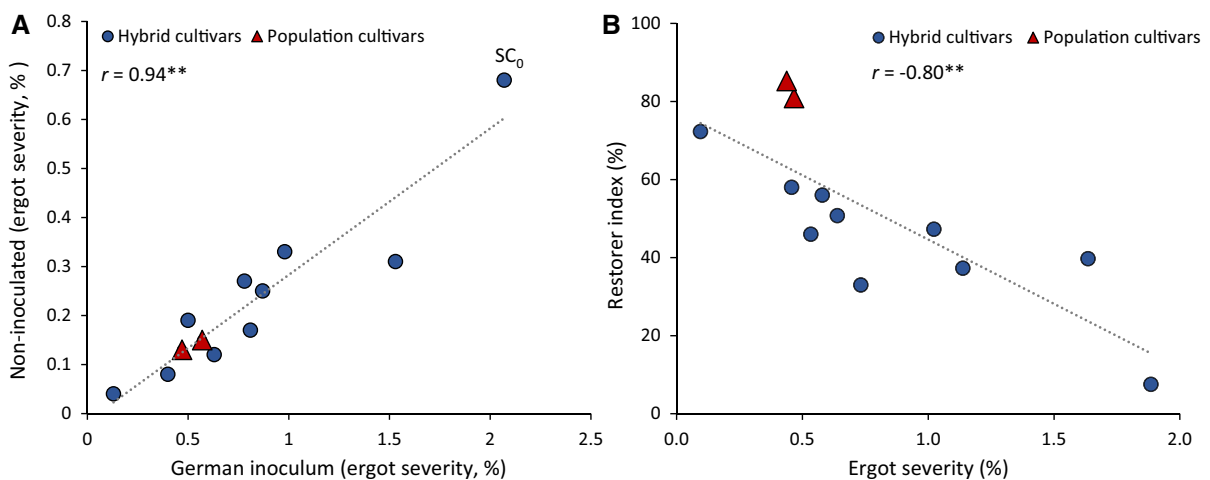


Fig. 2 Correlation between, **a** ergot severity of non-inoculated and German inoculum across eight locations and, **b** ergot severity and restorer index across seven locations and mean of

inoculation treatments for 12 winter rye genotypes (Exp. 1) (**significant at $P < 0.01$)

Table 2 Estimates of variance components, and entry-mean heritabilities for ergot severity (sq transf. = square root transformed), restorer index (RI), heading stage (HS), and plant

height (PH) of 12 winter rye genotypes for three levels of inoculation treatment (untreated, German inoculum, country-specific inoculum) across environments (Exp. 1)

Parameter	Ergot severity (%) (sq transf.)	RI (%)	HS (1–9)	PH (cm)
<i>No. of environments</i>	8	7	7	7
Variance components:				
Environment (E)	0.111**	498.53**	1.647**	140.35**
Inoculation treatment (I)	0.053*	– ^a	0.011	0.56
Genotype (G)	0.045**	420.16**	0.207	128.11**
G × E	0.045**	322.23**	1.577**	14.04**
G × I	0.009**	2.07	– ^a	0.77
E × I	0.102**	3.33	0.002	– ^a
G × E × I	0.024**	– ^a	0.022	– ^a
Error	0.036	140.07	0.443	18.89
Heritability	0.88	0.90	0.47	0.98

*, **Significant at $P < 0.05$ and $P < 0.01$, respectively^aNegative estimate**Table 3** Means of (1) ergot severity (%) across eight environments (location × year combinations) and (2) restorer index (%) across two environments for the combination of five female CMS lines and two male restorer lines with (Plus) and without (Minus) a non-adapted restorer gene after inoculation by *Claviceps purpurea* in set A (Exp. 2)

	Female line		Restorer line			
	R1 Plus	R1 Minus	R2 Plus	R2 Minus	Mean	Sign. ^a
(1) Ergot severity (%)						
CMS-1	0.51	0.56	0.31	1.16	0.63	a
CMS-2	0.78	0.58	0.34	0.83	0.63	a
CMS-3	0.71	0.65	0.51	1.28	0.79	b
CMS-4	0.48	0.53	0.38	0.53	0.48	c
CMS-5	0.46	0.44	0.31	0.54	0.44	c
Mean	0.59	0.55	0.37	0.87	0.59	
Sign. ^a	a	a	a	b		
(2) Restorer index (%)						
CMS-1	65.6	62.5	89.6	41.7	64.8	a
CMS-2	66.7	75	82.3	51.0	68.8	ab
CMS-3	61.5	65.6	86.5	54.2	66.9	a
CMS-4	76.0	86.5	87.5	67.7	79.4	c
CMS-5	77.1	67.7	86.5	60.4	72.9	b
Mean	69.4	71.5	86.5	55	70.6	
Sign. ^a	a	a	b	c		

^aTreatments with the same letter are not significantly different (Tukey test, $P < 0.05$)

differences were observed between plus and minus variants of lines R1 and R4. The female CMS lines also had significant differences for their ergot severity and restorer index in both sets when averaged across the restorer lines. The average infection level of genotypes from set B was much higher than of genotypes from set A (1.44 vs. 0.47% ergot severity)

when calculated across the orthogonal set of eight common environments (data not shown for brevity).

Close negative correlations were obtained between ergot severity and restorer index across locations for both, set A and B (Fig. 3).

Ergot severity showed significant variance components for all sources of variation in set A and B

Table 4 Means of (1) ergot severity (%) across eight environments (location × year combinations) and (2) restorer index (%) across two environments for the combination of five female CMS lines and two male restorer lines with (Plus) and without (Minus) a non-adapted restorer gene after inoculation by *Claviceps purpurea* in set B (Exp. 2)

Female line	Restorer line				Mean	Sign. ^a
	R3 Plus	R3 Minus	R4 Plus	R4 Minus		
(1) Ergot severity (%)						
CMS-10	0.37	2.29	0.49	0.49	0.91	a
CMS-11	0.51	3.50	1.03	1.42	1.62	c
CMS-12	0.42	4.32	3.20	2.76	2.68	d
CMS-14	0.24	1.56	1.81	1.62	1.31	bc
CMS-15	0.40	2.84	0.56	0.81	1.15	ab
Mean	0.39	2.90	1.42	1.42	1.53	
Sign. ^a	a	c	b	b		
(2) Restorer index (%)						
CMS-10	91.7	16.7	70.9	54.2	58.3	a
CMS-11	87.5	0	58.3	41.7	46.9	b
CMS-12	66.7	0	12.5	16.7	24.0	c
CMS-14	87.5	20.8	20.8	33.3	40.6	b
CMS-15	83.3	25	50	70.8	57.3	a
Mean	83.3	12.5	42.5	43.3	45.4	
Sign. ^a	a	c	b	b		

^aTreatments with the same letter are not significantly different (Tukey test, $P < 0.05$)

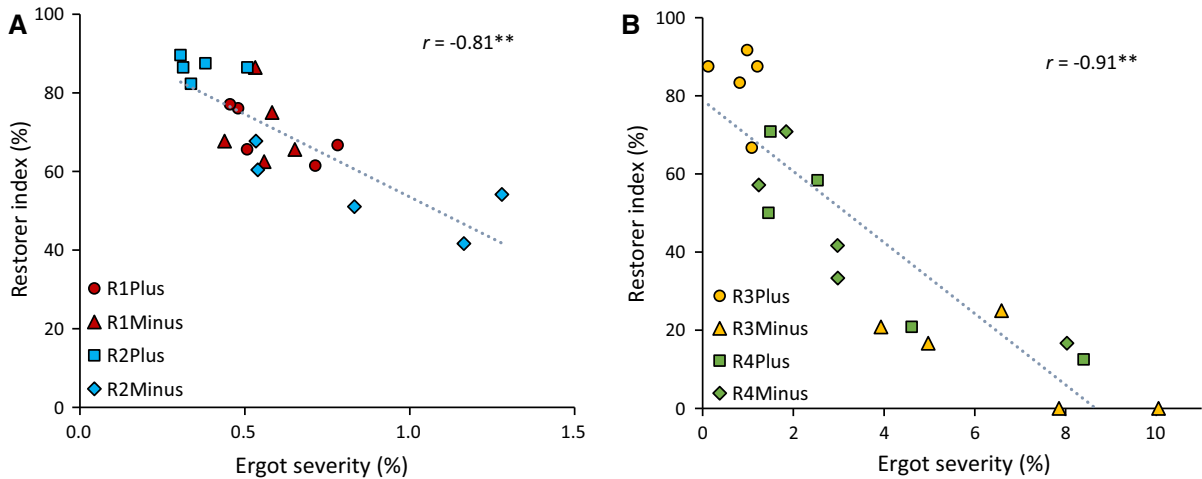


Fig. 3 Correlation between ergot severity and restorer index for two male restorer (Rx) lines with (Plus) and without (Minus) a non-adapted restorer gene and 5 female CMS lines for, **a** set A (8

environments) and, **b** set B (2 environments) after inoculation by *Claviceps purpurea* (r = coefficient of phenotypic correlation, **Significant at $P < 0.01$) (Exp. 2)

(Table 5). Clearly the GCA male variance and its interactions were more important than the GCA female variance in both, set A and B for ergot severity and restorer index. Estimates of variance components of ergot severity for the GCA male variance were about two and five times higher than for the GCA female variance in set A and B, respectively. For restorer indices, the differences were even larger. Also, the SCA interaction variance was significantly

different from zero for both traits in both sets. In set B, this source of variation was even more important than the GCA female variance. High heritabilities were found for both traits in set A and B (Table 5).

The GCA male and GCA female variances of all recorded traits (HS, PH) for both sets across locations were observed as significant, except the GCA male variance for heading stage in set A. SCA variances

Table 5 Estimates of variance components for general (GCA) and specific combining ability (SCA) and entry-mean heritabilities for set A and B, each of five female CMS lines and

four male restorer lines with (Plus) and without (Minus) a non-adapted restorer gene for ergot severity (sq transf. = square root transformed) and restorer index across environments

Parameter	Set A		Set B	
	Ergot severity (%) (sq transf. ($\times 10^{-2}$))	RI (%)	Ergot severity (%) (sq transf. ($\times 10^{-2}$))	RI (%)
<i>No. of environments</i>	16	8	8	2
Variance components:				
GCA males (M)	1.05**	156.61**	12.21**	821.99**
GCA females (F)	0.59**	24.35*	2.40*	161.24
SCA (M x F)	0.38**	13.23	3.41**	112.56*
M x Environment (E)	1.16**	53.02**	7.50**	34.74*
F x E	0.70**	47.21**	5.50**	60.92**
SCA x E	0.21	84.72**	3.48**	43.46
Error	1.77	196.90	5.77	106.36
Heritability	0.90	0.84	0.86	0.98

*, **Significant at $P < 0.05$ and $P < 0.01$, respectively

were not significant for heading stage and plant height in both sets (data not shown for brevity).

Discussion

Ergot (*Claviceps purpurea*) still causes problems in outcrossing rye caused by the contamination of harvested grain with toxic alkaloids. After inoculation, mean ergot severity was considerably higher than of naturally infected plants (Table 1). Inoculation is necessary for optimal differentiation in most environments as already shown in a previous experiment (Miedaner et al. 2010a). Also in our experiment 1, inoculation had a higher genetic coefficient of variation than natural infection (6.90 vs. 4.44). Despite this, differentiation among genotypes was also obtained in natural infection, most probably caused by the unusually high natural infection level in 2013 in Germany (BMEL 2013; Schwake-Anduschus 2018), Austria, and Denmark. A possible explanation of the low infection rate in Poland in all treatments might be the warm and dry weather that hinders ergot infections (Miedaner and Geiger 2015). The country-specific inoculum was more aggressive in all countries except Poland than the commonly used German inoculum. An assertion about the aggressiveness of a country-specific inoculum in another country cannot be made,

because the number of countries is too low and the inocula should also tested vice versa, e.g. the Austrian inoculum in Denmark. It would be informative to see in the future experiments, whether the aggressiveness is related to the collection site of a specific country. Menzies et al. (2017) had already shown the high pathogenic variation in wheat and also described significant differences among isolates according to geographic origin. Another interesting point is that the mean infection level varies very strongly between the countries, both under natural infection and artificial inoculations.

The high heritabilities across environments for ergot severity and RI revealed, that the multi-locational field testing system, as described in detail by Miedaner et al. (2010b) was appropriate for this study and resulted in a good differentiation among genotypes (Table 1). The genotypic coefficient of variation of inoculation variance was 1.5 fold higher than the non-inoculated variance illustrating the better differentiation. The two Open pollinated cultivars displayed full pollen fertility and were, therefore, less susceptible to ergot (Miedaner et al. 2010b, Fig. 2b). SC₁₀₀ was the genotype with the lowest ergot susceptibility, both untreated and under inoculation conditions. This illustrates that the introgression of a non-adapted restorer gene with a higher and environmentally stable restoration ability can boost pollen fertility

and consequently reduce the ergot reaction. SC_{50} did not significantly deviate from both population cultivars in ergot severity although its RI was significantly lower. Klotz (2002) already showed that, similar to our results, an introgression of already 25% of the IRAN IX gene reduced ergot severity by 70%. It should be noted, however, that hybrids with a maximum percentage of a non-adapted restorer gene suffer from a significant grain yield reduction in earlier backcross stages (Miedaner et al. 2017).

The variance components of genotype, inoculum, environment and their interactions were all significant, the environment was the most important factor for all traits. For ergot severity, the estimate of genotype \times environment interaction was of the same magnitude as the genotype illustrating that a higher number of environments is necessary to reliably test ergot incidence. Restorer index was used to estimate the pollen fertility of the genotypes, because plants with reduced pollen shedding are generally more susceptible to ergot (Miedaner and Geiger 2015). This negative correlation of the amount of pollen and ergot severity was also found in this study for both experiments (Figs. 2b, 3). Blending 5–10% population rye is often used to compensate a lower pollen shedding of commercial hybrid cultivars (Engelke 2002; Miedaner et al. 2005), but in this study, blending did not work as expected, because these supplemented hybrids (SU Stakkato, SU Satellit, SU Allawi) were still among the worst commercial hybrids (Miedaner et al. 2010b). SC_0 was designed to be male sterile and showed consequently the highest ergot severity. A highly significant negative correlation between ergot severity and plant height for natural infection as well as for artificial inoculation were observed in our study. Similarly, Gordon et al. (2015) found co-locating QTL for partial ergot resistance and plant height in wheat, however, being restricted to semi-dwarfing alleles at the *Rht* (reduced height) loci (Gordon et al. 2015).

In our factorial crosses (Exp. 2) the pollinators were derived from the pollen parent pool and possessed already restorer genes of European origin (Minus) in addition to the non-adapted restorer gene (Plus). The former were, however, obviously not good enough to fully restore R2 minus or R3 minus as shown in Tables 3 and 4. Indeed, the single crosses of these minus pollinator lines had significantly more ergot than those of the plus variant. In R4, the introgression of the non-adapted restorer gene did not properly

work, as shown by the fact that R4 plus and R4 minus had the same moderate amount of restoration and also their ergot severity was not significantly different. Both variants of R1 showed the same high amount of restoration, obviously R1 minus already possessed effective restorer genes. Despite this, narrow negative correlations existed between ergot severity and restorer index (Fig. 3).

In addition, the female CMS lines showed significant differences in ergot severity in both sets. This was partially due to an easier restoration, such as for CMS-4, CMS-10, and CMS-15 that display a higher amount of pollen across all male lines and in consequence a lower ergot severity. In contrast, CMS-12 was extremely hard to restore showing the highest ergot severity in the whole experiment. On the other hand, CMS-1 and CMS-3 and CMS-2 and CMS-5 had a significantly different ergot severity with a similar restoration ability. This could be explained by the physiological characteristics of the ovary or pistil (Miedaner and Geiger 2015) or as for CMS-2 and CMS-5 by different heading stages (HS: 5.58 vs. 4.79) that might lead to a “disease escape” mechanism (Willingale et al. 1986).

Partitioning of the genetic variance for ergot severity and RI showed that the GCA male variances were clearly of highest importance in both sets. Indeed, the GCA of males amounted to 52% (set A) and 68% (set B) of the total genetic variance. However, the GCA variances of the females and the SCA variances were also significant, each contributing between 13 and 29%. The interaction with environments played a tremendous role for all factors. This might be influenced by the high ecological range of locations from Denmark to Austria and from Poland to Southwestern Germany. However, the same hybrid cultivar might be registered in these countries, therefore, the breeder has to consider the whole rye growing area for ergot tests.

Because no fungicides are registered for ergot control and sorting out the sclerotia by color sorting machinery is expensive and time consuming (Engelke 2002; Miedaner and Geiger 2015), breeding strategies that ensure a high pollen fertility, especially when weather conditions are favorable for infection, are still the best way to reduce ergot. Therefore, effective restorer genes ensuring a high pollen ability, play an important role to guarantee high quality end products without ergot contamination. This study, however,

shows that there is also a potential for improvement of ergot susceptibility on the maternal side, which could be further exploited. When all hybrids are once well equipped with non-adapted restorer genes and show a high restorer index of $> 70\%$, this might be a chance to additionally reduce ergot susceptibility. A female effect, that could not be followed here, might be a physiological resistance to ergot infection on a quantitative basis. This was previously shown by achieving significant differences for ergot severity among fully male-sterile entries in isolated plots, *i.e.* without any availability of pollen in rye (Miedaner et al. 2010a) and in pearl millet (Willingale et al. 1986). In conclusion, this study illustrates that some parents are more suitable to reduce ergot susceptibility of cultivars and further improvements of ergot resistance by selecting a higher restoration ability is still possible. For reducing ergot, improving the pollen amount is still the most promising way. Although some new hybrids have similar low susceptibility to ergot than population cultivars (BSL 2019), rye as a crop is still more prone to ergot infection than the self-pollinating wheat. To proceed further, (1) maternal effects, although of lower importance, should be exploited for lower ergot incidence, (2) SCA effects could be exploited in practical breeding.

Acknowledgements Open Access funding provided by Projekt DEAL. The authors thank the teams at the respective locations for their excellent technical work. This study was partially financed by KWS LOCHOW GMBH, Bergen, Germany.

Funding Funding was provided by Federal Ministry for Economic Affairs and Energy (Grant No. 188EN/1).

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>.

References

- Bernal-Vasquez AM, Utz HF, Piepho HP (2016) Outlier detection methods for generalized lattices: a case study on the transition from ANOVA to REML. *Theor Appl Genet* 129:787–804. <https://doi.org/10.1007/s00122-016-2666-6>
- Betz HG, Mielke H (1996) Möglichkeiten zur Bekämpfung des Mutterkorns. *Die Mühle + Mischfüttertechnik* 44:726–728
- Beuerle T, Benford D, Brimer L, Cottrill B, Doerge D, Dusemund B, Farmer P, Fürst P, Humpf H, Mulder PPJ (2012) EFSA Panel on Contaminants in the Food Chain (CONTAM). Scientific opinion on Ergot alkaloids in food and feed. *EFSA J* 10:2798–2956
- BMEL (Bundesministerium für Ernährung und Landwirtschaft) (2013) Ernte 2013: Mengen und Preise. https://www.bmel.de/SharedDocs/Downloads/Landwirtschaft/Markt-Statistik/Ernte2013_Bericht+Anlagen.pdf?blob=publicationFile. Accessed 24 Sept 2019
- Bryła M, Ksieniewicz-Woźniak E, Waśkiewicz A, Podolska G, Szymczyk K (2019) Stability of ergot alkaloids during the process of baking rye bread. *LWT* 110:269–274. <https://doi.org/10.1016/j.lwt.2019.04.065>
- BSL (2019) Beschreibende Sortenliste für Getreide, Mais, Öl- und Faserpflanzen, Leguminosen, Rüben, Zwischenfrüchte [Descriptive variety list for cereals, maize, oil and fibre plants, pulse crops, beets, catch crops]. Bundessortenamt, Hannover
- Byrd N, Slaiding IR (2017) Final Project Report: Monitoring of mycotoxins and other contaminants in UK cereals used in malting, milling & animal feed. AHDB PR578. <https://ahdb.org.uk/final-project-report-contaminants-monitoring-150517>. Accessed 18 Feb 2020
- Cochran WG, Cox GM (1957) *Experimental designs*. Wiley, New York
- Communities EU (2002) Directive 2002/32/EC of the European Parliament and of the Council of 7 May 2002 on undesirable substances in animal feed. *Off J Eur Communities* 2002(L140):10–22
- Debegnach F, Patriarca S, Brera C, Gregori E, Sonogo E, Moracci G, De Santis B (2019) Ergot alkaloids in wheat and rye derived products in Italy. *Foods* 8:150. <https://doi.org/10.3390/foods8050150>
- Deutsches Brotinstitut e.V., Berlin: Historische Informationen. <https://www.brotinstitut.de/brotkultur/historische-informationen/>. Accessed 2 July 2019
- Engelke T (2002) Ansätze für eine integrierte Bekämpfung des Mutterkorns (*Claviceps purpurea* [Fr.] Tul.) im Roggen. Dissertation, University of Göttingen
- European Union (2015) Commission Regulation (EU) 2015/1940 of 28 October 2015 amending Regulation (EC) No 1881/2006 as regards maximum levels of ergot sclerotia in certain unprocessed cereals and the provisions on monitoring and reporting. *Off J Eur Union* 2015, L283/3
- FAO (2019) Food and Agriculture Organization of the United Nations. <https://www.fao.org/faostat/en/#data/QC>. Accessed 1 July 2019
- Fehr WR (1987) *Principles of cultivar development, theory and technique*, vol 1. Macmillan, New York

- Geiger HH, Miedaner T (2009) Rye breeding. In: Carena MJ (ed) Cereals (handbook of plant breeding). Springer, New York, pp 157–181
- Geiger HH, Morgenstern K (1975) Applied genetic studies on cytoplasmic pollen sterility in winter rye [Angewandt-genetische Studien zur cytoplasmatischen Pollensterilität bei Winterroggen]. *Theor Appl Genet* 46:269–276. <https://doi.org/10.1007/BF00281148>
- Geiger HH, Yuan Y, Miedaner T, Wilde P (1995) Environmental sensitivity of cytoplasmic genic male sterility (CMS) in *Secale cereale* L. In: Kück U, Wricke G (eds) Genetic mechanisms for hybrid breeding. Advances in plant breeding. Backwell Wissenschafts-Verlag, Berlin, pp 7–17
- Gordon A, Basler R, Bansept-Basler P, Fanstone V, Harinarayan L, Grant PK, Birchmore R, Bayles RA, Boys LA, O'Sullivan DO (2015) The identification of QTL controlling ergot sclerotia size in hexaploid wheat implicates a role for the Rht dwarfing alleles. *Theor Appl Genet* 128:2447–2460. <https://doi.org/10.1007/s00122-015-2599-5>
- Gordon A, Delamare G, Tente E, Boyd L (2019) Final Project Report: determining the routes of transmission of ergot alkaloids in cereal grains. AHDB PR603. <https://ahdb.org.uk/determining-the-routes-of-transmission-of-ergot-alkaloids-in-cereal-grains>. Accessed 18 Feb 2020
- Gordon A, McCartney C, Knox RE, Ereful N, Hiebert CW, Konkin DJ, Hsueh YC, Bhadauria V, Sgroi M, O'Sullivan DM, Hadley C, Boyd LA, Menzies J (2020) Genetic and transcriptional dissection of resistance to *Claviceps purpurea* in the durum wheat cultivar Greenshank. *Theor Appl Genet*. <https://doi.org/10.1007/s00122-020-03561-9>
- Hallauer AR, Carena MJ, Filho JBM (2010) Testers and combining ability. In: Quantitative genetics in maize breeding. Handbook of plant breeding. Springer, New York, pp 383–423. https://doi.org/10.1007/978-1-4419-0766-0_8
- Hulvová H, Galuszka P, Frébortová J, Frébort I (2013) Parasitic fungus *Claviceps* as a source for biotechnological production of ergot alkaloids. *Biotechnol Adv* 31:79–89. <https://doi.org/10.1016/j.biotechadv.2012.01.005>
- Kirchhoff H (1929) Beiträge zur Biologie und Physiologie des Mutterkornpilzes. *Centralbl Bakteriol Parasitenk Abt II* 77:310–369
- Klotz K (2002) Abhängigkeit des Befalls mit Mutterkorn (*Claviceps purpurea* [Fries] Tulasne) in Winterroggen (*Secale cereale* L.) bei unterschiedlicher Sortenstruktur und Prüfmethodik. Diploma thesis. University of Hohenheim, Stuttgart
- MacDonald SJ, Anderson WAC (2017) Final Project Report: a desk study to review current knowledge on ergot alkaloids and their potential for contamination to cereal grains. AHDB PR575. <https://ahdb.org.uk/a-desk-study-to-review-current-knowledge-on-ergot-alkaloids-and-their-potential-for-contamination-to-cereal-grains>. Accessed 18 Feb 2020
- Meier U (2001) Growth stages of mono- and dicotyledonous plants. *BBCH Monograph*. <https://www.julius-kuehn.de/media/Veroeffentlichungen/bbch%20epaper%20en/page.pdf>. Accessed 13 Nov 2019
- Meleard B (2016) Degradation and epimerization of wheat ergot alkaloids during French baking test. https://www.english.arvalisinstitutduvegetal.fr/file/galleryelement/pj/84/76/8a/c1/meleard_alkaloids_and_bread_mytox739905815900991417.pdf. Accessed 19 Feb 2020
- Menzies JG, Klein-Gebbinck HW, Gordon A, O'Sullivan D (2017) Evaluation of *Claviceps purpurea* isolates on wheat reveals complex virulence and host susceptibility relationships. *Can J Plant Pathol* 39:307–317. <https://doi.org/10.1080/07060661.2017.1355334>
- Miedaner T (2013) Roggenanbau. Eine erfolgreiche Alternative. DLG-Verlag, Frankfurt/M
- Miedaner T, Geiger HH (2015) Biology, genetics, and management of ergot (*Claviceps* spp.) in rye, sorghum, and pearl millet. *Toxins* 7:659–678. <https://doi.org/10.3390/toxins7030659>
- Miedaner T, Wilde P, Wortmann H (2005) Combining ability of non-adapted sources for male-fertility restoration in Pampa CMS of hybrid rye. *Plant Breed* 124:39–43. <https://doi.org/10.1111/j.1439-0523.2004.01038.x>
- Miedaner T, Dänicke S, Schmiedchen B, Wilde P, Wortmann H, Dhillon BS, Mirdita V (2010a) Genetic variation for ergot (*Claviceps purpurea*) resistance and alkaloid concentrations in cytoplasmic-male sterile winter rye under pollen isolation. *Euphytica* 173:299–306. <https://doi.org/10.1007/s10681-009-0083-5>
- Miedaner T, Mirdita V, Rodemann B, Drobeck T, Rentel D (2010b) Genetic variation of winter rye cultivars for their ergot (*Claviceps purpurea*) reaction tested in a field design with minimized interplot interference. *Plant Breed* 129:58–62. <https://doi.org/10.1111/j.1439-0523.2009.01646.x>
- Miedaner T, Herter CP, Goßlau H, Wilde P, Hackauf B (2017) Correlated effects of exotic pollen-fertility restorer genes on agronomic and quality traits of hybrid rye. *Plant Breed* 136:224–229. <https://doi.org/10.1111/pbr.12456>
- Mielke H (2000) Studien über den Pilz *Claviceps purpurea* (Fries) Tulasne unter Berücksichtigung der Anfälligkeit verschiedener Roggensorten. *Mitt Biol Bundesanst Land-u. Forstw* 375
- Pažoutová S (2002) The evolutionary strategy of *Claviceps*. In: White F, Bacon CW, Hywel-Jones NL (eds) Clavicipitalean fungi: evolutionary biology, chemistry, biocontrol and cultural impacts. Marcel Dekker, New York, pp 329–354
- R Core Team (2018) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, <https://www.R-project.org>. Accessed 5 June 2019
- RStudio Team (2016) RStudio: integrated development for R. RStudio, Inc., Boston. <https://www.rstudio.com/>. Accessed 5 June 2019
- Schwake-Anduschus C (2018) Mutterkorn und Ergotalkaloide—eine aktuelle sicherheitsrelevante Betrachtung. MRI—Mutterkorn und Ergotalkaloide -DAF e.V. Tagung Berlin. <https://www.agrarforschung.de/fileadmin/download/2018/Schwake-Anduschus.pdf>. Accessed 24 Sept 2019
- Thakur RP, Williams RJ (1980) Pollination effects on pearl millet ergot. *Phytopathology* 70:80–84. <https://doi.org/10.1094/Phyto-70-80>
- Tittlemier SA, Drul D, Roscoe M, Turnock D, Taylor D, Fu BX (2019) Fate of ergot alkaloids during laboratory scale

- durum processing and pasta production. *Toxins* 11:195. <https://doi.org/10.3390/toxins11040195>
- Tudzynski P, Tenberge K, Oeser B (1995) *Claviceps purpurea*. In: Kohmoto K, Singh US, Singh RP (eds) *Pathogenesis and host specificity in plant diseases: histopathological, biochemical, genetic and molecular bases, vol II, eukaryotes*. Elsevier Science, Oxford, pp 161–187
- Utz HF (2011) PLABSTAT: a computer program for statistical analysis of plant breeding experiments. Institute of Plant Breeding, Seed Science and Population Genetics. University of Hohenheim, Stuttgart
- Van Dongen PW, de Groot AN (1995) History of ergot alkaloids from ergotism to ergometrine. *Eur J Obstet Gynecol Reprod Biol* 60:109–116. [https://doi.org/10.1016/0028-2243\(95\)02104-Z](https://doi.org/10.1016/0028-2243(95)02104-Z)
- Wegulo SN, Carlson MP (2011) Ergot of small grain cereals and grasses and its health effects on humans and livestock. University of Nebraska, Extension, EC1880. <https://ianrpubs.unl.edu/live/ec1880/build/ec1880.pdf>. Accessed 21 June 2019
- Willingale J, Mantle PG, Thakur RP (1986) Postpollination stigmatic constriction, the basis of ergot resistance in selected lines of pearl millet. *Phytopathology* 76:536–539. <https://doi.org/10.1094/Phyto-76-536>

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.