Analyzing resistance to ergot caused by Claviceps purpurea [Fr.] Tul. and alkaloid contamination in winter rye (Secale cereale L.)

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List of publications included in the doctoral thesis:

[1)] Kodisch A, Wilde P, Schmiedchen B, Fromme FJ, Rodemann B, Tratwal A, Oberforster M, Wieser F, Schiemann A, Jørgensen LN, Miedaner T (2020) Ergot infection in winter rye hybrids shows differential contribution of male and female genotypes and environment. Euphytica 216:65. https://doi.org/10.1007/s10681-020-02600-2

[2)] Kodisch A, Oberforster M, Raditschnig A, Rodemann B, Tratwal A, Danielewicz J, Korbas M, Schmiedchen B, Eifler J, Gordillo A, Siekmann D, Fromme FJ, Wuppermann FN, Wieser F, Zechner E, Niewińska M, Miedaner T (2020) Covariation of Ergot Severity and Alkaloid Content Measured by HPLC and One ELISA Method in Inoculated Winter Rye across Three Isolates and Three European Countries. Toxins. 12(11):676. https://doi.org/10.3390/toxins12110676

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[4] Miedaner T, Kodisch A, Raditschnig A, Eifler J (2021) Ergot alkaloid contents in hybrid rye are reduced by breeding. Agriculture. 11(6): 526. https://doi.org/10.3390/agriculture11060526

ABBREVIATIONS

ANOVA	Analyses of variance
CMS	Cytoplasmic-male sterility
DMAT	4-(γ,γ-dimethylallyl)tryptophan
EAs	Ergot alkaloids
Eco	Ergocornine
Ecr	Ergocristine
EFSA	European food safety authority
Ekr	Ergocryptin
ELISA	Enzyme-linked immunosorbent assay
Em	Ergometrine
Es	Ergosine
Et	Ergotamine
ETI	Effector-triggered immunity
EU	European Union
FLD	Fluorescence detection
GCA	General combining ability
GMO	Genetically modified organism
HPLC	High performance liquid chromatography
HPLC-MS/MS or LC-MS	HPLC—tandem mass spectrometry
LSD	Lysergic acid diethylamide
MAMPs	Microbe-associated molecular patterns
P cytoplasm	Pampa cytoplasm
PAMPs	Pathogen-associated molecular patterns
PTI	PAMP-triggered immunity
PR proteins	Pathogenesis-related proteins
QTL	Quantitative trait loci

R proteins	Resistance proteins
Rf genes	Restorer-to-fertility genes
SCA	Specific combining ability
SRAP	Sequence-related amplified polymorphism
TDI	Tolerable daily intake
WA	Water agar
Wt.	Weight

1. General introduction

Plant diseases are a severe problem for the agricultural production worldwide because they reduce quality of products, causes substantial yield damages on average of approximately 17.2 - 30.0% depending on the crop species (Savary et al., 2019) and lead therefore to high economic losses. Plants can be damaged by biotic agents such as fungi, bacteria, nematodes, viruses and insects (Pandey, 1992) or by abiotic factors such as nutrient deficiency, drought, extreme temperature, radiation, or pollution (Freeman and Beattie, 2008). Although plants do not have an adaptive immune system, plant defense strategies are complex, specific (Spoel and Dong, 2012) and lead to many biochemical and physiological changes (Kombrink and Somssich, 1995). Plant immunity reaction can be distinguished into two categories: incomplete resistance (quantitative resistance) and complete resistance (qualitative resistance; Kushalappa et al., 2016). The first one is controlled by polygenes that can interact with the environment and with each other. Therefore, it is characterized by a quantitative distribution of the resistance values leading to a reduction but not an absence of the disease whereas ergot caused by *Claviceps purpurea* belongs to this category. Pyramiding of many quantitative trait loci (QTL) contributing more or less to the expression can increase the resistance level. The second one is mainly controlled by single genes affecting the expression of a qualitative heritable characteristic what can be illustrated by the zig-zag model of plant immunity (French et al., 2016). In a first step, signaling components and pathogenesis-related (PR) proteins are formed as response to pathogen-associated molecular patterns (PAMPs) and microbe-associated molecular patterns (MAMPs; Sudisha et al., 2012) in a PAMP-triggered immunity (PTI; Rajamuthiah and Mylonakis, 2014). During the infection process, pathogens secrete virulence factors (effector proteins) to suppress the host cellular defense processes. In response, plants have evolved resistance (R) proteins and provoke an effector-triggered immunity (ETI; Rajamuthiah and Mylonakis, 2014). Additionally, priming shortens response time or increases the magnitude of defense reaction (Paré et al., 2005) and epigenetic modifications seem to provide a long-lasting immune memory of pathogen attack (Spoel and Dong, 2012). So, plants are not defenseless, but must adapt consecutively to changing conditions due to ongoing evolution of the pathogens and effects of climate change (Juroszek et al., 2020).

Controlling plant diseases under practical conditions is complex due to balancing costs and benefits, efficiency and technical feasibility. For a successful disease management, a plenty of measures have to be considered (Chaube and Singh, 1991). In general, it includes appropriate application of chemical plant protection agents and fertilizers, agronomic measurements, and the use of resistant varieties that are developed by plant breeding. In the light of future challenges due to climate change, ongoing population growth and sustainable use of resources, disease resistance breeding will get an even more substantial importance (Miedaner and Juroszek, 2021a, 2021b).

1.1 Rye – a versatile and robust multi-talent

Winter rye (*Secale cereale* L.) is an important, major staple crop in the European Union (EU) grown on 2.3 million hectares with a production of 9.4 million tons in 2020 (Eurostat, 2021). Over seventy percent is produced in Germany (3.5 million tons), Poland (3.3 million tons), and Austria (231 thousand tons). The other large producers in the EU are Spain and Denmark. In Germany, important rye growing areas (2020) are: Brandenburg (172.800 ha), Lower Saxony (138.800 ha), Saxony-Anhalt (74.800 ha) and Mecklenburg-Western Pomerania (69.700 ha; DESTATIS, 2020).

Rye is characterized by a high tolerance to biotic and abiotic (drought, salt, aluminum) stress, a robust growth, and a high yield potential that is competitive to other cereals such as wheat or triticale not only under tough agroclimatic conditions but also on better soil types (Geiger and Miedaner, 2009). The possible uses are multifarious and comprise food production, animal feeding and energy production from biogas and bioethanol (Miedaner, 2013). In Germany, there is a long tradition and unique variety of bread making with different proportions of wheat and rye flour (Deutsches Brotinstitut e.v., 2021). For that reason, German bread culture was honored in 2014 by UNESCO as intangible cultural heritage (Deutsche UNESCO-Kommission, 2019). For the production of high-quality food and feed, it is essential that grain samples are free from visible impurities (stones, etc.) and other contaminations (ergot, etc.) to meet the mandatory legal requirements. In contrast, this does not apply for biogas and bioethanol production except when co-products were used as feeding material (Schmitz, 2003).

Rye is an outcrossing, allogamous species with a gametophytic self-incompatibility system (Lundqvist, 1956). There are two types of cultivars, the open-pollinated and hybrid cultivars. Open-pollinated cultivars are generated by random mating after self-incompatible families were carefully selected and are, therefore, fully pollen shedding. For hybrid cultivars, a single-cross seed parent is crossed with a two-line synthetic as pollen parent (A • B × Syn C,D; Geiger and Miedaner, 2009). The crosses are generated on the basis of cytoplasmic-male sterility (CMS) of the Pampa (P) cytoplasm and need for restoration of the pollen fertility in the progenies (commercial rye stand) restorer genes that are coming from the pollinator. The European restorer-to-fertility (*Rf*) genes which were used in early hybrids provided 30-50% pollen shedding and are additionally vulnerable to environmental changes. At the present day, non-adapted restorer genes from Iranian primitive rye and Argentinean landraces, so called exotic *Rf* genes, are used in some hybrid varieties, and lead to a considerably better restoration comparable to open-pollinated cultivars (Miedaner et al., 2008). Additionally, the pollen-fertility of the hybrids is also determined by the ease of restoration of the female (Miedaner and Geiger, 2015). The rye research group of the University of Hohenheim provided an enormous contribution to the hybrid breeding by discovering the P cytoplasm and exotic *Rf* genes, therefore, bringing the development of hybrids decisively forward (Geiger and Schnell, 1970). Furthermore, considerable heterosis can be exploited in hybrid rye breeding for important traits due to firmly established genetically distinct heterotic groups (Petkus and Carsten pools; Geiger and Miedaner, 2009). Thus, it is not surprising that in Germany almost 80% of the agricultural area is grown by hybrid cultivars (BMEL, 2019) whereas hybrids are also of increasing importance in Poland and Austria.

1.2 Claviceps purpurea: causal agent of ergot

1.2.1 Biology, epidemiology and (historical) importance of ergot disease

Ergot is caused by fungi of the genus *Claviceps* with the type species *Claviceps purpurea* [Fr.] Tul. and leads to a severe plant disease of the grass inflorescences (Wegulo and Carlson, 2011). The first symptom of an ergot infection is the emergence of a sugary, sticky, and yellow-brown liquid called honeydew on the head at or soon after flowering (Fig. 1A). After that, the characteristic, black-purpled, and up to 5 cm long ergot bodies, so-called sclerotia, are developing on the ear instead of the kernels (Fig. 1B). They extend out from the glume as

compact mass of fungal mycelium with a purplish-black outside layer (Pažoutová, 2002) that protects the fungal mycelia from desiccation, UV light and other adverse environmental conditions (Schumann, and Uppala, 2002). Honeydew and sclerotia can also occur simultaneously on the same ear (Fig. 1C) and lead to infections of rye stocks in the field (Fig. 1D) or in the polyethylene tunnel (Fig. 1D).

Fig. 1 Ergot on winter rye: characteristic disease symptoms on the ears in form of A) honeydew, B) matured sclerotium [origin: T. Miedaner], and C) simultaneously occurrence of honeydew and sclerotia; and general view of ergot disease in the D) field, and E) polyethylene tunnel.

The fungus is distributed worldwide with a large host range containing more than 400 grass species including important crops such as rye and forage grasses in the temperate regions. Interestingly, *Claviceps purpurea* grows faster than other *Claviceps* species (Irzykowska et al., 2012) what might be an explanation for the high relevance and wide host-range underlying the importance of the factor fungal strain or isolate for the ergot reaction of cultivars. Although *C. purpurea* is the type species for the genus and also economically the most

important one, about 45 other teleomorph *Claviceps* species have been described. Even more species are supposed to exist but are difficult to detect because they are probably present only in anamorphic (sphacelial) stage. The distribution area of the other *Claviceps* species is mostly in tropical or subtropical areas. Other prominent representatives of the genus are *C. sorghi, C. africana* (sorghum ergot), *C. fusiformis* (pearl millet ergot) and *C. gigantea* (maize ergot; Pažoutová, 2002).

The disease cycle of *C. purpurea* (Fig. 2) was for the first time fully described by the French mycologist Louis René Tulasne in 1853. In a first step, overwintered sclerotia of the last season produce perithecia at the periphery of the stroma with sexually produced ascospores that are inducing primary (initially) infections on wild species and forage grasses (Mantle et al., 1977). Imitating pollination, the germination hyphae exclusively colonize the ovary and grows from the pistil through the stigma until it get to the ovule (Kirchhoff, 1929; Mielke, 2000; Tenberge, 1999; Tudzynski et al., 1995). After colonization, honeydew is secreted by the heads and leads to secondary infections. Ergot spores are transmitted to other flowers by physical head-to-head contact, farming equipment, aerosols (Miedaner and Geiger, 2015), or insects crawling on the head caused by the attraction due to the syrupy honeydew (Schumann, and Uppala, 2002). After that, sclerotia are developing within four to five weeks (Miedaner and Geiger, 2015) substituting the kernels. During the harvest, the matured sclerotia fall down, overwinter on the field surface and the life cycle starts anew.

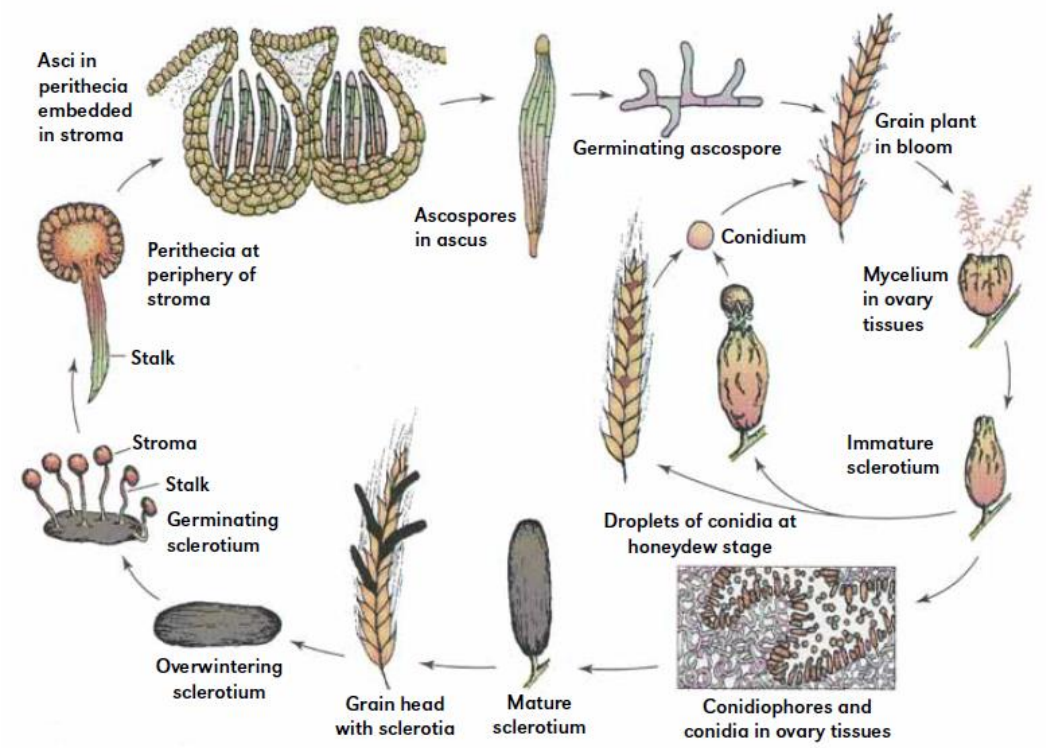

Fig. 2 Life cycle of ergot of small grain cereals and grasses (Schumann, and Uppala, 2002)

The time slot for a *C. purpurea* infection is only at or shortly after flowering because the fungus cannot grow through intact glumes, thus, making cross-pollinating crops such as rye particularly susceptible to ergot (Kirchhoff, 1929; Mielke, 2000; Tudzynski et al., 1995). Additionally, ergot does not appear randomly in the field but flowering, environmental and cultivation conditions and the presence of insects contribute to disease spreading (Dung et al., 2019).

The word ergot originates probably from argot (*old French* for cockspur) caused by the shape of the sclerotia (Schumann, and Uppala, 2002) and lead to a severe disease called ergotism also known as St. Anthony's fire or holy fire (Schiff, 2006). Ergotism can occur in two types: "convulsive" and "gangreneous" ergotism that is distinguishable by the symptoms (Hulvová et al., 2013). The first one comes together with fever, muscle spasms, tremor, paralysis and hallucinations due to the stimulation of the central nervous system by some ergot alkaloids (EAs) showing structural similarity to the neurotransmitter serotonin (Eadie, 2003). The other type, so-called gangreneous ergotism, is characterized by gangrene, peripheral pulses, violent burning, and shooting pain of distal organs (fingers, toes) up to losses of affected tissues after chronic intake due to vasoconstriction effects of some EAs and is eponymous for the disease

name “St. Anthony's fire” (Lee, 2009). One of the earliest recorded epidemics occurred in western Germany (857 AD) documented in the *Annales Xantenses* or in Limoges (France) in 944–945 AD leaving nearly 40,000 people dead (Engelke, 2002). Epidemic outbreaks in the Middle Ages resulted in death rates between 10 and 20% (Schumann, and Uppala, 2002) with thousands of dead people (Schiff, 2006). Sick people received help from the Hospital Brothers of Saint Anthony illustrated in “the Isenheim altarpiece” that can be visited in the Musée Unterlinden in Colmar (Musée Unterlinden, 2021). Also famous historical events like the Salem witchcraft trials of 1692 are suspected to be caused by ergot beside of other social, political, and psychological determinants (Caporael, 1976). Rye and ergot were so closely interwoven in previous times that early botanical drawings of rye included ergot in the outline. At this, people did not associate ergotism with ingestion of ergot-contaminated food. After assessing ergot as the disease cause of ergotism by the French physician Dr. Thuillier in 1670, severe epidemic events were getting rarer because the ergot bodies were sorted out before the milling process (Schumann, and Uppala, 2002). One of the last serious disease outbreaks with a total number of 47 fatalities occurred in 1977 in Ethiopia where local barley was infested by *Claviceps purpurea.* Here, more than 80% of the concerned people were between 5 and 34 years of age (Demeke et al., 1979). So, ergot was permanently of high concern especially for rural people that depend on rye as their primary source for bread making like it was the case in medieval times of Central and Eastern Europe (Schumann, and Uppala, 2002).

1.2.2 Ergot alkaloids with Janus face: toxic, but also of medical importance

Ergot alkaloids (EAs) are a diverse group of secondary metabolites (Money, 2016) known from several Ascomycetes species (Schiff, 2006) including pathogens (*C. purpurea*) or symbionts (*Neotyphodium*, *Epichloë*; Potter et al., 2008). They are defined as derivates of 4-(γ,γ-dimethylallyl)tryptophan (DMAT; (Florea et al., 2017)) and are grouped into clavine alkaloids, D-lysergic acid and its derivatives, and ergopeptines (Florea et al., 2017; Jakubczyk et al., 2014; Schardl et al., 2006; Young et al., 2015). More than 80 individual EAs are known in literature (Křen and Cvak, 1999; Schiff, 2006). In a communication of the European food safety authority (EFSA) ergometrine (Em), ergotamine (Et), ergosine (Es), ergocristine (Ecr), ergocryptin (Ekr), and ergocornine (Eco) and the corresponding -inine epimers were found to be the main alkaloids for *C. purpurea* (EFSA, 2012). The spectrum of the EAs is strongly influenced by the

host plant, geographic region and fungal isolate (Battilani et al., 2009; Krska and Crews, 2008; Malysheva et al., 2014; Schardl et al., 2006). In addition, the isolates seem to vary in their virulence depending on origin and geography of the host revealing complex relationships between host and fungus (Menzies et al., 2017). For *Festuca sinensis*, host of the systemic endophytic fungus *Epichloë*, even a seasonal-dependent variation of the EA formation was shown (Lin et al., 2019). Although EAs were investigated for a long time, the ecological function has not been sufficiently clarified yet. But there are studies indicating that EAs contribute to virulence (Panaccione and Arnold, 2017) and are necessary for the resistance of the fungus against insects (Potter et al., 2008), mammals (Panaccione et al., 2006), or microbes (Venkatesh and Keller, 2019). Additionally, pigmented fungi are known to be better adapted to withstand adverse environmental conditions (Diem, 1971; Durrell, 1964; Last and Deighton, 1965) what indicates that the pigmentation of ergot sclerotia might be an adaption mechanism against environmental stress factors such as heat or radiation. Interestingly, the EA content of a sclerotia is proportional to its pigment content making a toxicity analysis on base of pigment quantification imaginable (Flieger et al., 2019; Maríne Font et al., 1971; McClymont Peace and Harwig, 1982). However, EAs are light sensitive (Komarova and Tolkachev, 2001), thus, the correlation of EA and pigment content might be just a result of light-induced degradation of EAs in sclerotia with primary lower contents of pigments that are less protective (Flieger et al., 2019).

The yield loss due to ergot can amount to up to 5-10% and can be, therefore, a problematic issue in single years (Wegulo and Carlson, 2011). Nevertheless, the greatest threat is the contamination of the harvest by poisonous EAs leading to the severe symptoms of ergotism. The EAs are toxic for humans and warm-blooded animals (Hulvová et al., 2013) and can contaminate whole harvest lots by EA-containing dust caused by abrasion. Additionally, ergot bodies are fragile and the resulting fractions can be of similar size than the rye kernels which make separating expensive (Miedaner and Geiger, 2015). In contrast, the therapeutic effect of EAs has been known for a long time. Already around 350 BCE the effect that EAs trigger contractions at pregnant women was recorded in a sacred book of the Parsees (De Costa, 2002). In Germany, ergot was mentioned as a medical aid in childbirth by Adam Lonicer in 1582 for the first time (Mühle and Breuel, 1977) and was regularly used in obstetrics and inducing abortions in previous times. Since the 20th century, scientific research was started with isolation of ergotamine by Arthur Stoll in 1918 (Lee, 2009). After that milestones were

made such as suggesting ergotamine as drug for treating migraine by Hans Maier in 1926 or discovering Lysergic acid diethylamide (LSD) by Albert Hofmann in 1938 (Lee, 2010). Nowadays, EAs are used in modern medicine in several ways due to their pharmacological effects (Schardl et al., 2006; van Dongen and de Groot, 1995) as therapeutic drugs to treat a large number of human diseases like migraines, uterine hemorrhaging, or Parkinsonism (Florea et al., 2017; Sharma et al., 2016). For monitoring the toxicology of EAs in *e.g.* feeding studies in animal models, highly sensitive analytical methods are required (Shi and Yu, 2018; Strickland et al., 2011).

1.3 Detection methods for ergot alkaloids: pros and cons

EA concentration cannot be assessed by visual inspection (Shi and Yu, 2018), so, there are several methods for detecting and analyzing EAs based on different analytical techniques such as chromatography, immunology, spectroscopy or capillary electrophoresis (Crews, 2015; EFSA, 2012; Flieger et al., 1997; Scott, 2007; Shi and Yu, 2018). Nowadays, high performance liquid chromatography with fluorescence detection (HPLC-FLD) and HPLC—tandem mass spectrometry (HPLC-MS/MS or LC-MS) are routinely used as internationally validated standard in determining EA content in food and feed samples (EFSA, 2012; Schardl, 2015). Therefore, HPLC approaches are frequently applied in numerous screening studies (Debegnach et al., 2019; Malysheva et al., 2014; Meister and Batt, 2014; Mulder et al., 2012; Müller et al., 2009; Ruhland and Tischler, 2008; Wegulo and Carlson, 2011), particularly for the simultaneous screening of a large number of samples (Veršilovskis et al., 2019). Although HPLC determines EAs in a reliable, steady, repeatable and quantitative way (Shi and Yu, 2018), the analysis is time consuming and expensive because it requires on the one hand a well-equipped laboratory with well-trained employees (Beuerle et al., 2012; Ruhland and Tischler, 2008). On the other hand, time-consuming wet-chemical extraction and cleanup steps that rely on expensive and hazardous organic solvents (Senthilkumar et al., 2016) are needed. Furthermore, the choice of extraction solvents is of major importance, so, recurrent optimization of the experimental process has to be considered to gain maximum information from analytical data (Gemperline, 2006).

Besides, enzyme-linked Immunosorbent assays (ELISA) are also able to detect EAs because the mycotoxins can be discerned by the spatial structure in an antigen-antibody reaction (Shi and

Yu, 2018; Zheng et al., 2006). In this context, several commercial ELISA kits are available based on determining the content of lysergic acid and its derivatives, a common motif of the precursor group in the EA pathway (Cell Signaling Technology, 2021; LCTech, 2021; Veršilovskis et al., 2019). In comparison to other wet chemical methods such as HPLC, ELISA approaches are relatively fast, easy-to-use, and cheap (Cell Signaling Technology, 2021; Krska and Crews, 2008). Thus, making the tool a promising solution for a quick and financially sustainable screening assay for users in the daily routine. ELISA approaches were already applied for assessing EAs such as ergonovine in wheat and grass seeds (Shelby and Kelley, 1992) or the total EA content in native Turkish grasses (Tunali et al., 2000). Recent studies are using the technique more in the context of fescue toxicosis in livestock (Kenyon et al., 2018; Roberts et al., 2014; Schnitzius et al., 2001). Additionally, screening of EAs in a qualitative way was successful for two out of three ELISA kits for a low number of samples even though quantitative comparison with HPLC was difficult due to false positive and false negative results (Veršilovskis et al., 2019). This also illustrates major problems of this method. In particular, lower accuracy and specificity (Veršilovskis et al., 2019) and falsified results due to matrix effects or varying cross-reactivity (Coufal-Majewski et al., 2016; Krska and Crews, 2008; Shi and Yu, 2018) for instance against ergopeptines (*e.g.* ergovaline). This discrepancy may lead to divergent EA contents between different analytical methods due to under- or overestimation what is already reported in literature when comparing HPLC and ELISA (Roberts et al., 2014; Schnitzius et al., 2001).

1.4 Ergot-contamination in food and feed and consequent regulations

Numerous screening studies have shown that contamination of EAs in food and feed samples seems to occur regularly under natural infection conditions for all common cereals in different countries (Topi et al., 2017). EAs could be detected in a plethora of samples of rye and rye-based products (51% - 100%) with maximum levels between 61 to 1231 µg/kg whereas one rye feeding lot showed an extraordinary high EA content of 12340 µg/kg (Debegnach et al., 2019; Malysheva et al., 2014; Meister and Batt, 2014; Mulder et al., 2012; Ruhland and Tischler, 2008; Wegulo and Carlson, 2011). Additionally, other cereals like triticale or wheat showed similar maximum levels of EAs (123 and 1236 µg/kg) illustrating that ergot contamination is also a problem in other important crop species. So, screening of all cereals is

necessary to ensure food safety (Malysheva et al., 2014; Meister and Batt, 2014; Mulder et al., 2012; Müller et al., 2009; Ruhland and Tischler, 2008; Topi et al., 2017). But there is no uniform and consistent method for monitoring baked goods (Kniel et al., 2018) and EA content in individual sclerotia is variable (Schwake-Anduschus, 2018). Furthermore, EAs could be detected in ergot-cleaned samples what is probably caused by abrasion and arising dust formation during processing steps (Beuerle et al., 2012; Byrd and Slaiding, 2017; MacDonald and Anderson, 2017). Another explanation could be phloem movement because EAs could be detected in healthy grain that is evolved over- or underlying of the infected florets in wheat, barley and for a lesser extent in rye (Gordon et al., 2019). Nevertheless, a transfer over fungus-plant junction into healthy grain of the same ear seems not to be relevant in practical purposes as EAs could not be found in rye kernels of ergot infected ears (Mainka et al., 2007).

Nevertheless, the risk of an severe epidemic outbreak caused by ergot nowadays is low due to diversification of diets, cleaning possibilities (Miedaner and Geiger, 2015) and strict regulations of ergot within the EU (EFSA, 2012). In the EU, ergot sclerotia and sclerotial fragments in unprocessed cereals are restricted to 0.05% by weight (wt.) for human consumption (European Union, 2015) and to 0.1% by wt. for animal feeding (European Communities, 2002). Recent research gives the cause for a more critical evaluation of the regulatory limit because ergot doses close to the recommended limit of the tolerable daily intake (TDI) retained by the EFSA seem to induce alterations of the liver and intestine (Maruo et al., 2018). As a reason of the recurrent occurrence of EAs in food and feed and indications of an insufficient relationship between ergot amount and EA content (Bryła et al., 2018; Grusie et al., 2017; Kenyon et al., 2018; Mulder et al., 2012; Orlando et al., 2017; Roberts et al., 2014; Schwake-Anduschus et al., 2020), a reorganization of the regulations is expected to be enacted in the near future. At this, the limit will be adjusted to 0.02% by wt. (unprocessed rye). Furthermore, the maximum EA content will be limited to 250 µg/kg (as from 01.07.2023, until 30.06.2023: 500 µg/kg) for rye milling products and for infants and young children to 20 µg/kg on the base of the following 12 main EAs of *C. purpurea*: ergometrine, ergometrinine, ergosine, ergosinine, ergotamine, ergotaminine, ergocornine, ergocorninine, α-ergocryptine, α-ergocryptinine, ergocrystine, and ergocrystinine (A. Raditschnig, pers. commun. 2021).

1.5 Countermeasures, influencing factors and breeding strategies for reducing ergot

There are several factors for preventing ergot in a commercial field. First of all, agronomic measures such as supporting a well-developed, homogenous rye stand with an adequate density (Wegulo and Carlson, 2011), purification and controlling of weeds and grasses around the field (Mantle et al., 1977), crop rotation with non-susceptible species, deep plowing (Schumann, and Uppala, 2002), avoiding irrigation at the beginning of the flowering period (Alderman, 2006), and using certified seeds (Miedaner and Geiger, 2015) can be addressed by the farmers against ergot contamination. Chemical control of ergot disease via fungicides is not possible because no fungicides are registered on the market for this purpose (Engelke, 2002). When ergot occurs in the crop stand sclerotia and sclerotial fragments can be removed to a large extent by mechanical cleaning or optical color-sorting machines before milling what, however, increases the costs and slows down the processing speed (Miedaner and Geiger, 2015). Considerable reduction of EAs was observed in wheat and rye bread after baking (Bryła et al., 2018; Meleard, 2016) but not during durum pasta production (Tittlemier et al., 2019). Despite beer is not a key source of EA intake, EAs could be detected together with other mycotoxins after brewing in low levels but with high frequency (Bauer et al., 2016).

Another factor to prevent ergot is host resistance. It can be distinguished into passive mechanisms such as disease escape via stigma constriction (Willingale et al., 1986; Willingale and Mantle, 1985) or avoidance by cleistogamy of self-pollinating crops and active mechanisms like a real physiological resistance (Miedaner and Geiger, 2015). Although the existence is not validated yet few QTL associated with partial resistance to ergot are known in bread wheat influencing weight and size of sclerotia (Gordon et al., 2015) or in durum wheat having an effect on different components of the infection process like the hormonal pathway (Gordon et al., 2020).

Due to the fact that the fungus mimics pollination of a cereal, flowering biology is an essential factor influencing ergot contamination (Kirchhoff, 1929; Mielke, 2000; Tenberge, 1999; Tudzynski et al., 1995). Consequently, floral characteristics such as duration of flowering (Thakur and Williams, 1980), aperture angles, stigma size, duration of receptivity and drying time after pollination of stigmas are important factors as shown in sorghum (Bandyopadhyay et al., 1998; Cisneros-López et al., 2010; Dahlberg et al., 2001). Additionally, pollen availability

is crucial for preventing ergot and lead to a competition between pollen and fungal spores (Engelke, 2002; Miedaner and Geiger, 2015; Thakur and Williams, 1980). Consequently, a reduced pollen amount promotes ergot infection. This could arise because of partial restoration of a hybrid cultivar. Furthermore, rainy and moist weather with high humidity and low temperatures around flowering reduces on the one hand production, shedding, movement and viability of pollen negatively but increases on the other hand proliferation and development of the pathogen. In addition, warm and sunny weather conditions lead to thickened honeydew (McCrea, 1931; Menzies and Turkington, 2015; Miedaner and Geiger, 2015; Pažoutová, 2002). So, interactions between weather, fungus and host are complex making a high impact of genotype-by-environment interaction imaginable and lead to the necessity of testing ergot trials across several locations and years (Dhillon et al., 2010; Miedaner et al., 2010a, 2010b; Miedaner and Geiger, 2015; Mirdita and Miedaner, 2009).

Until now, breeding for reducing ergot focuses on the enhancement of the pollen shedding of the cultivars that should be environmental stable also under adverse conditions (Engelke, 2002; Miedaner and Geiger, 2015). At this, introgression of effective restorer genes to guarantee a high pollen ability is one of the most important tools for breeding companies and lead in practical conditions to a significant reduction of ergot contamination (Miedaner et al., 2008). In the descriptive list of varieties of Germany (grain utilization), nine population and 26 hybrid cultivars of winter rye were registered in 2020 and the susceptibility to ergot ranges between 3-6 on the 1-9 scale (1 = fully resistant, 9 = fully susceptible; Bundessortenamt, 2020). Despite population cultivars received on average a better ergot evaluation, the top grade (= 3) was assigned to population as well as hybrid cultivars. Consequently, farmers can choose varieties with a good ergot defense what was due to plant breeding progress.

2. Objectives

The aim of this research thesis was to investigate the relative importance of crucial factors (genotype (male, female), location, year, isolate) and their interactions on ergot infection and ergot alkaloid (EA) contents in multi-locational field trials across Central Europe, and to examine the covariation of high performance liquid chromatography (HPLC), enzyme-linked immunosorbent assay (ELISA) and ergot severity in a large-scale study.

In particular, the specific aims were to:

(I) Validate a harmonized method for testing resistance to ergot in winter rye cultivars considering different environmental conditions in Central Europe (Publication 1, 2);

(II) Evaluate the relative importance of the effects of male and female genotype, and environment for ergot severity (Publication 1) and the role the maternal component (Publication 3);

(III) Assess the effect of inocula from fungal isolates of different EU countries and to estimate potential changes of cultivars in rank order when using country-specific inocula (Publication 1, 2);

(IV) Analyse the EA contents regarding genotypes, locations, countries, years, isolates, and interactions (Publication 2, 4);

(V) Calibrate a commercial ELISA test kit (ErgoREAD; LCTech GmbH) for the total EA content by HPLC and determine the correlation among ELISA and HPLC values and ergot severity (Publication 2, 4).

3. Publication I: Ergot infection in winter rye hybrids shows differential contribution of male and female genotypes and environment[1)]

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

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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

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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•B × 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 × 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 Saatzucht 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. SC_0 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 m^2 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/m^2. 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 m^2 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 $\geq$ 4 were calculated according to the following formula: RI = 100 × (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). SC_{100}, both under natural and artificial infection, was even better than the population cultivars (Conduct, Dańkowskie Diament). SC_{50}, 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 SC_0 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

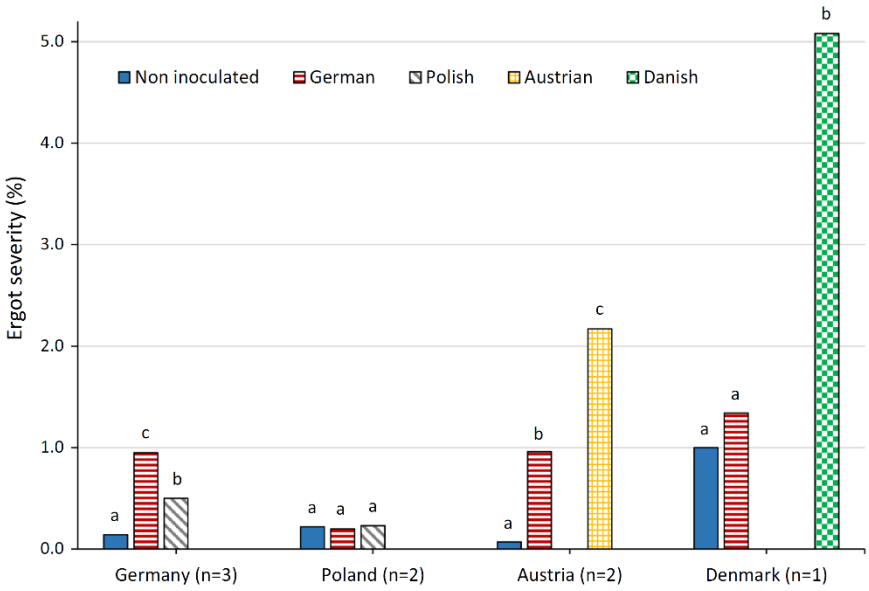

Fig. 1 Mean ergot severity (%) of 12 winter rye genotypes for each of three inoculation treatments across one to three locations in four countries in 2013 (n = number of locations, Exp. 1). Treatments with the same letter are not significantly different within individual countries (Tukey test, $P < 0.05$)

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**			

[a]P: population cultivar, H: hybrid cultivar

[b]10% population rye included

[c]From square root transformed (sq transf.) data for ergot severity

[d]Coefficient 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 × environment interaction variances. As expected, the inoculum treatment variance was significantly different from zero ($P < 0.05$) only for ergot severity. Genotype × inoculation treatment variance was negligible although significant, while environment × inoculation treatment variance had the second highest variance component. Entry-mean heritability was 0.88. The other traits also showed important genotypic and genotype × 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

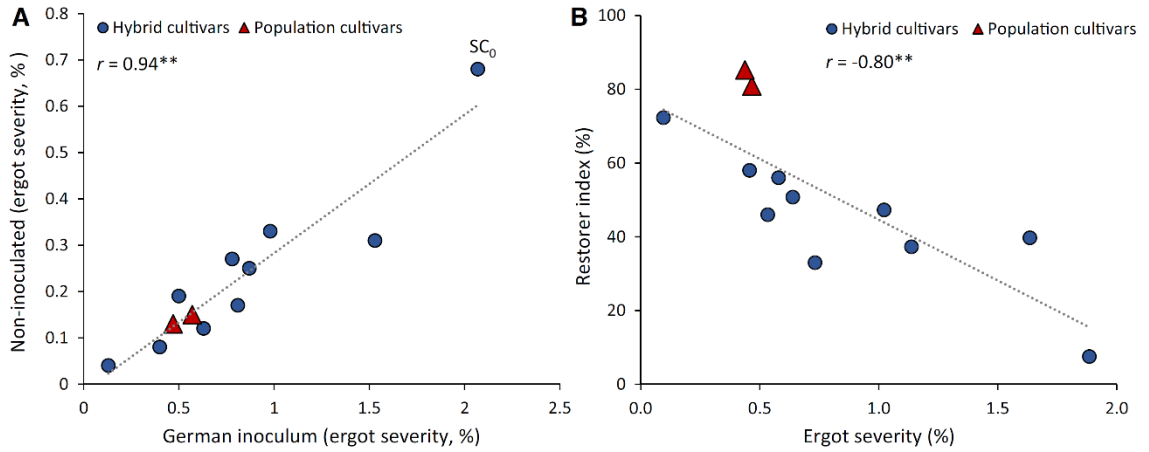

Fig. 2 Correlation between, **a** ergot severity of non-inoculated and German inoculum across eight locations and, **b** ergot severity and restorer index across seven locations and mean of inoculation treatments for 12 winter rye genotypes (Exp. 1) (**significant at $P < 0.01$)

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)
No. of environments	*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 x 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 x E x 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

[a]Negative 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		

[a]Treatments 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					
	R3 Plus	R3 Minus	R4 Plus	R4 Minus	Mean	Sign.[a]
(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		

[a]Treatments with the same letter are not significantly different (Tukey test, $P < 0.05$)

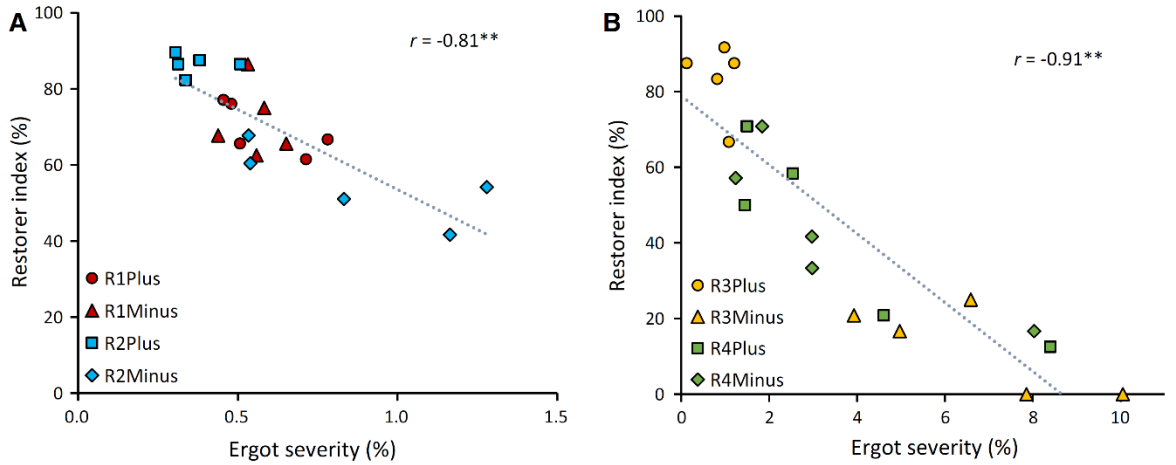

Fig. 3 Correlation between ergot severity and restorer index for two male restorer (Rx) lines with (Plus) and without (Minus) a non-adapted restorer gene and 5 female CMS lines for, **a** set A (8 environments) and, **b** set B (2 environments) after inoculation by *Claviceps purpurea* (r = coefficient 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 (%)
No. of environments	*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). SC_{100} 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 × 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.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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 + Mischfuttertechnik 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, Sonego 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 Winterrogen (*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 hostspecificity 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

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

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

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Supplemental materials

Table S1: Name of cultivars and single crosses (SC) with a varying percentage of a non-adapted restorer gene, type of cultivar and breeding company of Exp. 1

Entry	Type of cultivar[a]	Breeder
SU Satellit[b]	H	HYBRO GmbH & Co KG
SU Allawi[b]	H	HYBRO GmbH & Co KG
SU Stakkato[b]	H	HYBRO GmbH & Co KG
Dańkowskie Diament	P	"DANKO" Hodowla Roslin Sp. z o.o.
Brasetto	H	KWS LOCHOW GmbH
Palazzo	H	KWS LOCHOW GmbH
Visello	H	KWS LOCHOW GmbH
Conduct	P	KWS LOCHOW GmbH
SC_{100}	H	KWS LOCHOW GmbH
SC_{25}	H	KWS LOCHOW GmbH
SC_{0}	H	KWS LOCHOW GmbH
SC_{50}	H	KWS LOCHOW GmbH

[a]P: population cultivar, H: hybrid cultivar
[b]10% population rye included

Table S2: Country, location, and respective traits tested in Exp. 1 (x = trait observed)

Country	Location	Ergot severity (%)	Anther rating (1-9)	Ear emergence (1-9)	Plant height (cm)
	Number of environments	8	7	7	7
Germany	Oberer Lindenhof (48°28'25.5"N 9°18'17.9"E)	X	X	X	X
Germany	Braunschweig (52°16'33.4"N 10°34'09.3"E)	X	–	–	–
Germany	Petkus (51°58'50.3"N 13°21'01.7"E)	X	X	X	X
Germany	Wohlde (52°48'48.7"N 9°59'53.1"E)	–	X	X	X
Austria	Freistadt (48°29'23.6"N 14°29'47.6"E)	X	X	X	X
Austria	Zwettl (48°36'22.4"N 15°13'23.8"E)	X	X	X	X
Poland	Zybiszów (51°03'51.9"N 16°54'45.4"E)	X	X	X	X
Poland	Kościelna Wieś (51°46'28.7"N 18°00'58.0"E)	X	X	X	X
Denmark	Skejby (56°12'00.6"N 10°09'06.3"E)	X	–	–	–

Table S3: Country, year, location and respective traits tested in Exp. 2 (x = trait observed)

Country	Year	Location (GPS data)	Ergot severity (%)	Anther rating (1-9)	Ear emergence (1-9)	Plant height (cm)
			set A/set B			
		Number of environments[a)]	16/8	8/2	9/6	12/6
Germany	2014	Oberer Lindenhof (48°28'25.5"N 9°18'17.9"E)	x/x	x/x	x/x	x/x
Germany	2014	Braunschweig (52°16'33.4"N 10°34'09.3"E)	x/x	–/–	–/–	–/–
Germany	2014	Wohlde (52°48'48.7"N 9°59'53.1"E)	x/–	x/–	–/–	x/–
Germany	2014	Petkus (51°58'50.3"N 13°21'01.7"E)	x/–	x/–	x/–	x/–
Germany	2014	Wulfsode (53°03'45.7"N 10°14'02.5"E)	x/x	–/–	x/x	x/x
Germany	2014	Kleptow (53°21'54.9"N 14°00'04.4"E)	x/x	–/–	x/x	x/x
Poland	2014	Kościelna Wieś (51°46'28.7"N 18°00'58.0"E)	x/x (1 rep)	–/–	–/–	–/–
Denmark	2014	Skejby (56°12'01.3"N 10°09'32.0"E)	x/–	x/–	–/–	x/–
Germany	2015	Oberer Lindenhof (48°28'25.5"N 9°18'17.9"E)	x/x	x/x	x/x	x/x
Germany	2015	Braunschweig (52°16'33.4"N 10°34'09.3"E)	–/–	–/–	–/–	–/–
Germany	2015	Wohlde (52°48'48.7"N 9°59'53.1"E)	x/–	x/–	x/–	x/–
Germany	2015	Petkus (51°58'50.3"N 13°21'01.7"E)	x/–	x/–	x/–	x/–
Germany	2015	Wulfsode (53°03'45.7"N 10°14'02.5"E)	x/x	–/–	x/x	x/x
Germany	2015	Kleptow (53°21'54.9"N 14°00'04.4"E)	x/x	–/–	x/x	x/x
Poland	2015	Kościelna Wieś (51°46'28.7"N 18°00'58.0"E)	x/–	–/–	–/–	–/–
Poland	2015	Zybiszów (51°03'51.9"N 16°54'45.4"E)	x/–	–/–	–/–	–/–
Denmark	2015	Skejby (56°12'00.6"N 10°09'06.3"E)	x/–	x/–	–/–	x/–

[a)] Environment = location x year combinations

4. Publication II: Covariation of Ergot Severity and Alkaloid Content Measured by HPLC and One ELISA Method in Inoculated Winter Rye across Three Isolates and Three European Countries[2)]

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Covariation of Ergot Severity and Alkaloid Content Measured by HPLC and One ELISA Method in Inoculated Winter Rye across Three Isolates and Three European Countries

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Abstract: Ergot caused by *Claviceps purpurea* is a problem for food and feed security in rye due to the occurrence of toxic ergot alkaloids (EAs). For grain elevators and breeders, a quick, easy-to-handle, and cheap screening assay would have a high economic impact. The study was performed to reveal (1) the covariation of ergot severity (= percentage of sclerotia in harvested grain) and the content of 12 EAs determined by high performance liquid chromatography (HPLC) and (2) the covariation between these traits and results of one commercial enzyme linked immunosorbent assays (ELISA). In total, 372 winter rye samples consisting of a diverse set of genotypes, locations from Germany, Austria, and Poland over two years, and three isolates were analyzed. Ergocornine and α-ergocryptine were detected as major EAs. Ergocristinine occurred as a minor component. *Claviceps* isolates from different countries showed a similar EA spectrum, but different quantities of individual EAs. A moderate, positive covariation between ergot severity and EA content determined by HPLC was observed across two years ($r = 0.53$, $p < 0.01$), but large deviation from the regression was detected. ELISA values did neither correlate with the HPLC results nor with ergot severity. In conclusion, a reliable prediction of the EA content based on ergot severity is, at present, not possible.

Keywords: ergot alkaloids; *Claviceps purpurea*; HPLC; mycotoxins; ELISA; rye

Key Contribution: Ergot severity cannot be used to predict the EA content measured by HPLC because only a moderate correlation and a large deviation from the regression were detected. A commercial ELISA did neither correlate with ergot severity nor with HPLC data.

1. Introduction

The plant pathogen *Claviceps purpurea* ((Fr.: Fr.) Tul.) is distributed worldwide and can infect more than 400 grass species including rye (*Secale cereale* L.) [1]. Germany, Poland, and Austria cover 73% of the European Union rye production of 4.6 million metric tons in 2018 [2]. Ergot is a severe fungal disease and the over-wintering bodies called sclerotia contain toxic ergot alkaloids (EAs). The EAs are a group of secondary metabolites [3] that are defined as derivatives of 4-(γ,γ-dimethylallyl)tryptophan (DMAT) [4]. EAs are known from several Ascomycetes species [5], including *C. purpurea* as a plant pathogen, and symbionts like *Neotyphodium* and *Epichloë* [6]. The ecological function is not fully clarified yet, but there are indications that EAs contribute to virulence [7] and play an important role for the resistance of the fungus against insects [6], mammals [8], or microbes [9]. More than 80 individual EAs are known in literature [5,10], which can be grouped into Clavine alkaloids, D-lysergic acid and its derivatives, and ergopeptines [4,11–13]. For *Claviceps* spp., the main EAs are ergometrine (Em), ergotamine (Et), ergosine (Es), ergocristine (Ecr), ergocryptin (Ekr), and ergocornine (Eco) along with their corresponding -inine epimers [14,15]. The pattern of the produced EAs strongly differs depending on fungal strain, host plant, and the geographic region [12,14,16,17]. Furthermore, a seasonal-dependent variation of the EA formation was observed for *Festuca sinensis* [18]. Additionally, for pathogenic variation, significant differences were observed among isolates according to their geographic origin [19]. Kodisch et al. [20] showed that isolates may also differ in their ergot severity on winter rye. For determination of EAs, numerous methods are reported in literature based on immunology, chromatography, or capillary electrophoresis [15,21–23]. For determination of individual EAs in food and feed at relevant levels, chromatographic methods such as high performance liquid chromatography with fluorescence detection (HPLC-FLD) and high performance liquid chromatography—tandem mass spectrometry (HPLC-MS/MS or LC-MS) are internationally validated [15,24] and were commonly used in several screening studies [1,17,25–29], especially when a high number of samples is needed to be screened in parallel [30]. Enzyme-linked Immunosorbent Assays (ELISA) are based on the antigen-antibody reaction and could be used to determine the content of a precursor in the ergot biosynthetic pathway to obtain information about the EA content. Additionally, ELISAs are easy-to-use, fast, and relatively cheap [31] and, therefore, the ideal solution for a quick and financially sustainable screening assay. Several commercial ELISA kits for evaluating EAs detecting lysergic acid and its derivatives as a common motif of one precursor group of the EAs are available [30–32]. Three commercially available ELISA kits have been shown for a small number of samples to screen EAs correctly in a qualitative manner in two cases, but not in a quantitative manner caused by false positive and false negative results when comparing to an HPLC approach [30]. The ELISAs show further cross reactivity against ergopeptines (e.g., ergovaline), which is not covered by the 12 EAs analyzed with the HPLC approach. This may lead to a higher total ergot alkaloid content by ELISA in comparison with the HPLC analysis [32].

Rye is especially prone to ergot contamination because it is a cross-pollinating crop opening the flowers wide and allowing the ergot spores to be brought directly into the pistils by aerosol deposition or insects [33]. Ergot infection is favored by cool, rainy weather when the flowers have a prolonged opening period, but also by a low amount of pollen shedding that is genetically inherited [20,34]. Ergot-contaminated rye grain was caused in the Middle Ages' devastating epidemics in humans known as "St. Anthony's fire" or ergotism with severe pathological syndromes such as gangrene, neurological diseases, and, finally, death [35,36]. In contrast, some EAs are used in medicine as therapeutic drugs to treat migraines, uterine hemorrhaging, or Parkinsonism [4] due to the pharmacological activities

of these metabolites [12,36]. Since the second half of the 20th century, only single disease cases in humans are known and, currently, the risk of ergot as a fatal disease for humans and animals is low due to cleaning procedures, diversification of diets, and strict regulations of the ergot content within the European Union EU, [15,37]. Until now, the EU limits for ergot sclerotia and sclerotial fragments in unprocessed cereals are set to 0.05% by wt. for human consumption [38] and to 0.1% by wt. for animal feed [39]. However, reduction of the limit to 0.02% by wt. on unprocessed rye and maximum levels based on the EA alkaloid content as from 01.07.2022 onward to 250 µg/kg on rye milling products (until 30.06.2022: 500 µg/kg) and for infants and young children to 20 µg/kg are expected to be enacted for the 12 EAs ergometrine, ergometrinine, ergosine, ergosinine, ergotamine, ergotaminine, ergocornine, ergocorninine, α-ergocryptine, α-ergocryptinine, ergocrystine, and ergocrystinine (A. Raditschnig, pers. commun. 2020). However, not all milling companies are able to use photocell-based sorting machines because it enhances the costs and slows down the processing appreciably [33]. Furthermore, EAs could also be detected in ergot-cleaned grain samples likely caused by abrasion [40–42]. Gordon et al. [43] revealed that, in wheat and barley and, for a lesser extent, in rye, EAs also occurred in healthy grain that developed above and below the artificially infected individual florets. Therefore, it is not surprising that EAs occurred frequently in several studies in different countries and among all common cereals [44].

For rye and rye-based products, between 51% to 100% of all samples contained EAs with maximum levels ranging between 61 to 1231 µg/kg whereas, in one rye feeding lot, a maximum EA content of even 12,340 µg/kg was observed [1,17,25–29]. The maximum levels for other cereals such as wheat or triticale reached similar EA contents ranging between 123 and 1236 µg/kg [17,26–29,44], indicating the necessity of testing wheat and triticale for the presence of EAs. Furthermore, it is known that baking of wheat and rye flour reduces the EA content considerably [45,46] and also shifts the ratio between the epimeric forms toward the -inine forms [15]. However, this was not true for durum pasta production [47], so processing alone is not a guarantee to obtain alkaloid-free commodities. Although ergot often does not result in a large yield loss, ergot-contaminated grain can have a high economic impact on cereal production because of downgrading or rejecting grain lots contaminated with EAs [1,19,48]. The best measures for reducing ergot incidence and EA content is growing less susceptible cultivars, supporting the homogeneity of the rye stand, and controlling the weeds surrounding the fields [49]. However, up to now, it is not known whether physiological resistance exists in rye and breeding for lower susceptibility is difficult [33]. Only a few quantitative trait loci (QTL) were reported for assigning partial resistance to ergot, i.e., affecting the size and weight of sclerotia in bread wheat [50] or different components of the infection process like the hormonal pathway in durum wheat [51].

Regarding the recurring occurrence of EAs in rye and rye-based products, the future reduction of existing limits and expected maximum EU levels based on the EA content creates a need to clarify associated factors influencing EA content with the aim to develop and evaluate a quick screening method for farmers, small elevators, milling companies, and breeders. Otherwise, a high economic burden would arise for the industry, which likely would have an effect on the prize of the final products. Especially for selection for low EA content in breeding, cheap and quick tests are necessary because thousands of samples have to be screened in the short time frame between harvest and planting. The relationship of ergot severity and EA content is not fully clarified yet. Screening studies often consist of a low number of samples only and validation studies of HPLC and ELISA are rare in literature. Therefore, this correlation study was performed with a high number of samples to reveal (1) the covariation of ergot severity and EA content determined by HPLC in the light of different genotypes, environments (locations, years, countries), isolates, and infection levels and (2) the covariation between these traits and the commercial ErgoREAD ELISA test. For this, we analyzed 372 winter rye samples of several genotypes artificially infected by *C. purpurea* with three isolates at nine locations (Germany, Austria, Poland) in two years.

2. Results

2.1. Influence of Genotype, Environment, Isolate, and Interaction on Ergot Severity and EA Content

The environments showed wide ranges of ergot severity and EA content determined by HPLC and ELISA, varying from 0.06% to 6.46%, 0 to 97.85 mg/kg, and 0.52 to 3673.89 mg/kg, respectively. Huge differences between the locations and years were observed concerning ergot severity and EA content determined by HPLC (Figure 1). The Austrian locations EHO 2018 and HAG 2018 showed the highest ergot severity, whereas the Polish location ZYB revealed low levels for ergot severity and EA content in both years.

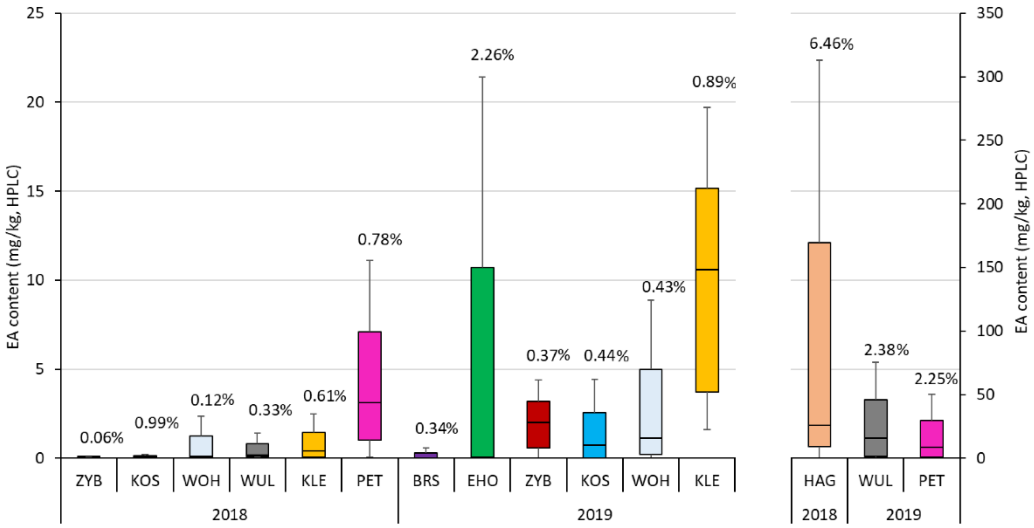

Figure 1. Boxplots of ergot alkaloids (EA) content determined by HPLC and mean ergot severity (% value above the respective column) of 15 environments for three winter rye genotypes (D.Amber, Elias, H_Hyb5) after inoculation with three isolates of *C. purpurea* of subset II (black line representing the median, error bars representing the standard error of means, WUL2019, PET2019, and HAG2018 are presented on a secondary y-axis due to their very high EA contents). For the abbreviations of the locations, please refer to Materials & Methods or Table S1.

Small differences were observed for ergot severity among three rye genotypes with cultivar Elias showing significantly less ergot than H_Hyb5 (Table S2). Differences among the cultivars of the EA levels were not statistically significant as measured by both methods. ELISA resulted, on average, in a 43-fold higher EA content than HPLC. Similar large differences were obtained for the distribution of the EA content determined by HPLC and ELISA (Figure 2). For HPLC, most of the samples (40%) contained EA concentrations ≤0.25 mg/kg. In contrast, only 3% of the ELISA samples reached this low EA concentration level. Vice versa, the minority of the samples (2%) measured by HPLC contained higher EA levels than 100 mg/kg. For ELISA, however, 28% of the samples fell in this high EA class.

The environment was the most important component of variance for all three traits (Table S3). For ergot severity, genotype, isolate, and genotype × environment were also important sources of variance. There was no significant genotype × isolate interaction. For EA content measured by HPLC, isolate variance was also significant. No significant genotypic effect was determined for EA content, neither for HPLC nor for ELISA analyses. High entry-mean heritabilities were observed for ergot severity and EA content measured by HPLC (0.91 and 0.82, respectively). EA content measured by ELISA had a considerably lower heritability (0.57).

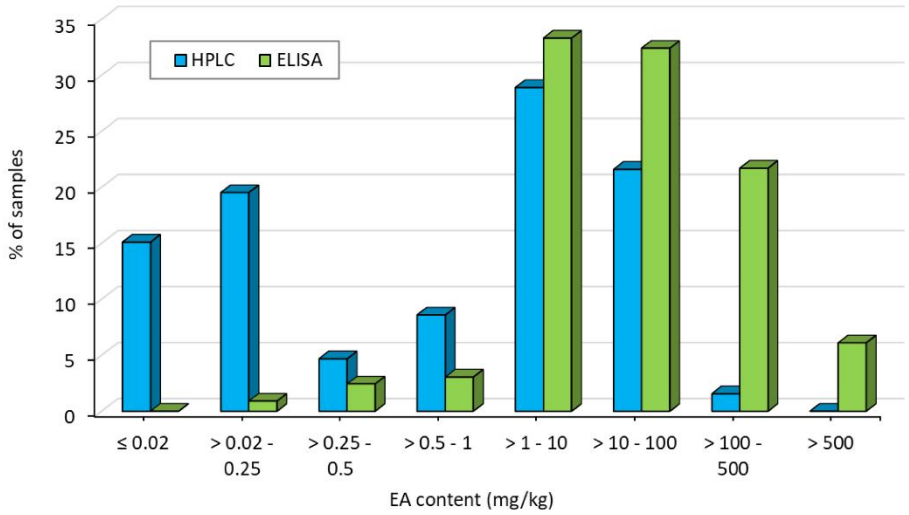

Figure 2. Distribution of ergot alkaloids (EA) content of subset I after inoculation with *C. purpurea* across 18 environments determined by HPLC (blue) and ErgoREAD ELISA (green, see Materials & Methods).

2.2. *Covariation of Ergot Severity and Alkaloid Content Measured by HPLC*

Ergot severity and EA content measured by HPLC showed good correlations of the respective two field plots (= replications) ranging from 0.70 to 0.91 (Figure S1) and illustrating that the genetic variation was high and the methods were reliable. A moderate positive covariation of ergot severity (%) and EA content determined by HPLC was obtained for subset I ($r = 0.53$, $p < 0.01$, Figure 3a) and subset II ($r = 0.65$, $p < 0.01$, data not shown). No grouping effects were observed when considering different factors such as isolate (Figure 3b), location (Figure 3c), and genotype (Figure 3d), i.e., the correlation did not improve. The correlations calculated within isolates ($n = 96$) and genotypes ($n = 96$) showed moderate to good correlations varying from $r = 0.45$ to $r = 0.90$. Splitting the samples in fractions of ergot severity <0.05% and ≥0.05% revealed low to medium covariations of $r = 0.26$ ($n = 145$) and $r = 0.49$ ($n = 183$), respectively.

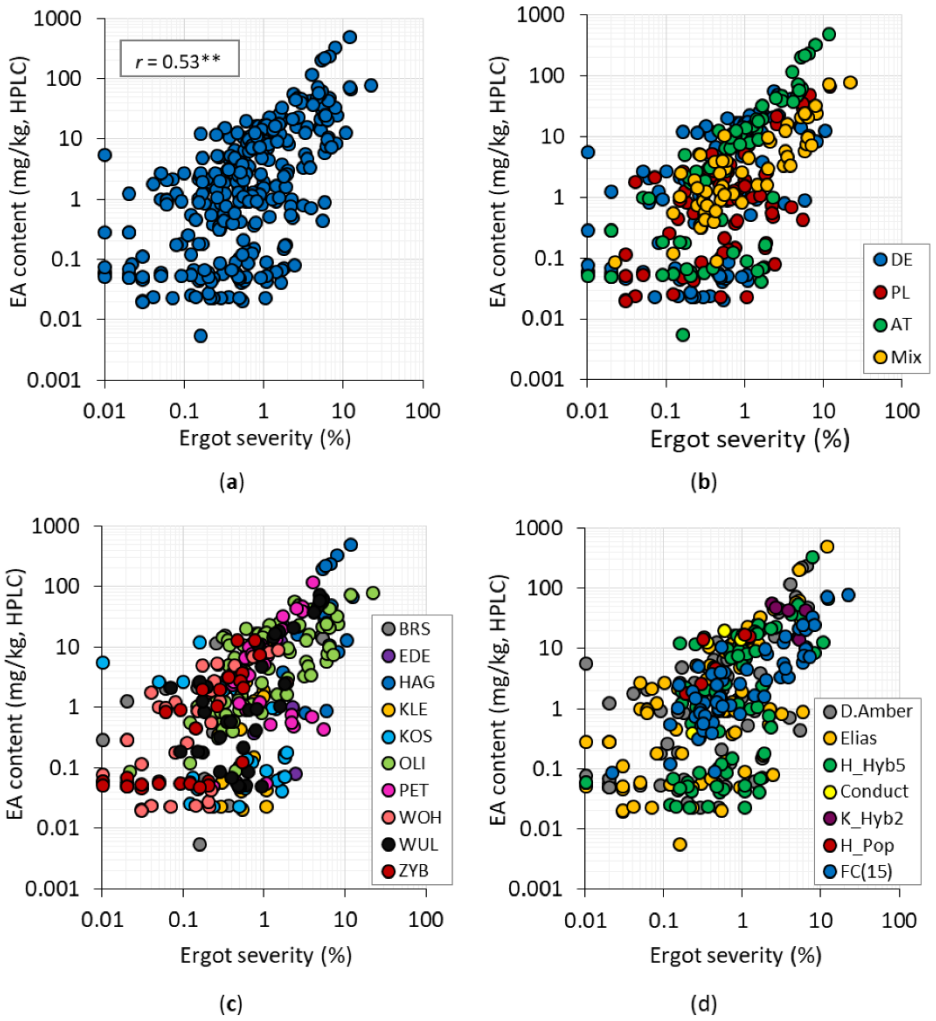

Figure 3. Correlation between ergot severity (%) and EA content determined by HPLC (mg/kg) of subset I after inoculation with *C. purpurea* (**a**) across all environments and isolates and colored by (**b**) origin of isolate (DE = German isolate, PL = Polish isolate, AT = Austrian isolate, Mix = Mix of all isolates [DE, PL, AT]), (**c**) location and (**d**) genotype (FC(15) = comprising 15 factorial crosses) (r = coefficient of correlation, **: significant at $p < 0.01$).

2.3. Covariations with ErgoREAD ELISA Values

The ELISA showed a good correlation between the two field plots (= replications) for both ergot severity and EA content (Figure S1). No correlation, however, was detected between ergot severity (%) and EA content for subset I ($r = -0.07$) (Figure S2a) and subset II ($r = -0.09$) (data not shown). Comparing both analytical methods for determining EA content revealed no covariation between ELISA and HPLC for subset I (Figure S2b) and subset II ($r = -0.04$) (data not shown). No improvement of the correlation could be detected when splitting the dataset in the factors isolate, location, genotype, or infection level (ergot severity <0.05% and ≥0.05%).

2.4. Effect of the Isolates on Ergot Infection and EA Content

Across the years, significant differences between the isolates were observed for all traits except for ergot severity in 2018 and ergot severity across both years (Table 1). EA contents measured by ELISA were for all isolates substantially higher in 2019 than in 2018. This was, however, not the case for measurements

by HPLC. The Austrian isolate always showed the highest EA contents measured by HPLC, which is significantly different from the Polish and German isolates. A table of ergot severity for the single rye genotypes separated by isolate and year could be found in the Supplementary Material (Table S4).

Table 1. Means and significance level of ergot severity and EA content determined by HPLC or ELISA across up to eight locations in two years and across years after inoculation with *C. purpurea* with three country-specific isolates (DE = German isolate, PL = Polish isolate, AT = Austrian isolate) for subset II.

Trait	2018			2019			2018 + 2019		
Isolate	**DE**	**PL**	**AT**	**DE**	**PL**	**AT**	**DE**	**PL**	**AT**
Ergot Severity (%)	2.04a [1]	2.07a	1.53a	0.67a	1.13b	1.72c	1.36a	1.60a	1.62a
EAs, HPLC (mg/kg)	1.10a	6.21a	37.64b	8.41b	0.80a	16.75c	5.00a	3.32a	26.50b
EAs, ELISA (mg/kg)	7.5a	26.8b	23.5b	862.9ab	1700.7a	254.8b	387.7a	824.8b	120.7a

[1] Treatments with the same letter within one row are not significantly different (Tukey test, $p < 0.05$).

2.5. Composition of the EA Spectrum

The pattern and distribution of all single EAs relative to the EA content determined by HPLC across all locations was mostly similar for both years (Figure 4a, subset I). Larger differences were found only for ergometrine and ergotamine. The amount of ergometrine was 6.3-fold higher in 2019 (19%) when compared to 2018 (3%), which is in contrast to the 30.6-fold decrease of ergotamine from 2018 (11%) to 2019 (0.36%). In almost all cases, the amount of the -inine forms of the respective EA was considerably lower than the -ine epimer, except for ergocristine (0.02%) and ergocristinine (0.11%) (2019, subset I) and for ergotamine (2%) and ergotaminine (17%) of the German isolate in 2019 (subset II), where the proportion of the -inine form was observed 8.5 fold higher than for ergotamine. Subset II showed a similar spectrum and ranking regarding the proportion across the three isolates (DE, PL, AT) and both years (2018, 2019) (Figure 4b). The EAs with the highest proportions were ergocornine and α-ergocryptine for both subsets except for the German isolate of 2019 in subset II. For subset II, α-ergocryptine followed by ergotaminine and ergometrine were detected as the most abundant EAs. Ergocristinine could be found only in traces. Ergocristine showed a higher amount in subset II than in subset I, especially for the German isolate in 2019 with a proportion of 7% on the EA content. A table with the content of all single EAs determined by HPLC of subset I after inoculation with *C. purpurea* from 2018 and 2019 could be found in the Supplementary Material (Table S5).

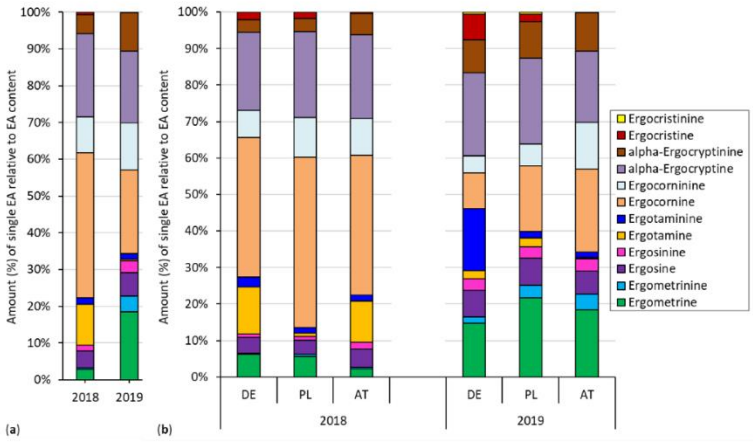

Figure 4. Amount (%) of single EAs relative to the EA content determined by HPLC after inoculation with *C. purpurea* of winter rye for 2018 and 2019 for (**a**): subset I and (**b**) subset II separated by three country-specific isolates (DE = German isolate, PL = Polish isolate, AT = Austrian isolate).

3. Discussion

For evaluating the covariation between ergot severity and EA content, artificial infection by spray inoculation was used. This is necessary to ensure a sufficiently high ergot infection and an even distribution across the experiment at all locations and years. Dung et al. [52] showed that natural ergot infection does not occur randomly within fields of Kentucky bluegrass (*Poa pratensis*) and that many factors, such as flowering, environmental or cultivation conditions, and occurrence of insects, contribute to disease proliferation. Nevertheless, Kodisch et al. [20] showed that ergot severity of genotypes after inoculation is highly correlated to that of natural infection. For our experimental set-up, it was previously described [34] that high heritabilities for ergot severity can be achieved. To achieve maximum variance necessary for calibration studies, we analyzed a dataset comprising nine locations in three countries, over two years, different rye genotypes, and three ergot isolates. We repeated each combination in the field experiment on two plots (replications).

3.1. Environment (Location × Year Combination) Has a High Impact

Ergot occurs naturally only sporadically and becomes a problem during wet and rainy weather conditions throughout the flowering stage [53]. Moist weather conditions around the time of meiosis have a negative effect on the production, viability, and movement of the pollen. Furthermore, honeydew becomes thick under warm and sunny conditions, which reduces ergot infections considerably [33]. Previous studies reported on a high influence of the environment on the ergot infection for sorghum (*Claviceps africana*) [54–56] and rye (*C. purpurea*) [20,34,57,58]. In our study, mean ergot severity showed a very large variation between environments ranging from 0.06% to 6.46% that might be caused by differences in weather despite artificial infection. These differences due to environments are highly significant, but clearly not predictable. The same location might have a low ergot severity in one year and a high severity in another year like WUL in our study (Figure 1). Therefore, we had highly differing ergot severities and EA contents according to the specific location × year combination. This explains, to some extent, the wide variation shown in Figure 1 and accords with the analysis of variance where the environment was the most important factor. The high genotype-by-environment interaction for ergot severity illustrates, once again, the necessity of multi-locational field trials [20,34,57,58]. One part of the environmental variation could be explained by the fact that ergot severity also depends on pollen shedding. Pollen shedding is affected by weather conditions as well [33]. With regard to the EA concentration, the mean ergot severity of an environment did not show any covariation with the mean level of EA concentration (Figure 1). For example, KOS 2019 and WOH 2019 nearly had the same ergot severity but differed in EA contents. On the other hand, the high EA contents of KLE 2019 or even PET and WUL 2019 are not mirrored in their ergot severity.

Ergot severity and EA content determined by HPLC had appreciable high heritabilities illustrating a strong genetic effect of these traits. EA contents determined by ELISA showed a moderate heritability only, which was likely caused by error-sensitive, adverse effects such as cross-reactivity or matrix effects. In conclusion, a clear influence of the environment affecting the ergot reaction and EA content measured by HPLC was detected.

3.2. Isolates Affect Ergot Severity and EA Formation in a Host-Unspecific Way

The isolates affected ergot severity and EA content measured by HPLC significantly, confirming earlier results [20]. The country-specific isolates in the balanced subset II in 2018 and across both years showed a similar ergot severity, but large differences concerning their EA quantity. Notably, the Austrian isolate produced a considerably higher EA quantity than the other isolates from Germany and Poland. In addition, Tittlemier et al. [59] showed that the isolate was a significant factor affecting EA concentrations in wheat sclerotia. Cagaš and Macháč [60] demonstrated for Kentucky bluegrass that isolates from different continents vary in their aggressiveness. Furthermore, Menzies et al. [19] showed a high pathogenic variation and significant differences among ergot isolates, according to the

geographic origin in wheat. In this study, no significant genotype-by-isolate interaction was detected for all traits, i.e., the genotypes reacted similarly to the three isolates. Thus, the origin of the isolate must not be considered with a high effort in future testing systems, but the aggressiveness of the inoculum is crucial for an optimal differentiation of the genotypes. Additionally, EAs do not seem to act as major virulence factors in the infection process because an increased EA content of an isolate does not reflect a higher disease severity. In this context, studies have to be done to investigate the virulence impact of EAs in the biology of the fungal infection process, e.g., with EA defective *Claviceps* strains. In the literature, it is discussed that EAs are important for resistance of the fungus against insects [6], mammals [8], or microbes [9]. However, the ecological function of the EAs is not entirely clarified yet. Therefore, further approaches are needed to reveal the general role of EAs for the lifestyle of the fungus. Remarkably, all three country-specific isolates showed a similar EA spectrum.

3.3. EA Spectrum Was Rather Stable across Years and Isolates

EA spectrum was rather similar for isolates and years. In the majority of cases, the amount of the -inine epimers of the respective EA was clearly lower compared to the -ine epimer, as already demonstrated by Franzmann et al. [61]. Despite the -inine forms being mentioned to be biologically inactive, EAs are able to epimerize under various conditions (alkaline, acidic) [15]. The -inine forms can also re-interconvert in the primary form during processing [15]. In contrast, Diana Di Mavungu et al. [62] demonstrated that epimerization was minimal during the analytical process. Additionally, some EAs seems to be more stable regarding epimerization than others [63]. In our study, ergocristinine and ergotaminine showed higher amounts than the respective -ine epimers in a few cases. The mechanisms of epimerization are not fully clarified yet [63], but this shift towards the - inine forms might be caused by specific conditions (heat, solvent solution, light) during the growing season, processing, or analysis. Nevertheless, it is known that the main EAs as well as their -inine forms are considered in future regulations to be caused by their ability for epimerization [15].

In the EA spectrum, a shift of ergometrine and ergotamine in subset I occurred across the years. Notably, higher amounts for ergometrine were detected in 2019 than in the previous year and, for ergotamine, the opposite occurred. An explanation could be different environmental, climate, or nutritional conditions during the growing season, which possibly affect the formation of individual EAs. The higher values of the ELISA analysis indicate a higher diversity for the EAs and metabolites when compared to the HPLC. Ergocornine and α-ergocryptine were detected as prime EAs in our HPLC study, whereas ergocristinine could be found only in traces. In literature, the abundance of individual EAs varies with the experiment. According to European Food Safety Authority (EFSA) [15], ergotamine, ergocristine, ergosine, and ergocornine were generally found to be more abundant than α-ergocryptine and ergometrine. Additionally, Mulder et al. [27] detected α-ergocryptine as major EA, followed by ergosine, ergocornine, and ergotamine. In commercially available rye flour and rye products, ergocristine and ergotamine were found to be the major EAs [28,61,64]. In diverse cereal samples, ergosine occurred most frequently, while the highest levels were observed for ergotamine, ergocristine, or ergosine, depending on the product type [62]. In contrast, Blaney et al. [65] determined ergotamine as the most prominent EA in Australian rye and ergocristine was only present in very small amounts. It is well known that different isolates can produce different types of alkaloids [33]. However, in our study, all three isolates had a similar pattern. In *Epichloë*, gains and losses of EA-influencing genes led to chemotypic variation [13]. Thus, changes and shifting of the EA spectrum seem to occur regularly.

3.4. Covariation of Ergot Severity and EA Content is Moderate

The covariation between ergot severity and EA content will get a great importance when the EU limits for ergot in cereals will be based on EA concentrations because their determination by HPLC is costly and time consuming. Therefore, there would be a high economic extra load, if all samples are determined by this approach. A reasonably narrow correlation would allow us to identify grain lots

with low ergot incidence and to concentrate the HPLC analyses on those samples with an EA level just above the limit. If this correlation is not useful, a rapid determination of EA concentrations by ELISA would be a good alternative. This technique could also be used by practical plant breeding companies to screen genotypes during the selection process. Therefore, we measured the EA content of our samples by both methods, the standard HPLC method, and one commercially available ELISA. HPLC analysis is a good and reliable tool for determining 12 EAs in a quantitative way [66], but the method needs a well-equipped laboratory with well-educated people [29,40]. In literature, ELISA approaches were already applied for evaluating EAs [67,68], but often with a lower number of samples and especially in the context of fescue toxicosis in livestock [69–71].

In our study, the results of the ErgoREAD ELISA analyses showed considerably higher EA contents for almost all samples when compared to HPLC with a notably high deviation in 2019. Accordingly, the distribution of the EA contents was completely different, as nearly all ELISA samples (92%) contained more than 1 mg/kg. Clearly, both methods measure EA concentrations in a completely different way. HPLC analysis determines the concentration of 12 main EAs, consisting of ergometrine, ergotamine, ergosine, ergocristine, α-ergocryptine, ergocornine, and the corresponding -inine epimers in a quantitative way [40]. In contrast, this ELISA is a qualitative method and measures lysergic acid as a progenitor in the EA pathway [32]. However, in the literature, >80 EAs are reported [5,10], so that, the individual 12 EAs analyzed by HPLC are only a subset of all existing EAs. Consequently, it is reasonable that the ELISA results overestimate the HPLC results. In this context, a varying consensus between both methods is also imaginable because the relative amount of the 12 analyzed EAs on all EAs can differ according to environmental conditions. Roberts et al. [70] already demonstrated that ergovaline concentrations determined by HPLC do not always correspond to the EA concentrations determined by ELISA. In addition, it is conceivable that matrix effects or cross-reactivity of components, which are comparable in structure falsify the results. Furthermore, the amount of cross-reactivity might vary between different groups of EAs [66] and usually ELISA approaches are less specific and accurate than HPLC methods [30]. However, all these factors cannot explain the 43-fold difference, on average, in EA concentrations between both methods (Table 1).

A comparison of three commercially available ELISA kits [30] showed that two out of three ELISA tests considerably overestimated the EA contents in flour in most cases when following the original protocol. The highest amounts were often found with the ErgoREAD ELISA. In addition, only one kit was reliable in a narrow range from 100–500 µg/kg [30]. In our study, the ErgoREAD Elisa was performed according to the manufacturer recommendations. Furthermore, the extraction procedure and processing seem to have a high impact on the output and the working ranges of the commercially available ELISA kits that are often not large enough even for natural infections [30]. However, a high dilution factor as always necessary for samples from artificial inoculation adds an additional error.

The covariation between EA content measured either by HPLC or ErgoREAD ELISA was basically zero in our study ($r = -0.04$). Schnitzius et al. [71] also demonstrated that no consistent pattern between ELISA and HPLC existed for samples from Kentucky blue grass. This indicates a discrepancy in the analytical detection due to a higher cross reactivity in the ELISA in comparison to the limited detection of EAs in the HPLC. Splitting the dataset into the factors isolate, environment, genotype, or infection level (ergot severity <0.05% and ≥0.05%) revealed no better relationship between both methods. A cause could be the different spectrum of chemical compounds analyzed by both methods. The HPLC focuses on the 12 selected EAs and cannot detect lysergic acid based on precursors and metabolites, which are not covered by the HPLC, as it is the case for ergopetides like ergovaline or higher protein conjugates, which could occur in nature. However, previous studies showed in *C. purpurea* that ergovalines seem to occur only in very low quantities [15]. Using the ELISA for a rapid screening of the EAs might give a higher security not to underestimate the EA concentration in a sample.

The samples in this approach were assembled by considering relevant criteria such as genotypes, environments (locations, years, country), and isolates to receive a maximal variation regarding ergot

severity and EA content. This is the usual procedure for calibration studies [66,72,73]. This study revealed only a moderate positive covariation between ergot severity and EA content determined by HPLC for both subsets, as previously demonstrated [27,69,74–76]. In our study, a large deviation from the regression reduced the coefficient of correlation to $r = 0.53$ (Figure S2a). A cause could be that, even in sclerotia of similar weight, the total amount of ergot alkaloids varied significantly [75]. The deviation is especially large for low ergot severities. For example, an ergot severity of 0.16% showed EA contents from 0.006 to 12.33 mg/kg. Conversely, with an EA content of 0.5 mg/kg, ergot severities varied from 0.01% to 9%. That implies that, for low EA concentrations, the prediction of the EA content based on the amount of ergot in grain is even more precarious, likely because the coefficient of (error) variation increases with declining EA concentrations [75]. Low EA concentrations, however, correspond even more to a practical situation than our study because screening studies of naturally infected rye and rye-derived products normally contain lower EA concentrations than those with artificial infection [1,17,26–29,36,77]. However, the grouping of samples after infection level (ergot severity <0.05% and ≥0.05%) did not improve the covariation in our study. The same is true for differentiating the samples according to genotype, environment, or isolate. Some locations showed high correlations, but this was often not stable across the years and is, therefore, not predictable for a single location. Although the Austrian isolate showed a high correlation for both years, this was not consistent for all other isolates. Therefore, a prognosis of the EA content based on the ergot amount is not possible because, in nature, there are always several isolates appearing.

In consequence, this study indicates that the EA content cannot be predicted in a reliable way based on ergot severity. Mulder et al. [27] and Schummer et al. [78] came to the same conclusion when analyzing a lower number of ergot samples from various cereals.

4. Conclusions

The isolates showed rather stable EA profiles even though they were obtained from three countries. There were slight differences among years. We got visible ergot infections in all environments. The ergot severity, however, cannot predict the EA concentrations in a given sample as measured by HPLC. This was even more clear for low ergot severities that are usually occurring in natural infections. Clearly, the EA contents have no relationship to disease severity and their absolute levels are not an important factor of the ergot infection in winter rye. Moreover, contents of individual EAs are most likely governed by different weather or physiological conditions than by ergot infection since only a moderate covariation could be found despite both traits having a similar high heritability when EA contents were measured by HPLC. To fulfill the future EU limits of 250 µg/kg for human consumption (except for baby food), HPLC analyses are necessary at least for those rye lots that visibly show some sclerotia. Many with a high sclerotia incidence will be rejected right away. However, even without visible infection in a sample, EAs might be present and may be caused by relocation within the ear or dust and abrasion from a few sclerotia during harvesting. The used ELISA did neither show a correlation to ergot severity nor to the EA concentrations measured by HPLC confirming previous findings. Further research is needed (1) to understand the factors influencing ergot infection more precisely as well as EA formation and their relationship and (2) to obtain a quick, cheap, and easy-to-use screening assay for the practical and commercial use. At the moment, the only recommendation could be for human consumption to concentrate on samples with minimal percentages of ergot sclerotia by visual means and to analyze them for their EA content by HPLC methods to meet the EU regulations.

5. Materials and Methods

5.1. Samples, Field Trials, and Inoculation Procedure

In total, 372 winter rye samples were used for this correlation study. The examined samples consisted of a diverse set of genotypes, locations, years, and isolates (Table 2). The following declaration is implemented in the study. Subset I consisted of all 372 winter rye samples and subset II consisted

of 288 winter rye samples orthogonally distributed across genotypes, isolates, and environments (location-year combinations). The experiment was performed in 2018 and 2019 at the following 10 locations: Oberer Lindenhof (OLI), Braunschweig (BRS), Wohlde (WOH), Petkus (PET), Wulfsode (WUL), and Kleptow (KLE) in Germany, Zwettl-Edelhof (EHO), and Hagenberg (HAG) in Austria, Kościelna Wieś (KOS), and Zybiszów (ZYB) in Poland. BRS could not be analyzed in 2018 due to missing infections, and HAG in 2019 due to severe hail destroying the crop shortly before harvest. A detailed list of locations and their characteristics can be found in the Supplementary Material (Table S1). The experiment was completely randomized with two replicates in a chessboard-like design. Therefore, each entry plot was surrounded by four plots of triticale (×Triticosecale Wittm.) as "border plots" [34]. Depending on the location, the size of the large-drilled plots ranged from 5.0 to 7.04 m^2. The varieties were supplied by the respective breeding companies (Table 2). Local triticale cultivars were grown as border plots.

Table 2. Genotype, breeding company, test environments (= location × year combinations), and inoculated isolate(s) of all 372 winter rye samples (= subset I) and the orthogonal samples (= subset II); FC(15) = 15 factorial crosses, DE = German isolate, PL = Polish isolate, AT = Austrian isolate, Mix = Mix of all isolates (DE, PL, AT).

Genotype	Breeding Company	Test Environments		Isolate(s)	No. of Samples
		Number	Name		
D.Amber [1]	"DANKO" Hodowla Roslin Sp. z o.o.	16	All [2]	DE, PL, AT	96
Elias [1]	Saatzucht LFS Edelhof	16	All [2]	DE, PL, AT	96
H_Hyb5 [1]	HYBRO Saatzucht GmbH & Co. KG	16	All [2]	DE, PL, AT	96
D.Amber	"DANKO" Hodowla Roslin Sp. z o.o.	2	OLI	DE	4
Elias	Saatzucht LFS Edelhof	2	OLI	DE	4
H_Hyb5	HYBRO Saatzucht GmbH & Co. KG	2	OLI	DE	4
Conduct	KWS LOCHOW GmbH	2	OLI	DE	4
K_Hyb2	KWS LOCHOW GmbH	2	OLI	DE	4
H_Pop	HYBRO Saatzucht GmbH & Co. KG	2	OLI	DE	4
FC(15)	KWS LOCHOW GmbH	2	OLI	Mix	60

[1] comprising subset II. [2] except BRS 2018, HAG 2019, OLI 2018, 2019.

Genotypes were inoculated with each of three inocula collected from sclerotia of infected rye in Germany (DE), Poland (PL), and Austria (AT) at nine locations in 2018 and 2019, comprising subset II. They were planted as split-plot design (main plot = isolate, subplot = genotype). Additionally, six other genotypes were inoculated at OLI in both years with the German isolate only and 15 factorial crosses (FC(15)) largely differing for their amount of pollen shedding with a mix of all three isolates (Table 2).

Seed density amounted to about 200 kernels/m^2 and sowing was done from mid-September to early October. Mineral fertilizers, herbicides, growth regulators, and fungicides were applied at each location in a conventional way. For inoculum production for both years of field testing, each country sent ergot samples in 2017 to the lab of B. Rodemann to ensure that the same inoculum was used for both years. The ergot samples were collected from rye stands from the main growing areas. As previously described in detail by Miedaner et al. [79], all inocula were produced for all location sites and years by Julius Kühn-Institute, Institute for Plant Protection in Field Crops and Grassland (Braunschweig, Germany) (JKI) by isolating *Claviceps purpurea*, according to Kirchhoff [80] and producing conidia suspension for inoculation on wheat-grain medium, according to Mielke [81] and Engelke [82]. Inoculation was started when the earliest 30% of the plots were fully flowering (BBCH 65, [83]) via spraying with a machine-driven field sprayer in the evening (5:30–9 p.m.) or in the morning (8–10:30 a.m.), repeated up to five times within a one-day to four-day interval, according to the temperature. The aim was that all plots were inoculated at least once at their respective flowering time. Harvesting was done by cutting a 1-m^2 subplot from the middle of the large plot by hand at the dough ripening stage (BBCH 85–89) using only primary tillers. Afterward, all heads of one plot were

air dried (30 °C) and threshed by a large single-head thresher (Pelz K 35, Saatzuchtbedarf Baumann, Waldenburg, Germany).

5.2. Sample Preparation

All sclerotia fragments were sorted out by hand or by an optical sorting machine (SATAKE Pikasen FMS 2000 F (2017/18)/ ANYSORT G64 #G64A-G112 (2018/19), Ruttmann, Hamburg, Germany) depending on the cooperation partner and the components (grain, ergot) were weighed to calculate ergot severity as a percentage of sclerotia relative to the total grain sample by weight. Afterward, a sub-sample of 200 g of grain and sclerotia were merged regarding the original ergot severity. This procedure was necessary because the samples from field plots were too large to handle. The 200-g sub-samples were milled (Ultra Centrifugal Mill ZM 200, 1 mm sieve, Retsch, Haan, Germany) and the flour was divided for HPLC and ELISA analyses.

5.3. High Performance Liquid Chromatography (HPLC) Analysis of EAs

HPLC analysis was conducted by the Austrian Agency for Health and Food Safety, Institute for Food Safety Linz, (AGES, Linz, Austria). Wet-chemical extraction, purification, and HPLC analysis were done according to BVL L 15.01/02-5:2012-01 [84] with some modifications. Briefly, EAs of 20 g flour were extracted with 100 mL of extraction solvent consisting of ethyl acetate ($C_4H_8O_2$)/methanol (CH_3OH)/ammonia (NH_3, 25%)/isopropyl alcohol (C_3H_8O) (75/5/7/7 (*v*/*v*/*v*/*v*)). Purifying of the extract with a solid-phase column (Alumina B) is followed by concentrating. EAs were separated using a Zorbax Eclipse XDB-C_{18}-HPLC-column (Agilent, Santa Clara, CA, USA) (Flow: 1 mL/min, Eluent: acetonitrile (C_2H_3N)/ ammonium carbamate ($CH_6N_2O_2$) solution 0.2 g/L, 50/50 (*v*/*v*)) and detected via Fluorescence detection (excitation λEx: 330 nm, emission λEm 415 nm). Calculating EA content was done by 3-point calibration and including multipliers according to the processing steps in the lab. The examined 12 EAs were: ergometrine, ergometrinine, ergosine, ergosinine, ergotamine, ergotaminine, ergocornine, ergocorninine, α-ergocryptine, α-ergocryptinine, ergocrystine, and ergocrystinine. For calculating the EA content, all individual EAs were summed up. In the following, the terms "ergot alkaloid content (determined) by HPLC" refer to the sum of these 12 EAs. All individual EA values below the limit of quantitation (LOQ < 0.02 mg/kg) were considered as zero. EHO 2018 showed no detectable level of EAs even though all samples were independently analyzed two times and was, therefore, not included in the respective analyses.

5.4. Enzyme-Linked Immunosorbent Assay (ELISA) of EAs

ErgoREAD ELISA, known as a competitive ELISA for detection of EAs in wheat, rye, and triticale samples, was purchased from LCTech GmbH (Daimlerstraße 4, Obertaufkirchen, Germany) and the analysis was performed at UHOH. A wet-chemical extraction of the EAs was carried out by mixing 20 g of the flour with 50 mL methanol (CH_3OH)/ phosphoric acid (H_3PO_4, 0.25%) (40/60 (*v*/*v*)) and shaking for 20 min (165 rpm, Orbital Shaker 3017, GFL Gesellschaft für Labortechnik GmbH, Burgwedel, Germany). The extract was filtered by rinsing through glass funnels with filter papers (Qualitative circles, 150 mm Ø, Cat No 1001 150, Whatman, Maidstone, United Kingdom). After dilution, the samples were filtered again with a syringe filter (pore size: 0.45 µmm, ø 15 mm, unster., PTFE, Rotilabo®, Roth, Karlsruhe, Germany) to remove all suspended solids. This was followed by proceeding the protocol of the ELISA Kit, measuring the extinction values by a microplate reader (Sunrise, Tecan Group Ltd., Männedorf, Switzerland) using the integrated Magellan software (Tecan Group Ltd., Männedorf, Switzerland) relative to the standard samples (0 ppb, 0.025 ppb, 0.1 ppb, 0.25 ppb, 0.5 ppb, 0.75 ppb, 1 ppb), covering the range from 0 to 5 ppm after taking the sample dilution factor into account. Higher concentrations of EAs could be analyzed by further sample dilution. Calculating the EA content was based on the company-owned software of LCTech GmbH (Obertaufkirchen, Germany). All samples were analyzed in duplicate.

5.5. Statistical Analyses

Single-plot data were used as a basis for all analyses. According to Bernal-Vasquez et al. [85], outlier tests were performed and detected outliers were considered as missing values. For conducting analyses of variance (ANOVA), a square-root transformation was done for all traits because the residuals were not normally distributed in any environment for biological reasons. ANOVA was first done for each location separately and, second, combined across locations for each trait using standard procedures [86]. For all ANOVAs, the effect of the factors 'genotype' and 'isolate' were considered as fixed and the factors 'replication' and 'environment' as random. A significance level of 0.05 or 0.01 was applied to all statistical calculations. The estimates of the ANOVA of the ratio of genotypic to phenotypic variance considering the number of replicates were used to calculate repeatability for each environment and entry-mean heritability (H^2) across all environments considering the number of replicates and environments, respectively [87]. The software packages R [88] and R-Studio (Version 3.5.1) [89] were used to perform the outlier test and ANOVAs for calculating the means and graphic visualization. The overall means reported in the tables were back-transformed. In the figures, the original means are reported. Multiple testing was conducted by a Tukey test as implemented in the R-studio.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6651/12/11/676/s1. Figure S1: Correlation of the replications (repeatability) of subset I of (a) ergot severity (%), and EAs measured with (b) HPLC, and (c) ELISA. Figure S2: Correlation of subset I after inoculation with *C. purpurea* across 18 environments between (a) ergot severity (%) and EA content determined by ELISA (mg/kg) and (b) EA content determined by HPLC and ELISA (mg/kg) (r = coefficient of correlation). Table S1: Location site, country, abbreviation, and characteristics of all field trials. Table S2: Means, ranges (in brackets), and least significant difference (LSD5%) for ergot severity (%), EAs (mg/kg) determined by HPLC, and ELISA for each genotype after inoculation with *C. purpurea* across 15 environments in subset II. Table S3: Estimates of variance components and entry-mean heritabilities for ergot severity (sq transf. = square root transformed) and EA contents determined by HPLC and ELISA after inoculation with *C. purpurea* across 15 environments in subset II. Table S4: Mean for ergot severity (%) and EAs (mg/kg) determined by HPLC and ELISA of subset II for each genotype and country-specific isolate (DE = German isolate, PL = Polish isolate, AT = Austrian isolate) across eight locations in 2018 and 2019. Table S5: Content (mg/kg, average) of single EAs determined with HPLC of subset I after inoculation with *C. purpurea* for 2018 and 2019.

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References

1. Wegulo, S.N.; Carlson, M.P. Ergot of Small Grain Cereals and Grasses and its Health Effects on Humans and Livestock. 2011. University of Nebraska, Extension, EC1880. Available online: http://ianrpubs.unl.edu/live/ec1880/build/ec1880.pdf (accessed on 9 March 2020).
2. Food and Agriculture Organization of the United Nations (FAO). FAOSTAT. 2020. Available online: http://www.fao.org/faostat/en/#data/QC (accessed on 12 March 2020).
3. Money, N.P. Fungi and biotechnology. In *The Fungi*, 3rd ed.; Watkinson, S.C., Boddy, L., Money, N.P., Eds.; Academic Press: Cambridge, MA, USA, 2016; ISBN 9780123820341. [CrossRef]

4. Florea, S.; Panaccione, D.G.; Schardl, C.L. Ergot alkaloids of the family Clavicipitaceae. *Phytopathology* **2017**, *107*, 504–518. [CrossRef]
5. Schiff, P.L. Ergot and its alkaloids. *Am. J. Pharm. Educ.* **2006**, *70*, 98. [CrossRef] [PubMed]
6. Potter, D.A.; Stokes, J.T.; Redmond, C.T.; Schardl, C.L.; Panaccione, D.G. Contribution of ergot alkaloids to suppression of a grass-feeding caterpillar assessed with gene knockout endophytes in perennial ryegrass. *Entomol. Exp. Appl.* **2008**, *126*, 138–147. [CrossRef]
7. Panaccione, D.G.; Arnold, S.L. Ergot alkaloids contribute to virulence in an insect model of invasive aspergillosis. *Sci. Rep.* **2017**, *7*, 8930. [CrossRef] [PubMed]
8. Panaccione, D.G.; Cipoletti, J.R.; Sedlock, A.B.; Blemings, K.P.; Schardl, C.L.; Machado, C.; Seidel, G.E. Effects of ergot alkaloids on food preference and satiety in Rabbits, as assessed with gene-knockout endophytes in perennial ryegrass (*Lolium perenne*). *J. Agric. Food Chem.* **2006**, *54*, 4582–4587. [CrossRef] [PubMed]
9. Venkatesh, N.; Keller, N.P. Mycotoxins in conversation with bacteria and fungi. *Front. Microbiol.* **2019**, *10*, 403. [CrossRef]
10. Křen, V.; Cvak, L. *Ergot: The Genus Claviceps*; Harwood Academic Publishers: Amsterdam, The Netherlands, 1999; pp. 173–200. [CrossRef]
11. Jakubczyk, D.; Cheng, J.Z.; O'Connor, S.E. Biosynthesis of the ergot alkaloids. *Nat. Prod. Rep.* **2014**, *31*, 1328. [CrossRef]
12. Schardl, C.L.; Panaccione, D.G.; Tudzynski, P. Ergot alkaloids—Biology and molecular biology. *Alkaloids. Chem. Biol.* **2006**, *63*, 45–86. [CrossRef]
13. Young, C.A.; Schardl, C.L.; Panaccione, D.G.; Florea, S.; Takach, J.E.; Charlton, N.D.; Moore, N.; Webb, J.S.; Jaromczyk, J. Genetics, genomics and evolution of ergot alkaloid diversity. *Toxins* **2015**, *7*, 1273–1302. [CrossRef]
14. Battilani, P.; Costa, L.G.; Dossena, A.; Gullino, M.L.; Marchelli, R.; Galaverna, G.; Pietri, A.; Dall'Asta, C.; Giorni, P.; Spadaro, D.; et al. Scientific Information on Mycotoxins and Natural Plant Toxicants. 2009. Scientific/Technical Report Submitted to EFSA. Available online: http://onlinelibrary.wiley.com/doi/10.2903/sp.efsa.2009.EN-24/pdf (accessed on 12 March 2020).
15. European Food Safety Authority (EFSA). Scientific Opinion on Ergot Alkaloids in Food and Feed. EFSA Panel on Contaminants in the Food Chain (CONTAM). 2012. EFSA 10: 2798. Available online: http://onlinelibrary.wiley.com/doi/10.2903/j.efsa.2012.2798/pdf (accessed on 12 March 2020).
16. Krska, R.; Crews, C. Significance, chemistry and determination of ergot alkaloids: A review. *J. Food Contam.* **2008**, *25*, 722–731. [CrossRef]
17. Malysheva, S.V.; Larionova, D.A.; Di Mavungu, J.D.; De Saeger, S. Pattern and distribution of ergot alkaloids in cereals and cereal products from European countries. *World Mycotoxin J.* **2014**, *7*, 217–230. [CrossRef]
18. Lin, W.; Kuang, Y.; Wang, J.; Duan, D.; Xu, W.; Tian, P.; Nzabanita, C.; Wang, M.; Li, M.; Ma, B. Effects of seasonal variation on the alkaloids of different ecotypes of *Epichloë* endophyte-*Festuca sinensis* associations. *Front. Microbiol.* **2019**, *10*, 1695. [CrossRef]
19. Menzies, J.G.; Klein-Gebbinck, H.W.; Gordon, A.; O'Sullivan, D. Evaluation of *Claviceps purpurea* isolates on wheat reveals complex virulence and host susceptibility relationships. *Can. J. Plant Pathol.* **2017**, *39*, 307–317. [CrossRef]
20. Kodisch, A.; Wilde, P.; Schmiedchen, B.; Fromme, F.J.; Rodemann, B.; Tratwal, A.; Oberforster, M.; Wieser, F.; Schiemann, A.; Jørgensen, L.N.; et al. Ergot infection in winter rye hybrids shows differential contribution of male and female genotypes and environment. *Eupyhtica* **2020**, *216*, 65. [CrossRef]
21. Crews, C. Analysis of ergot alkaloids. *Toxins* **2015**, *7*, 2024–2050. [CrossRef] [PubMed]
22. Flieger, M.; Wurst, M.; Shelby, R. Ergot alkaloids—Sources, structures and analytical methods. *Folia Microbiol.* **1997**, *42*, 3–30. [CrossRef] [PubMed]
23. Scott, P.M. Analysis of ergot alkaloids—A review. *Mycotoxin Res.* **2007**, *23*, 113–121. [CrossRef]
24. Schardl, C.L. Introduction to the toxins special issue on ergot alkaloids. *Toxins* **2015**, *7*, 4232–4237. [CrossRef]
25. Debegnach, F.; Patriarca, S.; Brera, C.; Gregori, E.; Sonego, E.; Moracci, G.; De Santis, B. Ergot alkaloids in wheat and rye derived products in Italy. *Foods* **2019**, *8*, 150. [CrossRef]
26. Meister, U.; Batt, N. Fusarium Toxins and Ergot Alkaloids in Rye of the Federal State Brandenburg Harvested 2013. In Proceedings of the 36th Mycotoxin Workshop, Göttingen, Germany, 16 June 2014; p. 131. Available online: http://www.mycotoxin-workshop.de/36th_Mycotoxin_Workshop_2014_-Proceedings.pdf (accessed on 12 March 2020).

27. Mulder, P.P.J.; van Raamsdonk, L.W.D.; van Egmond, H.J.; Voogt, J.; van Brakel, M.W.; van der Horst, G.M.; de Jong, J. Dutch Survey Ergot Alkaloids and Sclerotia in Animal Feeds. Report 2012.005. RIKILT. 2012. Available online: http://edepot.wur.nl/234699 (accessed on 12 March 2020).
28. Müller, C.; Kemmlein, S.; Klaffke, H.; Krauthaus, W.; Preiß-Weigert, A.; Wittkowski, R. A basic tool for risk assessment: A new method for the analysis of ergot alkaloids in rye and selected rye products. *Mol. Nutr. Food Res.* **2009**, *53*, 500–550. [CrossRef]
29. Ruhland, M.; Tischler, J. Determination of ergot alkaloids in feed by HPLC. *Mycotoxin Res.* **2008**, *24*, 73–79. [CrossRef] [PubMed]
30. Veršilovskis, A.; Pereboom-de Fauw, D.P.K.H.; Smits, N.; Mulder, P.P.J.; Mol, H.; de Nijs, M. EURL-MP-report_001. Screening of Ergot Alkaloids by ELISA Test Kits Available on the Market. 2019. EURL Mycotoxins and Plant Toxins, Wageningen Food Safety Research, Part of Wageningen University & Research. Available online: https://www.wur.nl/en/show/EURL-MP-report_001-Screening-of-ergot-alkaloids-using-ELISA-kits.htm (accessed on 12 October 2020).
31. Cell Signaling Technology (CST). Overview of Enzyme-Linked Immunosorbent Assay (ELISA). 2020. Available online: https://en.cellsignal.de/contents/_/overview-of-enzyme-linked-immunosorbent-assay-(elisa)/elisa-educational (accessed on 12 March 2020).
32. LCTech. ELISA Rapid Test ErgoREAD. 2020. Available online: https://www.lctech.de/en/products/elisa-rapid-test-ergoread.html (accessed on 11 March 2020).
33. Miedaner, T.; Geiger, H.H. Biology, genetics, and management of ergot (*Claviceps* spp.) in rye, sorghum, and pearl millet. *Toxins* **2015**, *7*, 659–678. [CrossRef] [PubMed]
34. Miedaner, T.; Mirdita, V.; Rodemann, B.; Drobeck, T.; Rentel, D. Genetic variation of winter rye cultivars for their ergot (*Claviceps purpurea*) reaction tested in a field design with minimized interplot interference. *Plant Breed.* **2010**, *129*, 58–62. [CrossRef]
35. Hulvová, H.; Galuszka, P.; Frébortová, J.; Frébort, I. Parasitic fungus *Claviceps* as a source for biotechnological production of ergot alkaloids. *Biotechnol. Adv.* **2013**, *31*, 79–89. [CrossRef]
36. Van Dongen, P.W.; de Groot, A.N. History of ergot alkaloids from ergotism to ergometrine. *Eur. J. Obs. Gynecol. Reprod. Biol.* **1995**, *60*, 109–116. [CrossRef]
37. Coufal-Majewski, S.; Stanford, K.; McAllister, T.; Blakley, B.; McKinnon, J.; Chaves, A.V.; Wang, Y. Impacts of cereal ergot in food animal production. *Front. Vet. Sci.* **2016**, *3*, 15. [CrossRef]
38. European Communities. 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.
39. European Union. 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.
40. Beuerle, T.; Benford, D.; Brimer, L.; Cottrill, B.; Doerge, D.; Dusemund, B.; Farmer, P.; Fürst, P.; Humpf, H.; Mulder, P.P.J. Scientific opinion on ergot alkaloids in food and feed. *EFSA J.* **2012**, *10*, 2798–2956. [CrossRef]
41. Byrd, N.; Slaiding, I.R. Final Project Report: Monitoring of Mycotoxins and Other Contaminants in UK Cereals Used in Malting, Milling and Animal Feed. AHDB PR578. 2017. Available online: https://ahdb.org.uk/final-project-report-contaminants-monitoring-150517 (accessed on 12 March 2020).
42. MacDonald, S.J.; Anderson, W.A.C. Final Project Report: A Desk Study to Review Current Knowledge on Ergot Alkaloids and Their Potential for Contamination to Cereal Grains. AHDB PR575. 2017. Available online: https://ahdb.org.uk/a-desk-study-to-review-current-knowledge-on-ergot-alkaloids-and-their-potential-for-contamination-to-cereal-grains (accessed on 12 March 2020).
43. Gordon, A.; Delamare, G.; Tente, E.; Boyd, L. Final Project Report: Determining the Routes of Transmission of Ergot Alkaloids in Cereal Grains. AHDB PR603. 2019. Available online: https://ahdb.org.uk/determining-the-routes-of-transmission-of-ergot-alkaloids-in-cereal-grains (accessed on 12 March 2020).
44. Topi, D.; Jakovac-Strajn, B.; Pavšič-Vrtač, K.; Tavčar-Kalcher, G. Occurrence of ergot alkaloids in wheat from Albania. *Food Addit. Contam. Part A* **2017**, *34*, 1333–1343. [CrossRef]
45. Bryła, M.; Ksieniewicz-Woźniak, E.; Waśkiewicz, A.; Podolska, G.; Szymczyk, K. Stability of ergot alkaloids during the process of baking rye bread. *LWT* **2019**, *110*, 269–274. [CrossRef]
46. Meleard, B. Degradation and Epimerization of Wheat Ergot Alkaloids during French Baking Test. 2016. Available online: https://www.english.arvalisinstitutduvegetal.fr/file/galleryelement/pj/84/76/8a/c1/meleardalkaloids_and_bread_mytox739905815900991417.pdf (accessed on 12 March 2020).

47. Tittlemier, S.A.; Drul, D.; Roscoe, M.; Turnock, D.; Taylor, D.; Fu, B.X. Fate of ergot alkaloids during laboratory scale durum processing and pasta production. *Toxins* **2019**, *11*, 195. [CrossRef]
48. Canadian Grain Commission. Harvest Survey of Canadian Grain Quality. 2013. Available online: http://www.grainscanada.gc.ca/quality-qualite/hsp-per/hs-er-eng.html (accessed on 12 March 2020).
49. Alderman, S. Ergot: Biology and Control. 2006. Available online: http://www.ars.usda.gov/SP2UserFiles/person/81/ErgotDVDtranscript.pdf (accessed on 4 September 2020).
50. Gordon, A.; Basler, R.; Bansept-Basler, P.; Fanstone, V.; Harinarayan, L.; Grant, P.K.; Birchmore, R.; Bayles, R.A.; Boys, L.A.; O'Sullivan, D.O. The identification of QTL controlling ergot sclerotia size in hexaploid wheat implicates a role for the *Rht* dwarfing alleles. *Theor. Appl. Genet.* **2015**, *128*, 2447–2460. [CrossRef] [PubMed]
51. Gordon, A.; McCartney, C.; Knox, R.E.; Ereful, N.; Hiebert, C.W.; Konkin, D.J.; Hsueh, Y.C.; Bhadauria, V.; Sgroi, M.; O'Sullivan, D.M.; et al. Genetic and transcriptional dissection of resistance to *Claviceps purpurea* in the durum wheat cultivar Greenshank. *Theor. Appl. Genet.* **2020**, *133*, 1873–1886. [CrossRef]
52. Dung, J.K.S.; Cheng, Q.; Kaur, N.; Walenta, D.L.; Cating, R.A.; Rondon, S.I.; Frost, K.E.; Alderman, S.C.; Hamm, P.B. Population biology and epidemiology of *Claviceps purpurea* in cool-season grass seed crops. In Proceedings of the 10th International Seed Conference, Corvallis, OR, USA, 12–19 May 2019; p. 78.
53. Menzies, J.G.; Turkington, T.K. An overview of the ergot (*Claviceps purpurea*) issue in western Canada: Challenges and solutions. *Can. J. Plant Pathol.* **2015**, *37*, 40–51. [CrossRef]
54. Wang, E.; Meinke, H.; Ryley, M.; Herde, D.; Henzell, B. On the relation between weather variables and sorghum ergot infection. *Aust. J. Agric. Res.* **2000**, *51*, 313–324. [CrossRef]
55. Workneh, F.; Rush, C.M. Evaluation of relationships between weather patterns and prevalence of sorghum ergot in the Texas panhandle. *Phytopathology* **2002**, *92*, 659–666. [CrossRef]
56. Workneh, F.; Rush, C.M. Weather factors associated with development of sorghum ergot in the Texas panhandle. *Plant Dis.* **2006**, *90*, 717–722. [CrossRef]
57. Dhillon, B.S.; Mirdita, V.; Miedaner, T. Preliminary evaluation of locations for conducting selection for resistance to ergot (*Claviceps purpurea*) in rye. *Indian J. Genet. Pl. Br.* **2010**, *23*, 265–268.
58. Mirdita, V.; Miedaner, T. Resistance to ergot in self-incompatible germplasm resources of winter rye. *J. Phytopathol.* **2009**, *157*, 350–355. [CrossRef]
59. Tittlemier, S.A.; Drul, D.; Roscoe, M.; Menzies, J.G. The effect of selected factors on measured ergot alkaloid content in *Claviceps purpurea*-infected hexaploid and durum wheat. *World Mycotoxin J.* **2016**, *9*, 555–564. [CrossRef]
60. Cagaš, B.; Macháč, R. Different pathogenicity of ergot isolates (*Claviceps purpurea* [Fr.] Tul.) on Kentucky bluegrass (*Poa pratensis* L.). *Plant Prot. Sci.* **2002**, *38*, 18–22. [CrossRef]
61. Franzmann, C.; Wächter, J.; Dittmer, N.; Humpf, H.S. Ricinoleic acid as a marker for ergot impurities in rye and rye products. *J. Agric. Food Chem.* **2010**, *58*, 4223–4229. [CrossRef] [PubMed]
62. Di Mavungu, D.J.; Malysheva, S.V.; Sanders, M.; Larionova, D.; Robbens, J.; Dubruel, P.; Peteghem, C.V.; De Saeger, S. Development and validation of a new LC–MS/MS method for the simultaneous determination of six major ergot alkaloids and their corresponding epimers. Application to some food and feed commodities. *Food Chem.* **2012**, *135*, 292–303. [CrossRef]
63. Schummer, C.; Zandonella, I.; van Nieuwenhuyse, A.; Moris, G. Epimerization of ergot alkaloids in feed. *Helyion* **2020**, 6. [CrossRef]
64. Kniel, B.; Meißner, M.; Koehler, P.; Schwake-Anduschus, C. Studies on the applicability of HPLC-FLD and HPLC–MS/MS for the determination of ergot alkaloids in rye-containing breads. *J. Consum. Prot. Food S.* **2018**, *13*, 69–78. [CrossRef]
65. Blaney, B.J.; Molloy, J.B.; Brock, I.J. Alkaloids in Australian rye ergot (*Claviceps purpurea*) sclerotia: Implications for food and stockfeed regulations. *Anim. Prod. Sci.* **2009**, *49*, 975–982. [CrossRef]
66. Shi, H.; Yu, P. Exploring the potential of applying infrared vibrational (micro)spectroscopy in ergot alkaloids determination: Techniques, current status, and challenges. *Appl. Spectrosc. Rev.* **2018**, *53*, 395–419. [CrossRef]
67. Shelby, R.A.; Kelley, V.C. Detection of ergot alkaloids from *Claviceps* species in agricultural products by competitive ELISA using a monoclonal antibody. *J. Agric. Food Chem.* **1992**, *40*, 1090–1092. [CrossRef]
68. Tunali, B.; Shelby, R.A.; Morgan-Jones, G.; Kodan, M. Endophytic fungi and ergot alkaloids in native Turkish grasses. *Phytoparasitica* **2000**, *28*, 375–377. [CrossRef]
69. Kenyon, S.A.; Roberts, C.A.; Kallenbach, R.L.; Lory, J.O.; Kerley, M.S.; Rottinghaus, G.E.; Hill, N.S.; Ellersieck, M.R. Vertical distribution of ergot alkaloids in the vegetative canopy of tall fescue. *Crop Sci.* **2018**, *58*, 925–931. [CrossRef]

70. Roberts, C.A.; Davis, D.K.; Looper, M.L.; Kallenbach, R.L.; Rottinghaus, G.E.; Hill, N.S. Ergot alkaloid concentrations in high- and low-moisture tall fescue silage. *Crop Sci.* **2014**, *54*, 1887–1892. [CrossRef]
71. Schnitzius, J.M.; Hill, N.S.; Thompson, C.S.; Craig, A.M. Semiquantitative determination of ergot alkaloids in seed, straw, and digesta samples using a competitive enzyme-linked immunosorbent assay. *J. Vet. Diagn. Invest.* **2001**, *13*, 230–237. [CrossRef] [PubMed]
72. Hu, Z.; Ao, D.; Mahadevan, S. Calibration experimental design considering field response and model uncertainty. *Comput. Methods Appl.* **2017**, *318*, 92–119. [CrossRef]
73. Raposo, F. Evaluation of analytical calibration based on least-squares linear regression for instrumental techniques: A tutorial review. *Trends Anal. Chem.* **2006**, *77*, 167–185. [CrossRef]
74. Bryła, M.; Ksieniewicz-Woźniak, E.; Podolska, G.; Waśkiewicz, A.; Szymczyk, K.; Jędrzejczak, R. Occurrence of ergot and its alkaloids in winter rye harvested in Poland. *World Mycotoxin J.* **2018**, *11*, 635–646. [CrossRef]
75. Grusie, T.; Cowan, V.; Singh, J.; McKinnon, J.; Blakley, B. Correlation and variability between weighing, counting and analytical methods to determine ergot (*Claviceps purpurea*) contamination of grain. *World Mycotoxin J.* **2017**, *10*, 209–218. [CrossRef]
76. Orlando, B.; Maumené, C.; Piraux, F. Ergot and ergot alkaloids in French cereals: Occurrence, pattern and agronomic practices for managing the risk. *World Mycotoxin J.* **2017**, *10*, 327–338. [CrossRef]
77. Byrd, N.; De Alwis, J.; Booth, M.; Jewell, K. Final Project Report: Monitoring the Presence of Ergot Alkaloids in Cereals and a Study of a Possible Relationship between Occurrence of Sclerotia Content and Levels of Ergot Alkaloids. Project Number FS516009.2014. Available online: https://www.food.gov.uk/sites/default/files/media/document/FS516009%20Final%20Ergot%20Alkaloid%20report%20(3).pdf (accessed on 18 June 2020).
78. Schummer, C.; Brune, L.; Moris, G. Development of a UHPLC-FLD method for the analysis of ergot alkaloids and application to different types of cereals from Luxembourg. *Mycotoxin Res.* **2018**, *34*, 279–287. [CrossRef] [PubMed]
79. Miedaner, T.; Dänicke, S.; Schmiedchen, B.; Wilde, P.; Wortmann, H.; Dhillon, B.S.; Mirdita, V. Genetic variation for ergot (*Claviceps purpurea*) resistance and alkaloid concentrations in cytoplasmic-male sterile winter rye under pollen isolation. *Euphytica* **2010**, *173*, 299–306. [CrossRef]
80. Kirchhoff, H. Beiträge zur Biologie und Physiologie des Mutterkornpilzes. *Centralbl. Bakteriol. Parasitenk. Abt. II* **1929**, *77*, 310–369.
81. Mielke, H. Untersuchungen zur Bekämpfung des Mutterkorns. *Nachr. Deut. Pflanzenschutzd.* **1993**, *45*, 97–102.
82. Engelke, T. Ansätze für eine integrierte Bekämpfung des Mutterkorns (*Claviceps purpurea* [Fr.] Tul.) im Roggen. Ph.D. Thesis, University of Göttingen, Göttingen, Germany, 2002.
83. Meier, U. Growth Stages of Mono- and Dicotyledonous Plants. BBCH Monograph. 2001. Available online: https://www.julius-kuehn.de/media/Veroeffentlichungen/bbch%20epaper%20en/page.pdf (accessed on 4 April 2020).
84. BVL L 15.01/02–5:2012–01. Untersuchung von Lebensmitteln—Bestimmung von Ergotalkaloiden in Roggen und Weizen—HPLC-Verfahren mit Reinigung an einer basischen Aluminiumoxid-Festphase. 2012. Available online: https://www.beuth.de/de/technische-regel/bvl-l-15--01--02--5/150736503 (accessed on 4 April 2020).
85. Bernal-Vasquez, A.M.; Utz, H.F.; Piepho, H.P. Outlier detection methods for generalized lattices: A case study on the transition from ANOVA to REML. *Theor. Appl. Genet.* **2016**, *129*, 787–804. [CrossRef] [PubMed]
86. Cochran, W.G.; Cox, G.M. *Experimental Designs*; Wiley: New York, NY, USA, 1957; ISBN 978-0-471-54567-5.
87. Fehr, W.R. *Principles of Cultivar Development, Theory and Technique*; Macmillan: New York, NY, USA, 1987; Volume 1, ISBN 0029499208.
88. R Core Team. *R: A Language and Environment for Statistical Computing*; R Foundation for Statistical Computing: Vienna, Austria, 2018; ISBN 3–900051–07–0.
89. RStudio Team. RStudio: Integrated Development for R. RStudio, Inc., Boston. 2016. Available online: https://www.rstudio.com/ (accessed on 4 March 2020).

Supplementary Materials: Covariation of Ergot Severity and Alkaloid Content Measured by HPLC and One ELISA Method in Inoculated Winter Rye across Three Isolates and Three European Countries

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Table S1. Location site, country, abbreviation and characteristics of all field trials.

Location Site	Country	Abbreviation	Soil Type	Mean Annual Temperature [°C]	Mean Precipitation [mm]
Ob. Lindenhof (48°28'25.5"N 9°18'17.9"E)	Germany	OLI	Brown soil	6.6	952
Braunschweig (52°16'33.4"N 10°34'09.3"E)	Germany	BRS	Sandy loam	9.3	570
Wohlde (52°48'48.7"N 9°59'53.1"E)	Germany	WOH	Sand	9.3	950
Petkus (51°58'50.3"N 13°21'01.7"E)	Germany	PET	Sand	8.5	596
Wulfsode (53°03'45.6"N 10°14'02.5"E)	Germany	WUL	Sand	9.3	852
Kleptow (53°21'54.9"N 14°00'04.8"E)	Germany	KLE	Loamy Sand	9.6	475
Zwettl-Edelhof (48°36'23.5"N 15°13'13.3"E)	Austria	EHO	Brown soil	7.7	657
Hagenberg (48°22'28.4"N 14°30'51.2"E)	Austria	HAG	Brown soil	9.1	772
Kościelna Wieś (51°46'28.7"N 18°00'58.0"E)	Poland	KOS	Brown soil	8.6	510
Zybiszów (51°03'51.9"N 16°54'45.4"E)	Poland	ZYB	Degraded black soil	9.1	571

Table S2. Means, ranges (in brackets) and least significant difference (LSD5%) for ergot severity (%), EAs (mg/kg) determined by HPLC and ELISA for each genotype after inoculation with Claviceps purpurea across 15 environments in subset II.

Genotype	Ergot Severity (%)	EAs, HPLC (mg/kg)	EAs, ELISA (mg/kg)
H_Hyb5	1.78 (0.01–9.3) a[1]	10.50 (0–337.13) a	473.9 (0.2–13,728.7) a
D.Amber	1.47 (0.02–10.5) ab	11.41 (0–239.32) a	441.2 (0.4–7595.9) a
Elias	1.32 (0.02–8.48) b	12.92 (0–498.40) a	494.5 (0.3–5387.6) a
Mean	1.53	11.61	468.9
LSD5%	0.32	7.44	218.8

[1] Treatments with the same letter are not significantly different (Tukey test, $p < 0.05$)

Table S3. Estimates of variance components and entry-mean heritabilities for ergot severity (sq transf. = square root transformed) and EA contents determined by HPLC and ELISA after inoculation with Claviceps purpurea across 15 environments in subset II.

Parameter	Degrees of Freedom	Ergot Severity (%) (sq Transf.)	HPLC (mg/kg) (sq Transf.)	ELISA (mg/kg) (sq Transf.)
Variance components: Environment (E)	14	6.122 **	67.04 **	3051.2 **
Genotype (G)	2	1.615 **	0.21	14.5
Isolate (I)	2	2.012 **	83.01 **	322.6
G × E	28	0.139 **	0.97	70.1
G × I	4	0.031	2.56	96.6
G × E × I	56	0.040	2.02	217.2 *
Error	120	0.037	1.57	130.9
Heritability		0.92	0.82	0.57

*, **: significant at $p < 0.1$, $p < 0.05$ and $p < 0.01$, respectively

Table S4. Mean for ergot severity (%) and EAs (mg/kg) determined by HPLC and ELISA of subset II for each 48 genotype and country-specific isolate (DE = German isolate, PL = Polish isolate, AT = Austrian isolate) across 8 49 locations in 2018 and 2019.

Trait	Year	Isolate	D.Amber	Elias	H_Hyb550
Ergot severity (%)	2018	DE	2.14	1.71	51 2.27
		PL	2.17	1.70	2.3452
		AT	1.20	1.63	1.75
	2019	DE	0.59	0.57	0.8553
		PL	1.16	0.84	1.39 54
		AT	1.58	1.49	2.09
HPLC (mg/kg)	2018	DE	1.23	0.43	1.6555
		PL	5.71	4.50	8.43
		AT	33.96	52.20	56 26.76
	2019	DE	7.43	6.33	11.4657
		PL	0.75	0.84	0.80 58
		AT	20.19	15.51	14.55
ELISA (mg/kg)	2018	DE	6.94	3.28	11.9859
		PL	26.66	20.84	32.51
		AT	23.77	27.96	60 18.68
	2019	DE	1195.29	1064.69	317.8861
		PL	1433.75	1490.36	2114.36
		AT	113.27	539.17	62 176.55

Table S5. Content (mg/kg, avarage) of single EAs determined with HPLC of subset I after inoculation with 66 Claviceps purpurea for 2018 and 2019.

Single EAs	Content (HPLC, mg/kg)	
	2018	2019
Ergometrine	538.93	1659.09
Ergometrinine	88.18	308.06
Ergosine	717.14	712.09
Ergosinine	227.05	341.01
Ergotamine	1916.17	133.38
Ergotaminine	300.92	572.89
Ergocornine	4783.26	1609.53
Ergocorninine	1192.67	821.94
alpha-Ergocryptine	2874.44	2367.83
alpha-Ergocryptinine	680.90	975.78
Ergocristine	1170.44	262.17
Ergocristinine	226.69	45.80

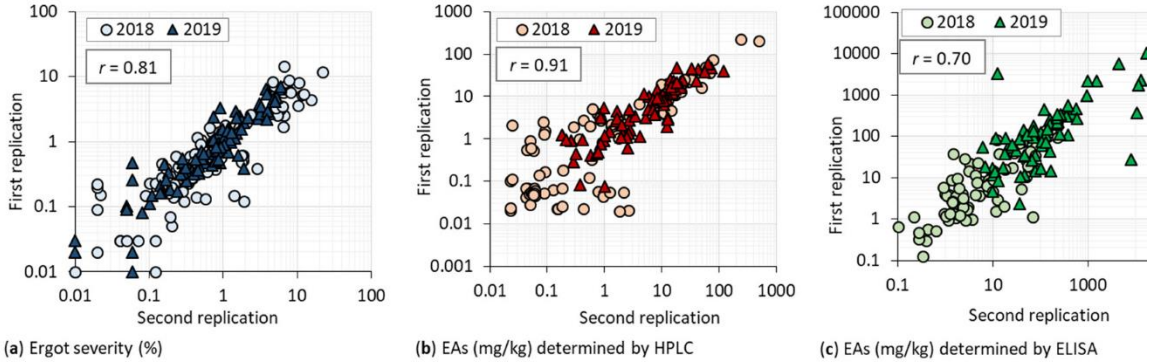

Figure S1. Correlation of the replications (repeatability) of subset I of (**a**) ergot severity (%), and EAs (mg/kg) determined by (**b**) HPLC, and (**c**) ELISA.

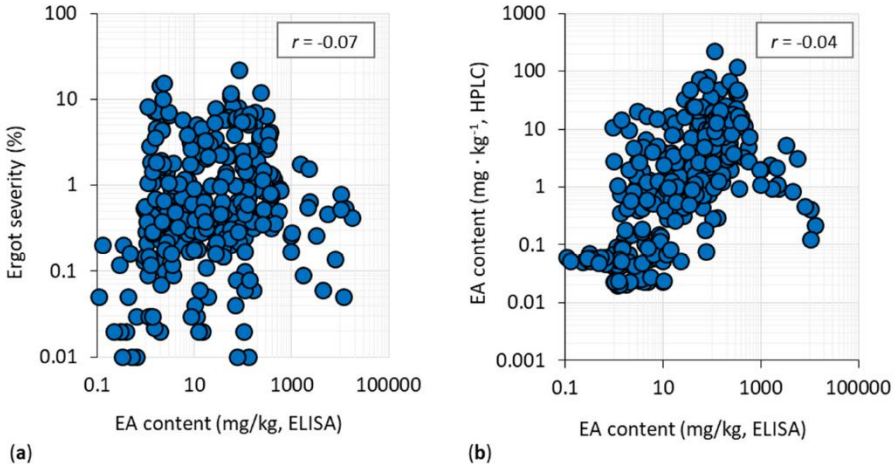

Figure S2. Correlation of subset I after inoculation with *Claviceps purpurea* across 18 environments between (**a**) ergot severity (%) and EA content determined by ELISA (mg/kg) and (**b**) EA content determined by HPLC and ELISA (mg /kg) (*r* = coefficient of correlation).

5. Publication III: Maternal differences for the reaction to ergot in unfertilized hybrid rye (*Secale cereale*)[3)]

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Maternal differences for the reaction to ergot in unfertilized hybrid rye (*Secale cereale*)

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Abstract *Claviceps purpurea* causing ergot maintains to be a problem in commercial cytoplasmic male sterile (CMS)-based hybrid rye growing. The fungal spores compete with pollen during flowering and ergot incidence is reduced in highly pollen-shedding stands. This study was carried out to identify maternal differences in ergot infection in the absence of pollen. Ten male-sterile single crosses were tested by needle and spray inoculation and kept unfertilized in up to four field sites (Germany, Austria) and three greenhouse experiments, respectively, in two years. A medium to high correlation was observed between field (needle inoculation) and greenhouse (spray inoculation) experiments. The environments (=location × year combinations) differed in their ergot severity and ergot incidence. Significant ($P \leq 0.05$) genotypic and genotype × environment interaction variances were detected for the unfertilized male-sterile single crosses in both test systems for both traits. The single cross K_4 showed a significantly lower ergot severity averaged across all environments, thus being more resilient to ergot than the other genotypes. In conclusion, spray and needle inoculation are suitable for testing unfertilized male-sterile rye materials, testing across several environments (locations, years) is definitely necessary. Selection of specific females might give the potential for further reducing ergot contamination in hybrid rye in future. The frequency of such genotypes within larger breeding populations needs to be analyzed.

Keywords *Claviceps purpurea* · Maternal effect · Resistance mechanism · Needle inoculation · Ergot · Rye

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Introduction

The plant pathogen *Claviceps purpurea* ((Fr.: Fr.) Tul.) is the causal fungus for a severe disease of the grass inflorescences called ergot. The fungus is distributed worldwide with a large host range containing more than 400 grass species (Wegulo & Carlson, 2011). After infection, instead of kernels sclerotia are produced (Pažoutová, 2002) that are a compact mass of fungal mycelium with a purplish-black outside layer (Wegulo & Carlson, 2011). The life cycle of ergot was already described in detail (Mielke, 2000; Schumann & Uppala, 2000; Tenberge, 1999; Tudzynski et al., 1995). *C. purpurea* cannot penetrate through intact glumes, so, an infection is only possible at or shortly after

flowering (Kirchhoff, 1929). Hence, cross-pollinated plants such as rye (*Secale cereale* L.) are particularly vulnerable to ergot (Mielke, 2000; Tudzynski et al., 1995). A high amount of pollen reduces ergot infection considerably (Kodisch et al., 2020a; Miedaner et al., 2010a; Miedaner et al., 2017; Thakur & Williams, 1980) due to the competitive situation of pollen and spores during flowering (Miedaner & Geiger, 2015). Cool and rainy weather conditions at flowering promote *Claviceps* infections because the opening of the unfertilized flowers is prolonged, pollen dispersal largely reduced and fungal infection is supported (Miedaner & Geiger, 2015). Recommendations for preventing ergot contamination are supporting the density and homogeneity of the rye stand by agronomic measures (early seeding, high seed density, appropriate nitrogen application), avoidance of lodging, grassy weed control within and around the fields including farm tracks, and growing of less susceptible cultivars (Alderman, 2006; Betz & Mielke, 1996; Thakur & Williams, 1980). Because no fungicides are registered on the market for ergot control, a resistance mechanism would be beneficial. However, just a few quantitative trait loci (QTL) related to partial resistance to ergot are known in wheat. These are influencing different components of the infection process like the hormonal pathway in durum wheat (Gordon et al., 2020) or size and weight of sclerotia in bread wheat (Gordon et al., 2015).

Ergot normally does not lead to an appreciable yield reduction, but the contamination of the grain by ergot alkaloids (EAs, Florea et al., 2017) is a serious threat, because they are toxic to humans and mammals (Hulvová et al., 2013). EAs occurred frequently in several studies in different countries and among all common cereals (Beuerle et al., 2012; Schwake-Anduschus et al., 2020; Topi et al., 2017). The European Union (EU) limits for ergot sclerotia and sclerotial fragments in unprocessed cereals are set to 0.5 g/kg for human consumption and 500 µg/kg for the sum of the six most common EAs (ergometrine, ergosine, ergotamine, ergocornine, α-ergocryptine and ergocrystine) and their –inine forms on rye milling products for consumers (European Union, 2021). For infants and young children, the maximum level is 20 µg EAs/kg. As from 01.07.2024 the limits for human nutrition will be further reduced to 0.2 g/kg on unprocessed rye and 250 µg EAs/kg on rye milling products for consumers. For animal feeding a guidance value of 1.0 g/kg is given (European Union, 2012). So, ergot-free rye lots have a high economic impact for breeders, farmers, and milling companies.

In 2021, rye was grown on 2.1 million hectares in the EU with a production volume of 8.5 million tons. The largest producers were Germany and Poland with 73% therefrom (Eurostat, 2022). In Germany, 70–80% of the area is grown by hybrid cultivars with increasing importance through the years (BMEL, 2021; BSL, 2020). Hybrid cultivars in rye are based on cytoplasmic-male sterility (CMS) induced by a special cytoplasm in the female parent, the male parent contributes nuclear-encoded genes of restorer-to-fertility (*Rf*) that should fully restore pollen shedding of the resulting hybrids (Miedaner & Laidig, 2019). In reality, however, restoration ability and, thus, pollen shedding ranges from poor to excellent due to the *Rf* genes used (Miedaner et al., 2005).

Besides pollen-fertility restoration, ergot infection in rye is affected by (1) morphological measures leading to a disease escape like closed flowering, small aperture angle of the glumes or a short glume opening period, (2) the ease of restoration by the female and (3) a resistance mechanism in the ovaries that has, however, not been fully proven, yet (Menzies & Turkington, 2015; Miedaner & Geiger, 2015). Recently, transcriptomic analyses in wheat showed that shortly after the conidia have reached the pistil, plant resistance is already activated (Boyd et al., 2020). Small, but significant differences of ergot severity were already shown for fully male-sterile entries under pollen isolation in rye (Miedaner et al., 2010b) and pearl millet (Willingale et al., 1986). To evaluate whether genetic differences occur due to the host resistance, we inoculated cytoplasmically-male sterile (CMS) rye without the availability of pollen, i.e. all flowers remained unfertilized. Our hypothesis is, that despite these extreme conditions, differences in their ergot reaction will occur that might lead in future to the detection of resistance mechanisms.

In particular, we aimed to (1) verify the role of the female parent by analyzing CMS single crosses that remain unfertilized during ergot infection, (2) partition genotypic and environmental variation and their interaction and (3) test the suitability of needle vs. spray inoculation. Ten CMS single crosses of winter rye were tested in eight field and six greenhouse trials in Germany and Austria with needle inoculation in the field and spray inoculation in the greenhouse without the availability of rye pollen in each trial.

Materials and methods

Plant material, experimental design, and cultivation conditions

This study was conducted with ten sterile CMS - single crosses of winter rye of two inbred lines (A × B) of the Petkus gene pool in the greenhouse and in field trials in each of three and four sites, respectively, in Germany and Austria in 2018 and 2019 (Table 1). Seeds were provided by the respective breeding companies: five CMS - single crosses by KWS LOCHOW GMBH (KWL, Bergen, Germany) (CMS_K1 – CMS_K5), and five CMS - single crosses by HYBRO Saatzucht GmbH & Co. KG (HYBRO, Schenkenberg, Germany) (CMS_H1 – CMS_H5). The same ten CMS single crosses were evaluated in all experiments.

In the greenhouse experiments, seeds of the ten CMS single crosses were previously sown in planting trays (53 × 40 × 9.5 cm^3) in September. In October, the entries were potted in a planting pot (13 × 13 × 13 cm^3, volume 1,5 l) or in a Kick-Brauckmann vessel (7 l volume, Vienna) and stored outside or in a cold greenhouse until February or April to fulfill the vernalization. In Austria (Vienna, Hagenberg), the trials were sown directly into the Kick-Brauckmann vessels. After that, the plants were handled in greenhouses. In the greenhouse experiments, the entries were grown according to a randomized complete block (RCB) design with four replications.

The field trials were grown on large-drilled plots ranging from 5.4 to 6.9 m^2 (dependent on the location) in a RCB design with four replications in a chessboard-like design. Each entry plot was surrounded by four plots of a locally grown Triticale (×*Triticosecale Wittm.*) as border plots. In Hagenberg, the field trials were conducted without border plots due to space limitations. Sowing was done in the end of September or early in October with a kernel density of 200 kernels m^{-2}. Application of mineral fertilizers, herbicides, growth regulators and fungicides was performed by each location in a conventional way.

Inoculation, harvesting and recorded traits

The inoculum was produced by the laboratory of Dr. B. Rodemann (Julius Kühn-Institute, Institute for Plant Protection in Field Crops and Grassland, Braunschweig, Germany) as described by Miedaner et al. (2010a). In short, *C. purpurea* samples were collected from Germany, Poland and Austria and isolation was done separately for each sample according to Kirchhoff (1929). Conidia for inoculation were produced on autoclaved wheat-grain medium colonized by *C. purpurea*. For preparing liquid inoculation, the colonized wheat was suspended in tap water by stirring on a plate stirrer for lab analysis (Ikamag®RCT, 60 min, room temperature) and the concentration was adjusted to $3{\cdot}10^6$ spores/ml and multiplied separately. For both approaches, three country-specific inocula (Germany, Poland, and Austria) were mixed directly before inoculation to ensure a broad ecological range of the inoculum.

In the greenhouse, spray inoculation was used during April outside the normal rye pollen season. Additionally, bagging of all ears was done shortly after heading to keep the ears unfertilized. In Hagenberg, previous bagging was not done because no other rye plants were in the greenhouse and a large distance to the next rye fields applied. At full flowering (BBCH 65), cellophane bags were removed, and the heads were inoculated by spraying with a low-volume flower sprayer. CMS rye in pollen isolation keeps the flowers open for several days and the stigma continues to grow until it reaches out of the glume. Thus, the conidia can directly be brought onto the stigma. The inoculated ears were covered by polyethylene (PE) bags right after inoculation to achieve maximum humidity for optimal infection conditions. After five days, the PE bags were exchanged by cellophane bags to guarantee that no sclerotia will be lost due to the remaining processing. The bags were sliced at the upper side to allow some aeration. Each entry was tested with 10 plants (5 pots × 2 plants) and two ears per plant in four replications. During the season, all following ears were constantly removed. In total, 40 plants/80 ears per entry were recorded.

For inoculation of the field trials, a needle inoculation device (Fig. 1) was used at BBCH 45- BBCH 49 (Meier, 2001), when heads were still in the leaf sheath. For this, 1% pre-cooked and cooled water agar (10 g/L, VWR International GmbH, Darmstadt, Germany) was added to the liquid spore suspension to enhance the adhesion of the spores onto the needles. Additionally, a sponge was stuck in the spaces between the needles to form a reservoir which can put more inoculum onto the plants while squeezing the needle gadget. After dipping the needle device in the inoculum solution, the inoculum was applied

Table 1 Overview of the experiments according to country (DE = Germany, AT = Austria), location with abbreviation (abbr.), cultivation, and evaluated traits (x = trait recorded) in 2018 and 2019

Country	Location (GPS data)	Abbr.	Test system	Ergot severity (g/ear)	Ergot incidence (number/ear)	Ear emergence (1–9)[b)]	Plant height (cm)[b)]
No. of environments [a)] (field/greenhouse)				8/6	5/5	8/–	8/–
DE	Ob. Lindenhof (48°28′25.5"N 9°18′17.9"E)	OLI	Field	X	–	X	X
DE	Hohenheim (48°42′51.1"N 9°12′31.3"E)	HOH	Greenhouse	X	X[c)]	–	–
DE	Petkus (51°59′14"N 13°21′ 22"O)	PET	Greenhouse, Field	X	X	X	X
DE	Wulfsode (53°02′57.5"N 10°14′51.8"E)	WUL	Field	X	X[d)]	X	X
AT	Hagenberg (48°22′28.4"N 14°30′51.2"E)	HAG	Field	X	X	X	X
AT	Wien (48°15′15.3"N 16°29′01.2"E)	VIE	Greenhouse	X	X	–	–

[a)] Environment = location x year combination

[b)] Recorded only in field

[c)] 1 year (recorded in 2019)

[d)] 1 year (recorded in 2018)

to the head by several needles. This should circumvent all escape mechanisms and mechanical barriers, such that a resistance of the ovary should get detectable. After inoculation, the ears were covered immediately by paper bags to eliminate possible pollination due to foreign pollen. About 2 weeks after flowering the bags were sliced on the upper edge for a better aeration and prevention of mold. For each entry, 40 randomly collected main ears per replication from the middle of the plot were inoculated.

Ergot severity, measured as weight of sclerotia per head (g) and ergot incidence as number of sclerotia per ear were recorded as resistance traits. Additionally, ear emergence (1–9, 1 = very late, 9 = very early at a specific date), and plant height (cm) were recorded in the field. Because we used non-restorer CMS rye and bagged the inoculated ears, no seed set was observed due to missing pollen.

Statistical analyses

Single-plot data were used for all analyses, i.e. data of all inoculated ears per replicate were averaged. Outlier tests were performed as described in Bernal-Vasquez et al. (2016) and detected outliers were considered as missing values. Firstly, ANOVA was conducted for each location separately and secondly combined across locations for each trait using standard procedures (Cochran & Cox, 1957). For all ANOVAs, the effect of the factor 'genotype' was considered as fixed and the factors 'replication' and 'environment' as random. A significance level (P) of 0.05 or 0.01 was applied to all statistical calculations. The estimates of ANOVA of the ratio of genotypic to phenotypic variance considering the number of replicates were used to calculate repeatability for each environment and entry-mean heritability (h^2) considering the number of replicates and environments across all environments, respectively (Fehr, 1987). The software R (R Core Team, 2018) and R Studio (Version 3.5.1) (RStudio Team, 2016) were used to perform outlier test, ANOVAs, calculating the means, doing graphic visualization and calculating the Pearson correlation coefficient (r). In the figures, the original means are reported. Multiple testing was performed by a Tukey test at $P = 0.05$ as implemented in the R - Studio.

Results

Influence of environment on ergot infection

Both inoculation methods, spray and needle inoculation, showed in all environments ergot infection (Fig. 2). The single locations largely differed for their ergot severity for field and greenhouse experiments. On average, the greenhouse approach showed higher ergot contamination in both years compared to the field trials with a higher mean ergot severity in 2018 for nearly all genotypes (Table S1). In the field, slightly higher ergot

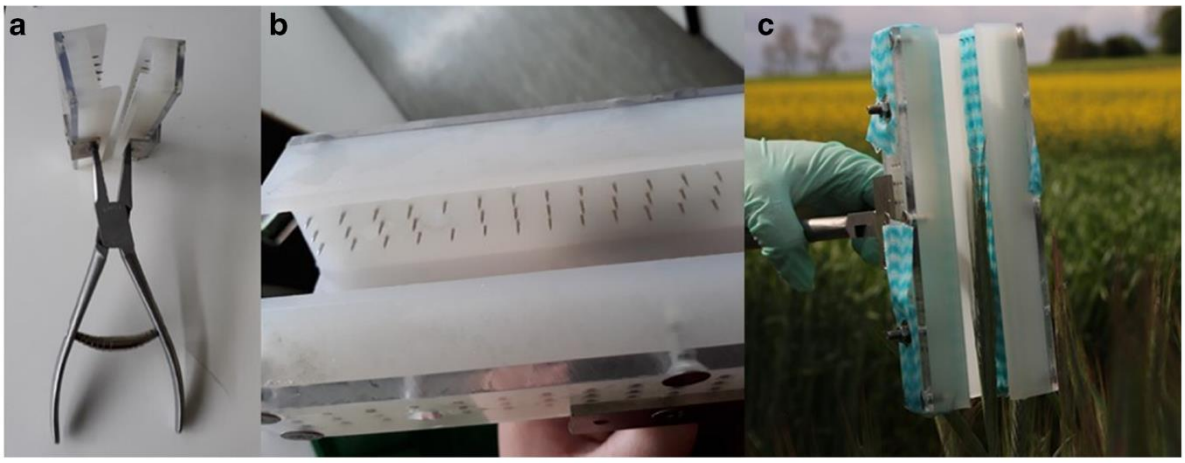

Fig. 1 Illustration of the needle inoculation device of the field trials; **A** plan view, **B** close up of the needles inside, and C: plan view with sponge (blue/white zigzag pattern) during usage in the field

severity was observed in 2019 compared to 2018 because of PET.

Influence of genotype on ergot and correlations to agronomic traits

Ergot incidence in the greenhouse was for all entries considerably higher than in the field (Table S2, S3). Mean of ergot severity and incidence of all genotypes separated by locations, years and test systems can be found in Tables S4 and S5, respectively. Although the differences were small, a good differentiation among genotypes was detected for the CMS - single crosses for both inoculation procedures and years, averaged across all locations (Table S1). Here, K_4 showed considerably the lowest ergot weight for both years and test systems. In contrast, a higher infection level was observed for K_5 and H_1 for each approach in 2018.

Correlation coefficients between greenhouse and field experiments were high in 2018 ($r = 0.72$, $P < 0.01$) and moderate in 2019 ($r = 0.59$) (Fig. 3). When leaving out one entry (H_3) that showed a large deviation from regression, the latter correlation coefficient reached $r = 0.84$ ($P < 0.01$). Correlating ergot severity with ergot incidence revealed in the field a slight negative, but not significant coefficient ($r = -0.19$, Fig. S1) and in the greenhouse a high coefficient (0.95, P < 0.01, Fig. 4). Ergot severity correlated also moderately with ear emergence ($r = -0.49$, P < 0.05) and plant height ($r = -0.54$, $P < 0.05$) in the field. The correlations between ergot incidence and ear emergence ($r = -0.35$) and plant height ($r = 0.04$) in the field were not significant.

Partitioning of genotypic and environmental variances

Repeatablilities of ergot severity and ergot incidence were largely varying due to locations and years (Table S6). In the combined analysis, all variance components were significantly different from zero for the main factors and their interaction for ergot severity (Table 2a) and ergot incidence (Table 2b). For the greenhouse experiments, genotype × environment interaction variance was the most important source of variation. In contrast, the environmental variance was the most relevant one for the field trials. The error variance relative to the genotypic variance was considerably lower in the greenhouse experiments than in the field experiments resulting in a higher entry-mean heritability for the greenhouse.

Discussion

Ergot infections of rye lots and accompanying contamination by toxic ergot alkaloids (EAs) is still a severe problem for food security. Current studies found only a moderate, positive correlation of ergot severity and EA content (Kodisch et al., 2020b; Schwake-Anduschus et al., 2020). Discovering a maternal resistance mechanism against ergot would have a high economic impact.

Comparison of greenhouse (spray inoculation) and field (needle inoculation) tests

Both inoculation methods and test systems resulted in considerable ergot infection. We had to weigh the

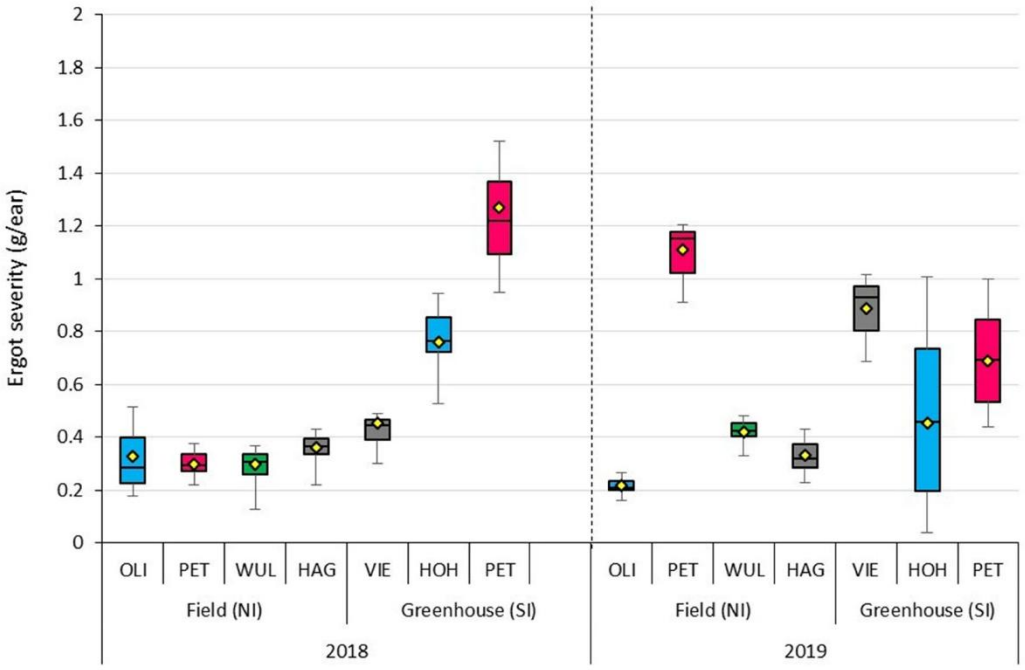

Fig. 2 Box-Whisker plots of 10 CMS single - crosses inoculated with *Claviceps purpurea* by needle inoculation (NI) in the field and spray inoculation (SI) in the greenhouse at three to four locations in 2018 and 2019; the boxplots show the distribution of ergot severity (black horizontal line representing the median, whiskers representing the 75% quantile, and yellow rhombus representing the mean; declaration of the abbreviations can be found in Tab 1)

sclerotia as resistance trait because no seed set occurred due to missing pollen. The inoculation method was confounded with the testing environment in our study, because the greenhouse experiments were inoculated by spray infection and the field experiments by needle infection. We could not do a spray infection in the field

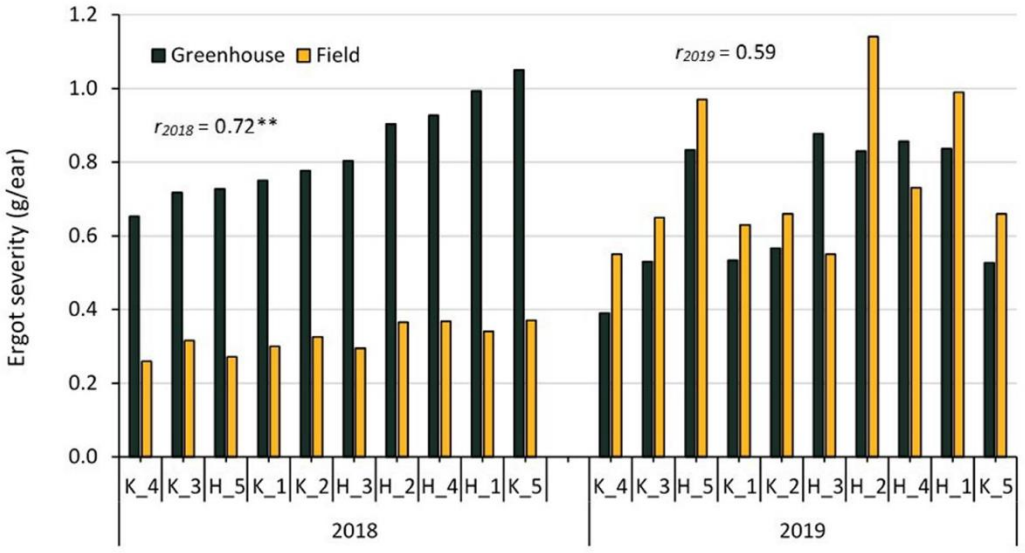

Fig. 3 Bar charts of 10 CMS single - crosses inoculated with *Claviceps purpurea* of ergot severity in field and greenhouse experiments at three locations in 2018 and 2019 (** significant for $P < 0.01$, r = coefficient of correlation)

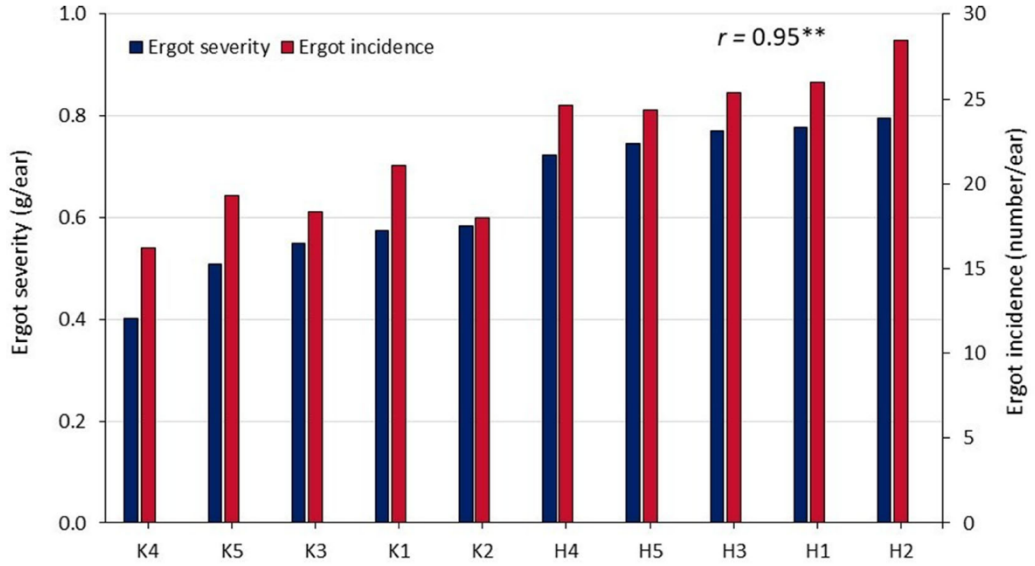

Fig. 4 Bar charts of 10 CMS single - crosses inoculated with *Claviceps purpurea* of ergot severity and ergot incidence in the greenhouse at three locations (HAG, PET, WUL) in 2018 and 2019 (** significant for $P < 0.01$, r = coefficient of correlation)

because of incomplete pollen isolation. Spray inoculation has to be done at full flowering when all surrounding plots are also pollen shedding. Even bagging of the inoculated heads could not inhibit fertilization, because the bags have to be removed for inoculation and in the meantime foreign pollen could enter the florets. The needle infection, however, is performed earlier when the heads are still in the leaf sheath and pollen shedding

Table 2 Estimates of variance components and entry-mean heritabilities of 10 CMS - single crosses after inoculation by *Claviceps purpurea* in the greenhouse by spray inoculation (SI) and in the field by needle inoculation (NI) for **a** ergot severity (g/ear), and **b** ergot incidence (number/ear); (df: degrees of freedom, Var. comp.: variance components, *P*: significance level)

Parameter	df	Greenhouse (SI)	*P*	df	Field (NI)	*P*
a Ergot severity						
No. of environments[a)]		6			8	
Variance components:						
Environment (E)	5	0.0093	**	7	0.0940	**
Genotype (G)	9	0.0125	**	9	0.0041	**
G×E	45	0.0242	**	58	0.0051	**
Error	175	0.0124		212	0.0101	
Heritability		0.73			0.66	
b Ergot incidence						
No. of environments[a)]		5			6	
Variance components:						
Environment (E)	4	397.6	**	5	365.8	**
Genotype (G)	9	2497.1	**	9	93.0	**
G×E	36	164.1	**	40	13.3	**
Error	145	21.8		158	8.4	
Heritability		0.83			0.71	

** significant for $P < 0.01$

[a)] Environment = location x year combinations

is obviously not yet occurring. Needles deliver ergot spores directly into developing ears, where the fungus can infect unfertilized ovaries regardless of morphological factors that affect flowering.

A decisive advantage of greenhouse trials is that experiments can also be performed in the winter season and are environmentally more independent than field trials when thinking of sclerotia losses due to storm, heavy rain or drought (Duarte-Galvan et al., 2012). This is also shown by the higher heritability and the lower contribution of the environments in greenhouse. However, also here a significant genotype × environment interaction occurred. A problem with greenhouse experiments is the restricted number of genotypes that can be tested or the high investment that is necessary to test a high number of entries.

Field approaches on the other hand are more suitable to reproduce practical conditions due to realistic ambient conditions, i.e. higher density of the rye stand, more uniform development of the plants with only one to two heads per plant. The higher error of field experiments found here might also be caused by the handling of the inoculation device. For generating better heritabilities in future, the needle method can be further refined and optimized. One crucial point is inserting the ears into the device. At this, it must be ensured that all ears were punctured similarly and on the right position, so, a mounting to fix the ears would be ideally. In this context, an integrated tank for the inoculum with a direct injection system via syringe would be beneficial because the inoculum run off quickly from the pure metal. For this, a sponge was used as "inoculum reservoir" in this study. However, the needle equipment used in this experimental design seems not yet to be suitable for a large number of genotypes due to the laborious and time-consuming inoculation process. A motorized machine called "Golden hamster" (in German "Goldhamster", Putoma AG Luzern, Switzerland, Fig. S2) was developed from the pharmaceutical industry to inoculate ergot spores professionally (Barsch & Klebs, 2017). This vehicle carried nozzles on the front side for needle inoculation and bristles for brushing off the ergot sclerotia from the ear and a collecting container on the back side for the mechanical harvesting process. With this method a "yield" of up to 600 kg sclerotia per hectare could be harvested on designated fields by using fully male-sterile hybrids in pollen isolation. Therefore, high-throughput technologies referring to the "Goldhamster" principle need to be considered, developed and adjusted to large-scaled ergot testing. Only the needle inoculation gives the advantage that male-fertile rye could be tested without participation of pollen, because the ears are already inoculated in the leaf sheath prior to flowering.

Nevertheless, the medium to high correlations for ergot severity between both approaches showed that the results gathered in the greenhouse can reproduce to some extent the outcome of the field and vice versa. Thus, both methods are appropriate and convertible for elaborating this kind of scientific question.

Influence of environment and agronomic traits on the ergot reaction

The tremendous role of environment and environmental interactions were already demonstrated for ergot severity (defined as the percentage of ergot sclerotia relative to the grain sample) in several previous studies (Kodisch et al., 2020a; Miedaner & Geiger, 2015; Dhillon et al., 2010). One cause is that the infection cycle of the fungus is clearly affected by weather (Wegulo & Carlson, 2011), another is the different flowering behavior of rye due to weather conditions. So, it is not surprising that also under pollen isolation the individual locations showed different infection levels and a large variation regarding their ergot reaction. From the variance components, the interaction of genotype by environment was the most important source of variation in the greenhouse for ergot severity. Remarkably, despite of small absolute differences, ergot severity and ergot incidence and also the differentiation among genotypes were in nearly all locations higher in the greenhouse compared to the field trials except of Petkus 2019 (Tables S3, S4, S5). The study also revealed that the factor year had a considerable high influence despite uniform handling and ambient conditions in the greenhouse. This shows once again that testing across several locations and years is definitely necessary as previously reported for other ergot resistance traits (Kodisch et al., 2020a; Miedaner et al., 2010a).

A significantly moderate negative correlation between ergot severity and plant height was previously found, whereas no correlation was reported between ergot severity and heading stage (Kodisch et al., 2020a). In this study, however, a moderate negative correlation between ergot severity and ear emergence was observed. The high positive correlation between ergot severity and incidence in the greenhouse leads to the hypothesis that highly contaminated entries contain

a high number of ergot bodies that are associated rather with small sclerotia due to a limited sink-source relationship. Thus, the greatest threat of ergot contamination would not come from genotypes forming a few large but from those supporting many small sclerotia. These are also more difficult to detect for indent cylinder separator due to a similar size like rye grain and thus, considerably slowing down artificial sorting processes (Miedaner & Geiger, 2015).

Genetic differences in ergot infection of unfertilized rye

Significant variation among unfertilized single crosses concerning ergot severity was observed despite of a low general infection level and a low number of genotypes. This confirms previous findings of significant differences of fully male-sterile entries under pollen isolation in rye (Miedaner et al., 2010b) and pearl millet (Willingale et al., 1986). Noticeably, in this study, the CMS-single cross with the lowest averaged ergot severity (K_4) was the same in the greenhouse as well as in the field and for both years of field testing (Fig. 3, Table S1). As a consequence, this might be an interesting candidate for further research. Thus, it is indicated that a maternal mechanism based on a resistance response of the ovary is existing because in our study the three other factors for the ergot reaction (1) influence of pollen, (2) ease of restoration of the female, and (3) morphological and/or escape mechanisms were excluded. This is supported by the fact that a significant differential gene expression at the base of the ovary was detected that included the activation of defense-related genes ahead of the arrival of the pathogen in the wheat-*Claviceps* interaction (Boyd et al., 2020).

In conclusion, this study illustrates the existence of significant genotypic variation among unfertilized CMS-single crosses for needle and spray inoculation, a moderate relationship between both test systems and in entry K_4 a promising candidate to further analyze maternal effects in ergot resistance. Consequently, the recurring risk of grain contamination by toxic EA (Topi et al., 2017) particularly in the light of tightened EU limits and the fact that even without visible infection in a sample toxic contamination can occur due to abrasion during handling and possible EA movement within the plant (Gordon et al., 2019) creates a need of further research on a resistance mechanism against ergot. This should include (1) to optimize the needle inoculation technique also for high-throughput applications and (2) to evaluate material for ergot resistance mechanisms with an increased set of carefully pre-selected entries and a subsequent transcriptome analysis for a deeper understanding of the underlying processes. If we aim for reducing ergot severity in rye to the level of self-pollinating crops, the maternal effects must be considered in future.

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Declarations

Human and animals rights No human and/or animal participants were involved in this research.

Informed consent All authors consent to this submission.

Conflict of interest The authors declare that they have no conflict of interest.

References

Alderman, S. (2006). *Ergot: biology and control*. Retrieved January 15, 2021, from https://www.ars.usda.gov/SP2UserFiles/person/81/ErgotDVDtranscript.pdf

Barsch, D. & Klebs, F. (2017). The ergot inoculation and harvesting machine "Goldhamster" [in German: Die Mutterkorn Impf- und Erntemaschine „Goldhamster"]. Retrieved January 18, 2022 from https://www.uni-hohenheim.de/117993?tx_ttnews%5Btt_news%5D=36083&cHash=92017e0a93912de06343f30c3e53d8dc

BSL. (2020). Descriptive variety list. Cereal, maize, large grained pulse crops, root crops (except potato). Bundessortenamt, Hannover (In German). https://www.bundessortenamt.de/bsa/media/Files/BSL/bsl_getreide_2020.pdf. Accessed 19 Jan 2021.

Bernal-Vasquez, A. M., Utz, H. F., & Piepho, H. P. (2016). Outlier detection methods for generalized lattices: A case study on

the transition from ANOVA to REML. *Theoretical and Applied Genetics, 129*, 787–804. https://doi.org/10.1007/s00122-016-2666-6

Betz, H. G. & Mielke, H. (1996). Possibilities to combat ergot [in German: Möglichkeiten zur Bekämpfung des Mutterkorns]. *Die Mühle + Mischfuttertechnik 44*, 726–728.

Beuerle, T., Benford, D., Brimer, L., Cottrill, B., Doerge, D., Dusemund, B., Farmer, P., Fürst, P., Humpf, H., & Mulder, P. P. J. (2012). EFSA panel on contaminants in the food chain (CONTAM). Scientific opinion on ergot alkaloids in food and feed. *EFSA Journal, 10*, 2798–2956.

BMEL (Bundesministerium für Ernährung und Landwirtschaft). (2021). Share of rye and maslin varieties by country, p. 39. In: Besondere Ernte- und Qualitätsermittlung (BEE) 2020-Reihe: Daten-Analysen. [in German: Anteil der Sorten von Roggen und Wintermenggetreide nach Ländern]. Retrieved January 18, 2022, https://www.bmel-statistik.de/fileadmin/daten/EQB-1002000-2020.pdf

Boyd, L. A., Tente, E., Ereful, N., Rodriguez, A. C., Grant, P., O'Sullivan, D. M. & Gordon, A. (2020). *Reprogramming of the wheat transcriptome in response to infection with Claviceps purpurea, the causal agent of ergot*, 16 December 2020, PREPRINT (Version 1) Retrieved February 11, 2021, from Research Square. https://doi.org/10.21203/rs.3.rs-126182/v1.

Cochran, W. G., & Cox, G. M. (1957). *Experimental designs*. Wiley.

Dhillon, B. S., Mirdita, V., & Miedaner, T. (2010). Preliminary evaluation of locations for conducting selection for resistance to ergot (*Claviceps purpurea*) in rye. *Indian Journal of Plant Genetic Resources, 23*, 265–268.

Duarte-Galvan, C., Torres-Pacheco, I., Guevara-Gonzalez, R. G., Romero-Troncoso, R. J., Contreras-Medina, L. M., Rios-Alcaraz, M. A. & Millan-Almaraz, J. R. (2012). Review. Advantages and disadvantages of control theories applied in greenhouse climate control systems. *Spanish Journal of Agricultural Research,* 10, 926–938. https://doi.org/10.5424/sjar/2012104-487-11.

European Union (2012). 012/154/EU: Commission Recommendation of 15 March 2012 on the monitoring of the presence of ergot alkaloids in feed and food Text with EEA relevance. *Official Journal of the European Union L 77,* 16.3.2012, p. 20–21. Retrieved January 18, 2022, from: http://data.europa.eu/eli/reco/2012/154/oj

European Union (2021). Commission Regulation (EU) 2021/1399 of 24 August 2021 amending Regulation (EC) No 1881/2006 as regards maximum levels of ergot sclerotia and ergot alkaloids in certain foodstuffs. *Official Journal of the European Union L 301,* 25.8.2021, p. 1–5. Retrieved January 18, 2022, from http://data.europa.eu/eli/reg/2021/1399/oj

Eurostat. (2022). *Data Browser*. Retrieved January 18, 2022, from https://ec.europa.eu/eurostat/databrowser/view/tag00049/default/table?lang=en

Fehr, W. R. (1987). *Principles of cultivar development, theory and technique* (Vol. 1) Macmillan.

Florea, S., Panaccione, D. G., & Schardl, C. L. (2017). Ergot alkaloids of the family Clavicipitaceae. *Phytopathology, 107*, 504–518. https://doi.org/10.1094/PHYTO-12-16-0435-RVW

Gordon, A., Basler, R., Bansept-Basler, P., Fanstone, V., Harinarayan, L., Grant, P. K., Birchmore, R., Bayles, R. A., Boys, L. A., & O'Sullivan, D. O. (2015). The identification of QTL controlling ergot sclerotia size in hexaploid wheat implicates a role for the *Rht* dwarfing alleles. *Theoretical and Applied Genetics, 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. Retrieved January 15, 2021, from https://ahdb.org.uk/determining-the-routes-of-transmission-of-ergot-alkaloids-in-cereal-grains

Gordon, A., McCartney, C., Knox, R. E., Ereful, N., Hiebert, C. W., Konkin, D. J., Hsueh, Y. C., Bhadauria, V., Sgroi, M., O'Sullivan, D. M., Hadley, C., Boyd, L. A., & Menzies, J. (2020). Genetic and transcriptional dissection of resistance to *Claviceps purpurea* in the durum wheat cultivar Greenshank. *Theoretical and Applied Genetics, 133*, 1873–1886. https://doi.org/10.1007/s00122-020-03561-9

Hulvová, H., Galuszka, P., Frébortová, J., & Frébort, I. (2013). Parasitic fungus *Claviceps* as a source for biotechnological production of ergot alkaloids. *Biotechnology Advances, 31*, 79–89. https://doi.org/10.1016/j.biotechadv.2012.01.005

Kirchhoff, H. (1929). Contributions to the biology and physiology of the ergot fungus [in German: Beiträge zur Biologie und Physiologie des Mutterkornpilzes]. *Zentralblatt für Bakteriologie und Parasitenkunde, 77*, 310–369.

Kodisch. A, Wilde, P., Schmiedchen, B., Fromme, F. J., Rodemann, B., Tratwal, A., Oberforster, M., Wieser, F., Schiemann, A., Jørgensen, L. N. & Miedaner, T. (2020a). Ergot infection in winter rye hybrids shows differential contribution of male and female genotypes and environment. Euphytica, 216, 65. https://doi.org/10.1007/s10681-020-02600-2.

Kodisch, A., Oberforster, M., Raditschnig, A., Rodemann, B., Tratwal, A., Danielewicz, J., Korbas, M., Schmiedchen, B., Eifler, J., Gordillo, A., Siekmann, D., Fromme, F. J., Wuppermann, F. N., Wieser, F., Zechner, E., Niewińska, M., & Miedaner, T. (2020b). Covariation of ergot severity and alkaloid content measured by HPLC and one ELISA method in inoculated winter rye across three isolates and three European countries. *Toxins, 12*, 676. https://doi.org/10.3390/toxins12110676

Meier, U. (2001). *Growth stages of mono- and dicotyledonous plants. BBCH Monograph*. Retrieved April 02, 2020, from https://www.julius-kuehn.de/media/Veroeffentlichungen/bbch%20epaper%20en/page.pdf

Menzies, J. G., & Turkington, T. K. (2015). An overview of the ergot (*Claviceps purpurea*) issue in western Canada: Challenges and solutions. *Canadian Journal of Plant Pathology, 37*, 40–51. https://doi.org/10.1080/07060661.2014.986527

Miedaner, T., & Geiger, H. H. (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. & Laidig, F. (2019). Hybrid breeding in rye (Secale cereale L.). In J. Al-Khayri, S. Jain, D. Johnson (Eds) *Advances in plant breeding strategies: Cereals*. (Vol. 5, pp. 343–372). Springer. https://doi.org/10.1007/978-3-030-23108-8_9.

Miedaner, T., Wilde, P., & Wortmann, H. (2005). Combining ability of non-adapted sources for male-fertility restoration

in Pampa CMS of hybrid rye. *Plant Breeding, 124*, 39–43. https://doi.org/10.1111/j.1439-0523.2004.01038.x

Miedaner, T., Mirdita, V., Rodemann, B., Drobeck, T., & Rentel, D. (2010a). Genetic variation of winter rye cultivars for their ergot (*Claviceps purpurea*) reaction tested in a field design with minimized interplot interference. *Plant Breeding, 129*, 58–62. https://doi.org/10.1111/j.1439-0523.2009.01646.x

Miedaner, T., Dänicke, S., Schmiedchen, B., Wilde, P., Wortmann, H., Dhillon, B. S., & Mirdita, V. (2010b). 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., Herter, C. P., Goßlau, H., Wilde, P., & Hackauf, B. (2017). Correlated effects of exotic pollen-fertility restorer genes on agronomic and quality traits of hybrid rye. *Plant Breeding, 136*, 224–229. https://doi.org/10.1111/pbr.12456

Mielke, H. (2000). Studies on the fungus *Claviceps purpurea* (Fries) Tulasne considering the susceptibility of different rye cultivars [in German: Studien über den Pilz *Claviceps purpurea* (Fries) Tulasne unter Berücksichtigung der Anfälligkeit verschiedener Roggensorten]. *Mitteilungen aus der Biologischen Bundesanstalt für Land- und Forstwirtschaft, 375*.

Pažoutová, S. (2002). The evolutionary strategy of Claviceps. In: F. White, C. W. Bacon, N. L. Hywel-Jones, & S. W. Spatafora. (Eds) *Clavicipitalean fungi: Evolutionary biology, chemistry, biocontrol and cultural impacts*. (pp 329–354). Marcel Dekker.

R Core Team. (2018). *R: A language and environment for statistical computing*. R Foundation for Statistical Computing, . ISBN 3-900051-07-0. Retrieved April 07, 2020, from https://www.R-project.org

R Studio Team. (2016). *RStudio: Integrated development for R. RStudio*, Inc., Boston. Retrieved April 07, 2020, from https://www.rstudio.com/

Schumann, G. L. & Uppala, S. (2000). *Ergot of rye*. Updated 2017. The Plant Health Instructor. Retrieved January 18, 2022 from https://www.apsnet.org/edcenter/disandpath/fungalasco/pdlessons/Pages/Ergot.aspx

Schwake-Anduschus, C., Lorenz, N., Lahrssen-Wiederholt, M., Lauche, A., & Dänicke, S. (2020). German monitoring 2012–2014: Ergot of *Claviceps purpurea* and ergot alkaloids (EA) in feeding stuffs and their toxicological relevance for animal feeding. *Journal of Consumer Protection and Food Safety, 15*, 321–329. https://doi.org/10.1007/s00003-020-01298-7

Tenberge, K. B. (1999). *Biology and life strategy of the ergot fungi*. In V. Křen, L. Cvak (Eds.), *Ergot: The genus Claviceps*. (pp 25–56) Harwood Academic Publishers.

Thakur, R. P., & Williams, R. J. (1980). Pollination effects on pearl millet ergot. *Phytopathology, 70*, 80–84. https://doi.org/10.1094/Phyto-70-80

Topi, D., Jakovac-Strajn, B., Pavšič-Vrtač, K., & Tavčar-Kalcher, G. (2017). Occurrence of ergot alkaloids in wheat from Albania. *Food Additives & Contaminants: Part A, 34*, 1333–1343. https://doi.org/10.1080/19440049.2017.1307528

Tudzynski, P., Tenberge, K., Oeser, B. (1995). Claviceps purpurea. In: K. Kohmoto, U. S. Singh, R. P. Singh. (Eds.) *Pathogenesis and host specificity in plant diseases: Histopathological, biochemical, genetic and molecular bases* (Vol. II, eukaryotes pp 161–187). Elsevier Science.

Wegulo, S. N. & Carlson, M. P. (2011). *Ergot of small grain cereals and grasses and its health effects on humans and livestock*. University of Nebraska, extension, EC1880. Retrieved January 14, 2021, from https://ianrpubs.unl.edu/live/ec1880/build/ec1880.pdf

Willingale, J., Mantle, P. G., & Thakur, R. P. (1986). Post pollination 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

Supplemental materials

Maternal differences for the reaction to ergot in unfertilized hybrid rye (*Secale cereale*)

Anna Kodisch · Brigitta Schmiedchen · Jakob Eifler · Andres Gordillo · Dörthe Siekmann · Franz Joachim Fromme · Michael Oberforster · Thomas Miedaner

Tab. S1: Means and significances (Sign.) of ergot severity (g/ear) for the individual years (2018, 2019) and test systems (greenhouse, field) of 10 CMS - single crosses after inoculation by *Claviceps purpurea* (SI: spray inoculation, NI: needle inoculation)

CMS single-cross	Greenhouse (SI)				Field (NI)			
	2018	Sign.[a]	2019	Sign.[a]	2018	Sign.[a]	2019	Sign.[a]
K_4	0.65	a	0.39	a	0.26	a	0.55	a
K_3	0.72	ab	0.53	b	0.32	abcd	0.65	ab
H_5	0.73	ab	0.83	c	0.27	ab	0.97	c
K_1	0.75	ab	0.53	b	0.30	abcd	0.63	ab
K_2	0.78	b	0.57	b	0.33	abcd	0.66	ab
H_3	0.80	bc	0.88	c	0.30	abc	0.55	a
H_2	0.90	cd	0.83	c	0.37	cd	1.14	c
H_4	0.93	d	0.86	c	0.37	cd	0.73	b
H_1	0.99	de	0.84	c	0.34	bcd	0.99	c
K_5	1.05	e	0.53	b	0.37	d	0.66	ab
Mean	0.83		0.68		0.32		0.69	
$LSD_{5\%}$	0.10		0.22		0.08		0.18	

[a] Treatments with the same letter are not significantly different within one column (Tukey test, $P < 0.05$)

Tab. S2: Means and significances (Sign.) of ergot incidence (number/ear) for the individual years (2018, 2019) of 10 CMS - single crosses after inoculation by *Claviceps purpurea* (SI: spray inoculation, NI: needle inoculation)

CMS single-cross	Greenhouse (SI)				Field (NI)			
	2018	**Sign.[a]**	**2019**	**Sign.[a]**	**2018**	**Sign.[a]**	**2019**	**Sign.[a]**
K3	23.41	a	21.31	b	7.61	e	12.43	e
K1	25.22	ab	19.85	ab	5.47	abcd	7.75	abc
K4	26.83	bc	16.57	a	6.94	de	11.05	de
H2	26.90	bc	28.49	c	4.81	abc	10.22	cde
H5	29.20	cd	28.23	c	3.72	a	6.48	ab
K2	29.30	cd	21.57	b	6.66	cde	11.97	e
H1	30.82	de	29.56	c	4.52	ab	11.50	de
K5	32.93	e	19.12	ab	7.35	e	13.23	e
H3	33.02	e	30.63	cd	3.93	a	6.11	a
H4	33.22	e	33.80	cd	5.91	bcde	8.72	bcd
Mean	29.01		24.91		5.69		10.09	
$LSD_{5\%}$	3.35		3.92		1.85		2.39	

[a] Treatments with the same letter are not significantly different within one column (Tukey test, $P < 0.05$)

Tab. S3: Mean of ergot severity (g/ear) and incidence (number/ear) for the individual locati
(2018, 2019) and cultivation system (greenhouse, field) of 10 CMS - single crosses after ino
by *Claviceps purpurea*

Locations	Cultivation	Ergot severity		Ergot incidence	
		2018	2019	2018	2019
Ob. Lindenhof	Field	0.32	0.22	-	-
Hohenheim	Greenhouse	0.76	0.45	-	-
Petkus	Field	0.3	1.11	8.54	11.7
Petkus	Greenhouse	1.27	0.69	39.06	26.75
Wulfsode	Field	0.3	0.42	3.32	9.94
Hagenberg	Field	0.36	0.33	5.22	7.33
Wien	Greenhouse	0.45	0.89	19.11	27.8

Tab. S4: Mean of ergot severity (g/ear) of the genotypes, location, years (2018, 2019) and test system (greenhouse, field) of 10 CMS - single crosses (SC) after inoculation by *Claviceps purpurea* (SI: spray inoculation, NI: needle inoculation)

SC	Greenhouse (SI)						Field (NI)							
	2018			2019			2018				2019			
	HOH	PET	VIE	HOH	PET	VIE	OLI	PET	WUL	HAG	OLI	PET	WUL	HAG
K1	0.6	1.3	0.4	0.16	0.6	0.8	0.2	0.3	0.3	0.4	0.3	1.2	0.46	0.26
K2	0.7	1.2	0.5	0.37	0.5	0.8	0.2	0.3	0.4	0.5	0.3	1.2	0.43	0.39
K3	0.8	1.0	0.4	0.31	0.5	0.8	0.3	0.3	0.3	0.4	0.2	1.2	0.45	0.32
K4	0.5	1.1	0.3	0.04	0.4	0.7	0.2	0.3	0.3	0.3	0.2	0.9	0.49	0.23
K5	0.8	1.9	0.4	0.09	0.6	0.9	0.3	0.4	0.4	0.4	0.2	1.2	0.42	0.36
H1	0.9	1.5	0.5	0.55	0.9	1.0	0.6	0.2	0.3	0.2	0.2	1.0	-	-
H2	0.9	1.4	0.4	0.79	0.8	1.0	0.5	0.3	0.2	0.4	0.2	1.1	-	-
H3	0.9	1.1	0.5	0.78	0.9	1.0	0.2	0.3	0.3	0.3	0.2	1.0	0.33	0.3
H4	0.7	1.3	0.7	0.72	0.9	1.0	0.4	0.4	0.3	0.4	0.2	1.3	0.41	0.47
H5	0.8	1.0	0.5	0.74	0.8	1.0	0.3	0.3	0.1	0.4	0.2	1.1	0.39	-
Mean	0.8	1.3	0.5	0.45	0.7	0.9	0.3	0.3	0.3	0.4	0.2	1.1	0.42	0.33
$LSD_{5\%}$	0.1	0.2	0.1	0.14	0.2	0.1	0.1	0.1	0.2	0.1	0.1	0.3	0.1	0.09

Tab. S5: Mean of ergot incidence (number/ear) of the genotypes, location, years (2018, 2019) and test system (greenhouse, field) of 10 CMS - single crosses (SC) after inoculation by *Claviceps purpurea* (SI: spray inoculation, NI: needle inoculation)

SC	Greenhouse (SI)						Field (NI)							
	2018			2019			2018				2019			
	HOH	PET	VIE	HOH	PET	VIE	OLI	PET	WUL	HAG	OLI	PET	WUL	HAG
K1	-	42	20	3.7	29	24	-	7.7	3.4	5.4	-	12	8.46	4.19
K2	-	37	21	17.38	23	24	-	7.8	5.1	7.8	-	17	11.12	9.86
K3	-	38	15	23.36	19	22	-	6.1	4.2	6.6	-	11	12.69	7.34
K4	-	38	16	2.57	25	22	-	10	5.1	5.2	-	17	15.61	5.7
K5	-	36	17	2.29	26	29	-	5.3	4.5	6.4	-	15	13.16	9.33
H1	-	34	22	22.58	33	33	-	7.6	2.3	3.6	-	10	-	-
H2	-	41	20	31.87	25	29	-	7	2.1	4.5	-	7	-	-
H3	-	40	20	30.98	34	29	-	12	2.2	3.5	-	12	5.86	7.16
H4	-	39	23	36.94	30	34	-	11	2.8	4.9	-	5	6.90	7.72
H5	-	40	20	34.25	22	30	-	11	1.5	4.4	-	12	5.71	-
Mean	-	39	19	20.6	27	28	-	8.5	1.7	5.2	-	12	9.94	7.33
$LSD_{5\%}$	-	9.4	3.9	7.66	6.9	4.6	-	4	0.8	1.9	-	2.4	2.1	4.31

Tab. S6: Repeatability of ergot severity and incidence for the locations and the respective te of 10 CMS - single crosses after inoculation by *Claviceps purpurea* in 2018 and 2019

Locations	Cultivation	Repeatability			
		Ergot severity		Ergot incidence	
		2018	2019	2018	2019
Ob. Lindenhof	Field	0.91	0.78	-	-
Hohenheim	Greenhouse	0.86	0.97	-	0.96
Petkus	Field	0.53	0.45	0.62	0.96
Petkus	Greenhouse	0.93	0.82	0.21	0.77
Wulfsode	Field	0.15	0.42	0.12	0.41
Hagenberg	Field	0.43	0.87	0.79	0.45
Wien	Greenhouse	0.83	0.84	0.75	0.86

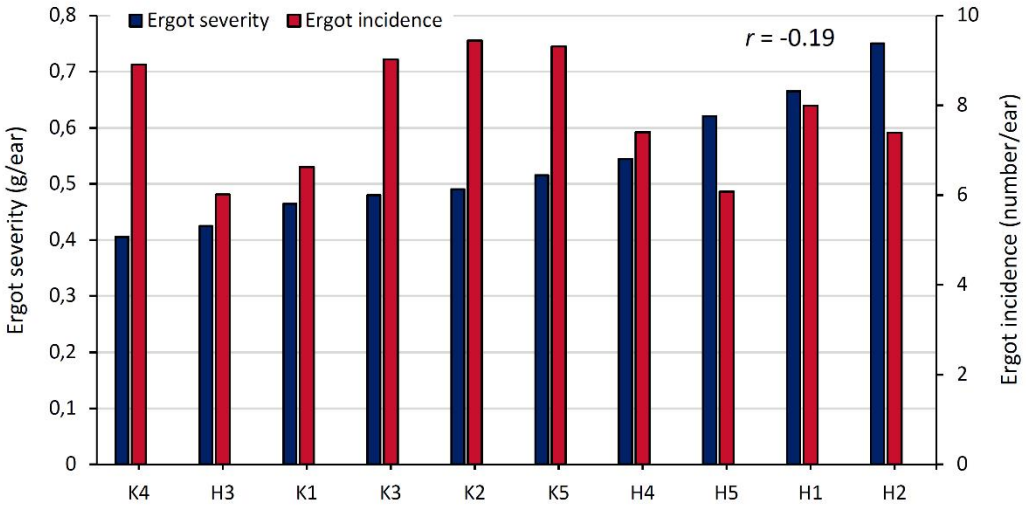

Fig. S1: Bar charts of 10 CMS single-crosses inoculated with *Claviceps purpurea* of ergot severity and ergot incidence in the field at three locations (HAG, PET, WUL) in 2018 and 2019 (*r* = coefficient of correlation)

Fig. S2: Motorized machine called "Golden hamster" (in German "Goldhamster", Putoma AG Luzern, Switzerland) for mechanically inoculating ergot spores and harvesting ergot sclerotia in the field (University of Hohenheim, German Agricultural Museum, Angelika Emmerling)

6. Publication IV: Ergot alkaloid contents in hybrid rye are reduced by breeding[4)]

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Article

Ergot Alkaloid Contents in Hybrid Rye are Reduced by Breeding

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Abstract: Contamination by ergot caused by the phytopathogenic fungus *Claviceps purpurea* is a constant threat to the whole rye value chain. Ergot alkaloids (EA) produced within the fungal sclerotia are toxic for humans and animals and are subjected to strict regulations in human food. Our main objective was to analyze whether less susceptible rye cultivars with a lower content of sclerotia also contain fewer ergot alkaloids (EA). We analyzed 15 factorial single crosses in multi-environmental trials with artificial inoculation for their ergot severity, the content of twelve EAs by HPLC, and the total ergot content by ELISA. The genotypes displayed a wide range of pollen shedding from fully sterile to fully fertile, of ergot severity expressed as percentage of sclerotia relative to the harvest (0.22–11.47%), and of EA contents when analyzed by HPLC (0.57–45.27 mg/kg. Entry-mean heritabilities were high throughout (0.87–0.98). The factorial analysis yielded a preponderance of male general combining ability (GCA) variances, the estimates for the females were smaller, although significant. EA contents measured by ELISA were, on average, seven times larger. The correlation between ergot severity and EA contents determined by HPLC was $r = 0.98$ ($p \leq 0.01$) and only somewhat lower when analyzed by ELISA. In conclusion, less ergot prone rye genotypes also support lower EA contents.

Keywords: *Claviceps purpurea*; ELISA; ergot severity; HPLC; pollen; *Secale cereale*

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1. Introduction

Ergot is a century-old problem in rye (*Secale cereale* L.) cultivation and the disease-causing pathogen *Claviceps purpurea* (Fr.) Tul. has as generalist a wide host range with over 400 different grass species [1]. The fungus colonizes the unfertilized ovaries during flowering [2] and causes the plant to form large overwintering organs, the purple-black sclerotia [3]. They contain over 80 ergot alkaloids (EAs; [4]) that are dangerous to humans and animals [5]. Because the fungus competes with pollen for the stigma, any conditions where even only little pollen is available are conducive for infection. This includes cool, damp weather at flowering that further promotes fungal infection [6]. Cultivation of hybrid varieties based on cytoplasmic-male sterility (CMS) has also resulted in increased ergot incidence since the mid-1980s because the required restorer-to-fertility (*Rf*) genes provided only 30–50% pollen shedding at that time [7,8]. Nowadays, non-adapted restorer genes from Iranian primitive rye and Argentinean landraces are used in some hybrid varieties, which cause a considerably better restoration and, thus, a significant reduction in ergot content [9]. In the Descriptive List of Varieties of Germany, the susceptibility to ergot varies between ratings of 3–6 on the 1–9 scale (1 = fully resistant, 9 = fully susceptible; [10]).

Total ergot alkaloid (EA) contents ranging from 3 to 300 µg/kg have been detected in randomly collected rye samples from natural infections in Germany [11]. Because toxic EAs occur in food and feed samples consistently, there are strict European Union limits on the use of rye for human consumption with a maximum of 0.5 g sclerotia fragments/kg, and

these limits are planned to be reduced to 0.2 g/kg as from 1 July 2023 [12]. In this context, the six most frequent EAs (ergometrine, ergotamine, ergosine, ergocristine, ergocryptin, ergocornine; [13]) with their inine epimers are to be regulated in unprocessed rye to a maximum total content of 250 µg/kg from that date. Rye is used in Northern Europe mainly for bread making, feeding, and bioenergy production and for these purposes the occurrence of ergot sclerotia is harmful. Therefore, the question arises whether less susceptible rye cultivars with a lower content of sclerotia also contain fewer alkaloids. For this, we used CMS inbred lines to create 15 single crosses differing maximally in pollen shedding, tested them in multi-site field trials with artificial infection, and measured the alkaloid content using High Performance Liquid Chromatography (HPLC) analysis and a commercial Enzyme-Linked Immunosorbent Assay (ELISA).

2. Materials and Methods

2.1. Field Trials and Inoculation Procedure

Fifteen factorial single crosses were produced by crossing four female lines (SE) of the Petkus gene pool in the cytoplasmic-male sterility (CMS) inducing Pampa cytoplasma with four male lines (PE) by KWS LOCHOW GMBH, Bergen, Germany. Two of the male lines (PE2, PE4) had non-adapted *Rf* genes, one (PE3) had European *Rf* genes, all being from the Carsten gene pool, and one line (PE5) was a non-restorer from the Petkus gene pool, providing only non-pollen shedding progeny as negative control. In 2018 and 2019, the field trials were conducted at the following 6 locations: Oberer Lindenhof (OLI; 48°28′25.5′′ N 9°18′17.9′′ E), Braunschweig (BRS; 52°16′33.4′′ N 10°34′09.3′′ E), Wohlde (WOH; 52°48′48.7′′ N 9°59′53.1′′ E), Petkus (PET; 51°59′14′′ N 13°21′22′′ E) in Germany, Kościelna Wieś (KOS; 51°46′28.7′′ N 18°00′58.0′′ E), and Zybiszów (ZYB; 51°03′51.9′′ N 16°54′45.4′′ E) in Poland. Data of BRS 2018 were lacking because of missing infections. The field trials were grown in a chessboard-like design with locally grown triticale (×*Triticosecale* Wittm.) as surrounding border plots [14] completely randomized with two replications. The size of the large-drilled plots varied between 5.0 and 6.9 m^2 according to the location. Sowing was carried out in September/October with a seed density of 200 kernels/m^2, all chemical treatments (herbicide, fertilizer, fungicide, growth regulator) were applied conventionally at the different locations.

After collecting *C. purpurea* samples from Germany, Poland, and Austria, the laboratory of Dr. B. Rodemann (Julius Kühn-Institute, Institute for Plant Protection in Field Crops and Grassland, Braunschweig, Germany) produced the inoculum as previously described in detail by Miedaner et al. [15]. Briefly, each sample was isolated separately according to Kirchhoff [16] and autoclaved wheat-grain medium colonized by *C. purpurea* was used to produce conidia for inoculation. Suspending the colonized wheat in tap water and adjusting the concentration to 3×10^6 spores/mL was done directly before inoculation. For inoculation, a mix of three country-specific inocula from Germany, Poland, and Austria were used to guarantee a wide ecological range of the inoculum, whereas the inoculation procedure was performed as described in Kodisch et al. [17].

Visual scoring of the anthers (size, dehiscence) in the field on a scale of 1–9 was done to evaluate the degree of pollen fertility as described in Geiger and Morgenstern [7] due to a highly positive correlation ($r > 0.9$, $p \leq 0.01$) between this anther score and pollen amount [8]. This scale varied from male-sterile (score: 1–3), partially male-fertile (score: 4–6) and male-fertile (score: 7–9) plants, whereas the classes were categorized by increasing anther size. Scorings were done several times at mid-flowering of the respective plot. At dough ripening stage (BBCH 85–89; [18]), a subplot of 1 m^2 from the center of the large plot was harvested by hand while avoiding secondary tillers. After drying (30 °C), all heads of one plot were threshed by a large single-head thresher (Pelz K 35, Saatzuchtbedarf Baumann, Waldenburg, Germany). The sclerotia and sclerotia fragments were sorted out by hand, grain and ergot samples were separately weighed and calculated as percentage of ergot sclerotia relative to the total grain sample by weight (=ergot severity).

2.2. Sample Preparation

For preparing the samples for chemical analyses, grain and sclerotia were merged in sub-samples of 200 g according to the respective ergot severity due to a better handling. Afterwards, the sub-samples were milled (Ultra Centrifugal Mill ZM 200, 1 mm sieve, Retsch, Haan, Germany) and the flour was used for all analyses. Samples of OLI in 2018 and 2019 were analyzed by HPLC and ELISA.

2.3. High Performance Liquid Chromatography (HPLC) Analysis of EAs

HPLC analysis was performed as described in detail by Kodisch et al. [17] according to BVL L 15.01/02-5:2012-01 [19] with some minor alterations. In short, HPLC was performed for the samples from two locations (OLI18, OLI19) by the Austrian Agency for Health and Food Safety, Institute for Food Safety (AGES, Linz, Austria). Twelve EAs were quantified: ergometrine, ergometrinine, ergosine, ergosinine, ergotamine, ergotaminine, ergocornine, ergocorninine, α-ergocryptine, α-ergocryptinine, ergocrystine, and ergocrystinine. The EA content was determined as the sum of all individual EAs, individual EA values lower than the quantitation limit (LOQ < 0.02 mg/kg) were taken as zero. Subsequently, the term "(total) EA content (determined) by HPLC" is related to the sum of these 12 EAs.

2.4. Enzyme-Linked Immunosorbent Assay (ELISA) of EAs

ELISA analysis was performed as previously described in detail by Kodisch et al. [17]. Briefly, the competitive ErgoREAD ELISA (LCTech GmbH, Obertaufkirchen, Germany) was conducted at the University of Hohenheim. After extraction and filtration, the company's internal protocol was performed and, afterwards, the extinction values were determined by a microplate reader ('Sunrise') with integrated Magellan software (Tecan Group Ltd., Männedorf, Switzerland) relative to the standard samples (0, 0.025, 0.1, 0.25, 0.5, 0.75, 1 $\mu g \cdot kg^{-1}$). Higher EA concentrations were accordingly diluted to fit into this range. The EA content was calculated using the proprietary software of LCTech GmbH (Obertaufkirchen, Germany), all samples were analyzed as duplicates.

2.5. Statistical Analyses

For all analyses, single-plot data were used. Outliers were identified according to Bernal-Vasquez et al. [20] and handled in the following as missing values. For ergot severity and EA contents, a square-root transformation was performed because the residuals did not follow a normal distribution in any environment for biological reasons. After performing the analyses of variance (ANOVA) for each location independently, the ANOVA was computed combined across locations for each trait using the methods described in [21]. The effect of the factor 'genotype' was taken as fixed and the factors 'replication' and 'environment' as random. For all statistics, significance levels of 0.05 or 0.01 were used. Entry-mean heritability (H^2_G) across all environments was calculated as the ratio of genotypic to phenotypic variance considering the number of replicates end environments [22]. The software R [23] and R-Studio (Version 3.5.1) [24] were used for all analyses including the Pearson correlation coefficient (r). In the tables and figures, the original means are reported. Multiple testing was performed by a Tukey test at $p = 0.05$ as implemented in R-Studio.

3. Results

Fifteen single crosses consisting of four female and four male lines were tested for anther score, ergot severity, and EA contents determined by HPLC and ELISA (Table 1).

Table 1. Means of anther score (1–9), ergot severity (%), and ergot alkaloid (EA) contents determined by HPLC and ELISA (mg/kg) across two environments for the combination of four female CMS lines and four male lines after inoculation by *Claviceps purpurea*.

Female	**Male Line**					
Line	**PE2**	**PE3**	**PE4**	**PE5**	**Mean**	**Sign.** [a]
Anther score (1–9):						
SE2	8.25	8.25	7.75	2.00	6.56	*a*
SE3	8.00	-	7.50	3.00	6.17	*a*
SE4	7.75	4.75	7.25	1.50	5.31	*b*
SE5	7.75	4.00	7.75	1.75	5.31	*b*
Mean	7.94	5.67	7.56	2.06	5.84	
Sign. [a]	*a*	*b*	*a*	*c*		
Ergot severity (%):						
SE2	0.44	0.57	0.54	11.47	3.26	*a*
SE3	0.25	-	0.43	4.16	1.61	*b*
SE4	0.22	1.63	0.37	5.12	1.84	*b*
SE5	0.31	3.09	0.39	6.76	2.64	*ab*
Mean	0.31	1.76	0.43	6.88	2.34	
Sign. [a]	*a*	*b*	*a*	*c*		
EAs HPLC (mg/kg):						
SE2	5.25	1.51	1.02	45.27	13.26	*a*
SE3	0.57	-	1.72	11.28	4.52	*c*
SE4	0.92	6.48	1.54	17.27	6.55	*b*
SE5	1.91	9.99	2.25	16.35	7.63	*b*
Mean	2.16	5.99	1.63	22.54	7.99	
Sign. [a]	*a*	*b*	*a*	*c*		
EAs ELISA (mg/kg):						
SE2	32.64	18.30	28.40	75.82	38.79	*a*
SE3	20.94	-	43.67	133.09	65.90	*b*
SE4	22.13	51.29	27.28	126.99	56.92	*ab*
SE5	55.85	112.99	27.06	88.48	71.10	*b*
Mean	32.89	60.86	31.60	106.10	58.18	
Sign. [a]	*a*	*a*	*a*	*b*		

[a] Treatments with the same letter are not significantly different (Tukey test, $p \leq 0.05$). The other letters(a,b) indicate the significance.

The crosses displayed the whole range of pollen shedding from full male sterility (AS 1.50) to full male fertility (AS 8.25) by the variation of the males possessing none (PE5), only European (PE3), and additionally non-adapted *Rf* genes (PE2, PE4). Accordingly, they differed significantly ($p \leq 0.05$) in their mean performance with anther scores ranging from 2.06 to 7.94 on the 1–9 scale. Also, the female lines showed a significant ($p \leq 0.05$), although much smaller difference in AS ranging from 5.31 to 6.56. Especially, SE2 could be fully restored by the European *Rf* line PE3, while this male led only to low anther scores with SE4 and SE5, illustrating a specific combining ability. Ergot severities ranged widely from 0.22% to 11.47% across 11 environments. Highly positive and significant correlations were observed for ergot severities ($r = 0.97$; $p \leq 0.001$, Figure 1a and anther scores ($r = 0.98$; $p \leq 0.001$, Figure 1b) when comparing all 11 environments and the two test environments (OLI18, OLI19) used for alkaloid analyses illustrating the representativeness of the test environments.

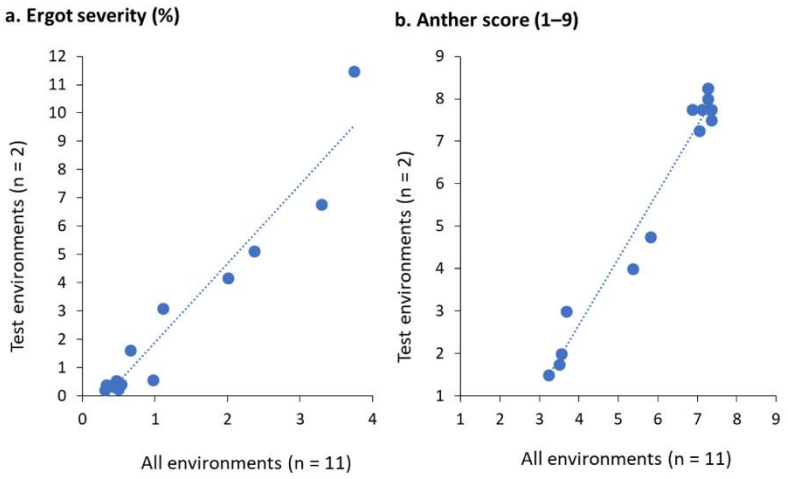

Figure 1. Association across all environments vs. test environments for 15 single crosses after inoculation with *C. purpurea* for (**a**): ergot severity (%) and (**b**): anther score (1–9).

A clear negative association between ergot severity and anther scores was found for the two test environments ($r = -0.87$, $p \leq 0.01$). The same narrow association between anther score and ergot severity was also found across all 11 environments ($r = -0.91$, $p \leq 0.01$). The factorial crosses also differed strongly for their EA contents ranging from 0.57 to 45.27 mg/kg when analyzed by HPLC. The combination SE2 × PE5 had an unusually high EA content. The EA contents measured by ELISA were, on average, seven times higher than analyzed with HPLC. Nevertheless, the correlation between both analytical methods was significant ($r = 0.53$, $p \leq 0.05$). When the combination SE2 × PE5 was omitted from this analysis, the coefficient of correlation between both methods was raised to $r = 0.87$ ($p \leq 0.01$). The correlations between ergot severity and EA contents determined by HPLC and ELISA were $r = 0.98$ ($p \leq 0.01$, Figure 2) and $r = 0.63$ ($p \leq 0.05$, without SE2 × PE5: $r = 0.84$, $p \leq 0.01$), respectively.

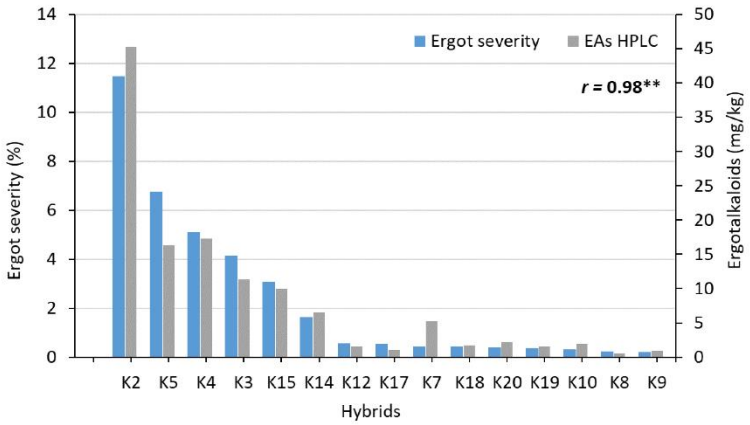

Figure 2. Association between ergot severity (%) and ergot alkaloids (EAs, mg/kg) determined by HPLC across 2 environments (OLI18, OLI19) for 15 single crosses after inoculation with *C. purpurea* (r = coefficient of correlation, **: significant at $p < 0.01$).

The analyses of variance showed very high genotypic entry-mean heritabilities ranging from 0.80 to 0.98 (Table 2).

Table 2. Estimates of variance components for general (GCA) and specific combining ability (SCA) and genotypic entry-mean heritability (H^2_G) of four female CMS lines and four male restorer lines for ergot severity, anther score (AS), and ergot alkaloid (EA) contents analyzed by HPLC and ELISA across all environments (n = 11) and the test environments (n = 2).

	All Environments (n = 11)				Test Environments (n = 2)							
Parameter	**Ergot Severity [a]**		**Anther Score**		**Ergot Severity [a]**		**Anther Score**		**EA Content HPLC [a]**		**EA Content ELISA [a]**	
Variance components:												
GCA male (M)	13.07	***	271.98	***	13.39	***	115.5	***	1429	***	17612	***
GCA female (F)	0.37	**	5.64	***	0.38	***	6.08	**	182	***	3849	*
SCA	0.25	**	4.04	***	0.57	***	3.69	**	242	***	2845	*
M × environment (E)	1.32	***	17.44	***	0.68	***	0.06		831	***	3586	*
F × E	0.17	**	1.13		0.16	*	0.58		158	***	3243	
F × M × E	0.14	**	1.26	*	0.15	*	1.25		190	***	3032	*
Error	0.08		0.83		0.05		1.01		5		1159	
H^2_G	0.87		0.89		0.95		0.88		0.98		0.8	

*, **, *** Significant at p = 0.05, 0.01, 0.001, respectively. [a] Data have been square-root transformed.

The estimates for ergot severity and anther scores across the test environments were of the same magnitude of those calculated across all environments. The variances of the male lines were of highest importance for all traits. The shares of the female lines and that of the female × male interaction (SCA, specific combining ability) were of equal size. The interactions with environments were significant for ergot severities and EA content measured by HPLC but of lesser importance for the other traits. Calculated across the genetic variance, the share of the male parent was 93% and 92% for ergot severity and anther score and 77% and 73% for EA contents measured by HPLC and ELISA, respectively. The female and SCA effects had also clearly an impact on alkaloid contents.

The fifteen single crosses did not show large deviations in their amount (%) of the individual EA profile relative to the total EA content (Supplementary Table S2). The most important individual EA was ergocristin, followed by alpha-ergocryptin and ergotamine. The amount of the –inine epimers were in all cases lower than the respective –in form.

4. Discussion

Ergot alkaloids are clearly an important concern in the total rye value chain. Although high concentrations are rarely found in food samples, the stricter EU regulations expected in 2023 [12] and general concerns of consumers on food security cast a poor image upon the acceptance of rye. Even worse, home-grown rye is not controlled and can be suspected to have even higher alkaloid concentrations despite the high sensitivity of some livestock [13]. Because no fungicides against ergot are registered on the EU market [25,26], it is of utmost importance to reduce ergot sclerotia in the rye harvest by breeding. It was shown previously that cultivars with a high pollen shedding lead always to a lower ergot contamination [27,28], thus confirming the tight correlations between anther score and ergot severity found here among 15 single crosses.

In our study, ergocristin and alpha-ergocryptin were the main EAs. In the literature, the abundance of the single EAs differed strongly with the experiments [29–31], therefore, it was shown again that shifting of the EA profile seems to happen regularly. Only the fact seems to be consistent that the amounts of the –inine epimers are lower than the respective –in forms, which was also the case here. These –inine epimers are probably biologically inactive but can also be contributing to the toxicity due to epimerization conversions [13].

In recent studies, only a moderate correlation between ergot severity and EA content was found in rye [11]. Additionally, a large screening approach of 372 winter rye samples across three cultivars, three isolates, eleven locations in three countries, and two years resulted in a similar moderate, positive covariation between ergot severity and EA content determined by HPLC (r = 0.53, $p < 0.01$, [17]). Obviously, EA contents are highly affected by the interactions with isolates, locations, and years. However, when we concentrate on genotypic differences such as in this study, correlations between ergot severity and

EA content become obviously much closer. An explanation could be the low number of environments in this study. However, the two locations used for the chemical analysis differed strongly for ergot severity as well as for anther score while both traits correlated nearly perfectly with the full number of environments. So, the data are representative and could be probably extrapolated, but of course, it has to be verified in future datasets. This also confirms the findings of Tittlemier et al. [32] where a strong linear relationship of ergot sclerotia and EA concentration could be detected in wheat.

Ergot severities at the two test environments were considerably higher than among the 11 environments. This was probably caused by the fact that the test location OLI has a very high amount of yearly precipitation [33] and the year 2018 was extremely dry in other locations, which is well known to hinder ergot infection [6]. This shows once again the necessity of having suitable locations for ergot testing [34]. Another important point for correlation analyses is, that we had here a maximum range of ergot severity and EA contents among the 15 single crosses from 0.22% to 11.47% and 0.57 to 45.27 mg/kg, respectively. This was not given in the earlier calibration study where we tested only three genotypes with only a small difference regarding their ergot reaction [17]. In this earlier study, the main differences among EA contents came from isolate and environmental factors. Screening approaches that are fast, cheap, and easy to handle were found to be not yet convincing because of a poor relationship between ELISA and HPLC results in recent calibration studies [28,35]. The correlation of ELISA and HPLC found in this study was moderate, which could also be caused by the higher range for ergot reaction of the genotypes here.

The high preponderance of the male GCA variance for ergot severity illustrates that it is promising to reduce ergot susceptibility in hybrid rye by breeding for high pollen shedding, e.g., by introgressing non-adapted *Rf* genes [9] or by using alternative CMS cytoplasms that are easier to restore [36]. However, when a high pollen shedding is integrated in all rye hybrid breeding programs, the only way to make further achievements in reducing ergot towards the low percentages known from wheat is an additional resistance of the female parent for which first indications have been shown in this study and elsewhere [15,37]. This might be also of great importance for reducing EA contents in future breeding programs because the female lines contributed to the EA contents 10% to 16% of the total genetic variance compared to only 2.6% for ergot severity.

5. Conclusions

We could demonstrate that hybrid cultivars with a low proportion of sclerotia in the harvest also contain less EA content, which is auspicious for farmer, milling, and breeding companies as well as consumers. In future, this tight correlation between ergot severity and EA content should be substantiated by a larger number of hybrids that show a distinct reaction to ergot, and additionally maternal effects should be further exploited in breeding programs with an increased effort to reduce EA content to meet the future limits.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agriculture11060526/s1, Table S1: Means of anther score (1–9). ergot severity (%). and ergot alkaloid contents (mg/kg) measured by HPLC and ELISA of 15 single-cross hybrids across all environments and the test environments after inoculation by *Claviceps purpurea*, Table S2: Ergot severity (%) and individual and total ergot alkaloid contents (mg/kg) measured by HPLC of 15 hybrids across two environments.

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References

1. Wegulo, S.N.; Carlson, M.P. Ergot of Small Grain Cereals and Grasses and its Health Effects on Humans and Livestock. 2011. University of Nebraska, Extension, EC1880. Available online: http://ianrpubs.unl.edu/live/ec1880/build/ec1880.pdf (accessed on 9 March 2021).
2. Pažoutová, S. The evolutionary strategy of *Claviceps*. In *Clavicipitalean Fungi: Evolutionary Biology, Chemistry, Biocontrol and Cultural Impacts*; White, F., Bacon, C.W., Hywel-Jones, N.L., Eds.; Marcel Dekker: New York, NY, USA, 2002; pp. 329–354.
3. Schumann, G.L.; Uppala, S. Ergot of rye. The Plant Health Instructor. 2000. Updated 2017. Available online: https://www.apsnet.org/edcenter/disandpath/fungalasco/pdlessons/Pages/Ergot.aspx (accessed on 10 February 2021).
4. Schiff, P.L. Ergot and its alkaloids. *Am. J. Pharm. Educ.* **2006**, *70*, 98. [CrossRef]
5. Hulvová, H.; Galuszka, P.; Frébortová, J.; Frébort, I. Parasitic fungus *Claviceps* as a source for biotechnological production of ergot alkaloids. *Biotechnol. Adv.* **2013**, *31*, 79–89. [CrossRef] [PubMed]
6. Miedaner, T.; Geiger, H.H. Biology, genetics, and management of ergot (*Claviceps* spp.) in rye, sorghum, and pearl millet. *Toxins* **2015**, *7*, 659–678. [CrossRef] [PubMed]
7. Geiger, H.H.; Morgenstern, K. Applied genetic studies oncytoplasmic pollen sterility in winter rye (Angewandt-genetische Studien zur cytoplasmatischen Pollensterilität bei Winterroggen). *Theor. Appl. Genet.* **1975**, *46*, 269–276. (In German) [CrossRef] [PubMed]
8. Geiger, H.H.; Yuan, Y.; Miedaner, T.; Wilde, P. Environmental sensitivity of cytoplasmic genic male sterility (CMS) in *Secale cereale* L. In *Genetic Mechanisms for Hybrid Breeding (Advances in Plant Breeding, 18)*; Kück, U., Wricke, G., Eds.; Paul Parey Scientific Publishers: Berlin, Germany, 1995; pp. 7–17.
9. Miedaner, T.; Wilde, P.; Wortmann, H. Combining ability of non-adapted sources for male-fertility restoration in Pampa CMS of hybrid rye. *Plant Breed.* **2005**, *124*, 39–43. [CrossRef]
10. BSL (Beschreibende Sortenliste) Cereals, Maize, Oil and Fiber Plants, Legumes, Beets, Cover Crops [Getreide, Mais, Öl- und Faserpflanzen, Leguminosen, Rüben, Zwischenfrüchte] (In German). Hannover. 2020. Available online: https://www.bundessortenamt.de/bsa/sorten/beschreibende-sortenlisten/download-bsl-im-pdf-format (accessed on 28 March 2021).
11. Schwake-Anduschus, C.; Lorenz, N.; Lahrssen-Wiederholt, M.; Lauch, A.; Dänicke, S. German monitoring 2012–2014: Ergot of *Claviceps purpurea* and ergot alkaloids (EA) in feeding stuffs and their toxicological relevance for animal feeding. *J. Consum. Prot. Food Saf.* **2020**, *15*, 321–329. [CrossRef]
12. Raditsching, A.; Austrian Agency for Health and Food Safety (AGES), Institute for Food Safety, Linz, Austria. Personal communication, 2020.
13. European Food Safety Authority (EFSA). Scientific Opinion on Ergot Alkaloids in Food and Feed. EFSA Panel on Contaminants in the Food Chain (CONTAM). 2012. EFSA 10: 2798. Available online: http://onlinelibrary.wiley.com/doi/10.2903/j.efsa.2012.2798/pdf (accessed on 12 March 2021).
14. Miedaner, T.; Mirdita, V.; Rodemann, B.; Drobeck, T.; Rentel, D. Genetic variation of winter rye cultivars for their ergot (*Claviceps purpurea*) reaction tested in a field design with minimized interplot interference. *Plant Breed.* **2010**, *129*, 58–62. [CrossRef]
15. Miedaner, T.; Dänicke, S.; Schmiedchen, B.; Wilde, P.; Wortmann, H.; Dhillon, B.S.; Mirdita, V. Genetic variation for ergot (*Claviceps purpurea*) resistance and alkaloid concentrations in cytoplasmic-male sterile winter rye under pollen isolation. *Euphytica* **2010**, *173*, 299–306. [CrossRef]
16. Kirchhoff, H. Contributions to the biology and physiology of the ergot fungus [Beiträge zur Biologie und Physiologie des Mutterkornpilzes] (In German). *Centralbl. Bakteriol. Parasitenk. Abt. II* **1929**, *77*, 310–369.
17. Kodisch, A.; Oberforster, M.; Raditschnig, A.; Rodemann, B.; Tratwal, A.; Danielewicz, J.; Korbas, M.; Schmiedchen, B.; Eifler, J.; Gordillo, A.; et al. Covariation of ergot severity and alkaloid content measured by HPLC and one ELISA Method in inoculated winter rye across three isolates and three European countries. *Toxins* **2020**, *12*, 676. [CrossRef] [PubMed]
18. Meier, U. Growth Stages of Mono- and Dicotyledonous Plants. BBCH Monograph. 2001. Available online: https://www.julius-kuehn.de/media/Veroeffentlichungen/bbch%20epaper%20en/page.pdf (accessed on 4 March 2021).

19. BVL L 15.01/02–5:2012–01. Investigation of food-determination of ergot alkaloids in rye and wheat-HPLC method with purification on a basic aluminum oxide solid phase [Untersuchung von Lebensmitteln—Bestimmung von Ergotalkaloiden in Roggen und Weizen—HPLC-Verfahren mit Reinigung an einer basischen Aluminiumoxid-Festphase]. 2012. Available online: https://www.beuth.de/de/technische-regel/bvl-l-15-01-02-5/150736503 (accessed on 4 June 2021). (In German)
20. Bernal-Vasquez, A.M.; Utz, H.F.; Piepho, H.P. Outlier detection methods for generalized latt ices: A case study on the transition from ANOVA to REML. *Theor. Appl. Genet.* **2016**, *129*, 787–804. [CrossRef] [PubMed]
21. Cochran, W.G.; Cox, G.M. *Experimental Designs*; Wiley: New York, NY, USA, 1957; ISBN 978-0-471-54567-5.
22. Fehr, W.R. *Principles of Cultivar Development, Theory and Technique*; Macmillan: New York, NY, USA, 1987; Volume 1, ISBN 0029499208.
23. R Core Team. *R: A Language and Environment for Statistical Computing*; R Foundation for Statistical Computing: Vienna, Austria, 2018; ISBN 3–900051–07–0.
24. RStudio Team. *RStudio: Integrated Development for R*; RStudio, Inc.: Boston, MA, USA, 2016; Available online: https://www.rstudio.com/ (accessed on 4 March 2021).
25. Alderman, S. Ergot: Biology and Control. 2006. Available online: https://www.ars.usda.gov/SP2UserFiles/person/81/ErgotDVDtranscript.pdf (accessed on 26 March 2021).
26. Engelke, T. Approaches for an Integrated Control of Ergot (*Claviceps purpurea* [Fr.] Tul.) in Rye [Ansätze für eine integrierte Bekämpfung des Mutterkorns (*Claviceps purpurea* [Fr.] Tul.) im Roggen]. Ph.D. Thesis, University of Göttingen, Göttingen, Germany, 2002. (In German)
27. Miedaner, T.; Wilde, P. Selection strategies for disease-resistant hybrid rye. In *Advances in Breeding Techniques for Cereals*; Ordon, F., Friedt, W., Eds.; Burleigh Dodds Science Publishing: Cambridge, UK, 2019; pp. 223–246, ISBN 978-1786762443.
28. Kodisch, A.; Wilde, P.; Schmiedchen, B.; Fromme, F.J.; Rodemann, B.; Tratwal, A.; Oberforster, M.; Wieser, F.; Schiemann, A.; Jørgensen, L.N.; et al. Ergot infection in winter rye hybrids shows differential contribution of male and female genotypes and environment. *Eupyhtica* **2020**, *216*, 65. [CrossRef]
29. Blaney, B.J.; Molloy, J.B.; Brock, I.J. Alkaloids in Australian rye ergot (*Claviceps purpurea*) sclerotia: Implications for food and stockfeed regulations. *Anim. Prod. Sci.* **2009**, *49*, 975–982. [CrossRef]
30. Kniel, B.; Meißner, M.; Koehler, P.; Schwake-Anduschus, C. Studies on the applicability of HPLC-FLD and HPLC–MS/MS for the determination of ergot alkaloids in rye-containing breads. *J. Consum. Prot. Food Saf.* **2018**, *13*, 69–78. [CrossRef]
31. Müller, C.; Kemmlein, S.; Klaffke, H.; Krauthaus, W.; Preiß-Weigert, A.; Wittkowski, R. A basic tool for risk assessment: A new method for the analysis of ergot alkaloids in rye and selected rye products. *Mol. Nutr. Food Res.* **2009**, *53*, 500–550. [CrossRef] [PubMed]
32. Tittlemier, S.A.; Drul, D.; Roscoe, M.; McKendry, T. Occurrence of ergot and ergot alkaloids in western Canadian wheat and other cereals. *J. Agric. Food Chem.* **2015**, *63*, 6644–6650. [CrossRef] [PubMed]
33. Agrarmeteorologie Baden-Württemberg. Available online: https://www.wetter-bw.de (accessed on 10 March 2021).
34. Dhillon, B.S.; Mirdita, V.; Miedaner, T. Preliminary evaluation of locations for conducting selection for resistance to ergot (*Claviceps purpurea*) in rye. *Indian J. Plant Genet. Resour.* **2010**, *23*, 265–268.
35. Veršilovskis, A.; Fauw, D.P.K.H.P.; Smits, N.; Mulder, P.P.J.; Mol, H.; de Nijs, M. *Screening of Ergot Alkaloids by ELISA Test. Kits available on the Market. EURL Mycotoxins and Plant Toxins*; Wageningen Food Safety Research, Part of Wageningen University & Research: Wageningen, The Netherlands, 2019; EURL-MP-report_001; Available online: https://www.wur.nl/en/show/EURL-MP-report_001-Screening-of-ergot-alkaloids-using-ELISA-kits.htm (accessed on 12 March 2021).
36. Vendelbo, N.; Mahmood, K.; Sarup, P.; Kristensen, P.; Orabi, J.; Jahoor, A. Genetic architecture of male fertility restoration in a hybrid breeding system of rye (*Secale cereale* L.). *Res. Sq.* **2021**. reprinted in *Res. Sq.* **2021**. Available online: https://assets.researchsquare.com/files/rs-275908/v1_stamped.pdf (accessed on 31 March 2021). [CrossRef]
37. Kodisch, A.; Schmiedchen, B.; Eifler, J.; Gordillo, A.; Siekmann, D.; Fromme, F.J.; Oberforster, M.; Miedaner, T. Maternal differences for the reaction to ergot in unfertilized hybrid rye (*Secale cereale*). *Eur. J. Plant. Pathol.* **2021**, submitted.

Ergot alkaloid contents in hybrid rye are reduced by breeding

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Supplementary Materials

Table S1. Means of anther score (1-9). ergot severity (%). and ergot alkaloid contents (mg/kg) measured by HPLC and ELISA of 15 single-cross hybrids across all environments and the test environments after inoculation by *Claviceps purpurea*

	All environments (N=11)		Test environments (N=2)			
Hybrid	AS (1-9)	Ergot severity (%)	AS (1-9)	Ergot severity (%)	EA content HPLC (mg/kg)	EA content ELISA (mg/kg)
K2	3.55 c	3.74 a	2.00 de	11.47 a	45.27 a	75.82 bcd
K5	3.50 c	3.29 a	1.75 de	6.76 b	16.35 b	88.48 abc
K4	3.23 c	2.37 b	1.50 e	5.12 bc	17.27 b	126.99 a
K3	3.68 c	2.01 b	3.00 cd	4.16 c	11.28 c	133.09 a
K15	5.36 b	1.11 c	4.00 bc	3.09 e	9.99 c	112.99 ab
K14	5.82 b	0.66 cde	4.75 b	1.63 e	6.48 de	51.29 cde
K12	7.27 a	0.97 cd	8.25 a	0.57 e	1.51 ef	18.30 e
K17	7.12 a	0.46 de	7.75 a	0.54 e	1.02 ef	28.40 de
K7	7.27 a	0.46 de	8.25 a	0.44 e	5.25 ef	32.64 de
K18	7.36 a	0.53 cde	7.50 a	0.43 e	1.72 ef	43.67 cde
K20	6.87 a	0.33 e	7.75 a	0.39 e	2.25 ef	27.06 de
K19	7.05 a	0.39 de	7.25 a	0.37 e	1.54 ef	27.28 de
K10	7.36 a	0.44 de	7.75 a	0.31 e	1.91 ef	55.85 cde
K8	7.27 a	0.49 de	8.00 a	0.25 e	0.57 f	20.94 de
K9	7.32 a	0.31 e	7.75 a	0.22 e	0.92 ef	22.13 e
Mean	6.00	1.17	5.82	2.38	8.22	57.66

Table S2. Ergot severity (%) and individual and total ergot alkaloid contents (mg/kg) measured by HPLC of 15 hybrids across two environments

Geno-type	Ergot severity (%)	Ergot alkaloid content HPLC (mg/kg)												
		Ergo-metrin	Ergo-metrinin	Ergo-sin	Ergo-sinin	Ergo-tamin	Ergo-taminin	Ergo-cornin	Ergo-corninin	α-Ergo-cryptin	α-Ergo-cryptinin	Ergo-cristin	Ergo-cristinin	T
K2	3.74	1.79	0.27	1.09	0.28	8.93	1.35	3.23	0.66	5.45	1.19	18.20	2.82	4
K5	3.29	0.80	0.09	0.56	0.14	2.06	0.44	1.81	0.37	3.30	0.67	5.45	0.67	1
K4	2.37	0.94	0.16	0.52	0.15	2.51	0.47	1.33	0.30	2.68	0.69	6.69	0.84	1
K3	2.01	0.56	0.08	0.35	0.10	1.67	0.32	0.72	0.15	1.64	0.41	4.53	0.75	1
K15	1.11	0.41	0.06	0.30	0.06	1.55	0.40	0.92	0.16	1.38	0.24	4.07	0.44	
K12	0.97	0.08	0.00	0.09	0.02	0.18	0.04	0.38	0.08	0.34	0.07	0.21	0.02	
K14	0.66	0.47	0.11	0.27	0.08	1.08	0.22	0.63	0.17	1.06	0.30	1.87	0.23	
K18	0.53	0.17	0.02	0.09	0.02	0.19	0.04	0.17	0.04	0.35	0.09	0.48	0.07	
K8	0.49	0.04	0.00	0.02	0.00	0.17	0.03	0.04	0.01	0.09	0.03	0.12	0.02	
K17	0.46	0.08	0.00	0.05	0.01	0.11	0.03	0.13	0.02	0.28	0.06	0.21	0.03	
K7	0.46	0.20	0.05	0.08	0.01	1.10	0.30	0.11	0.03	0.26	0.05	2.73	0.33	
K10	0.44	0.31	0.11	0.14	0.04	0.06	0.00	0.22	0.06	0.73	0.16	0.07	0.01	
K19	0.39	0.14	0.02	0.11	0.03	0.07	0.02	0.37	0.10	0.42	0.12	0.13	0.02	
K20	0.33	0.13	0.02	0.16	0.05	0.12	0.02	0.60	0.13	0.57	0.16	0.25	0.03	
K9	0.31	0.07	0.00	0.04	0.01	0.14	0.04	0.07	0.01	0.13	0.03	0.37	0.04	

7. General discussion

7.1 Establishment of a harmonized method for testing ergot-related traits

For establishment of a harmonized testing system for ergot-related traits, five steps should be validated regarding their efficiency, suitability and practicability (Figure 1):

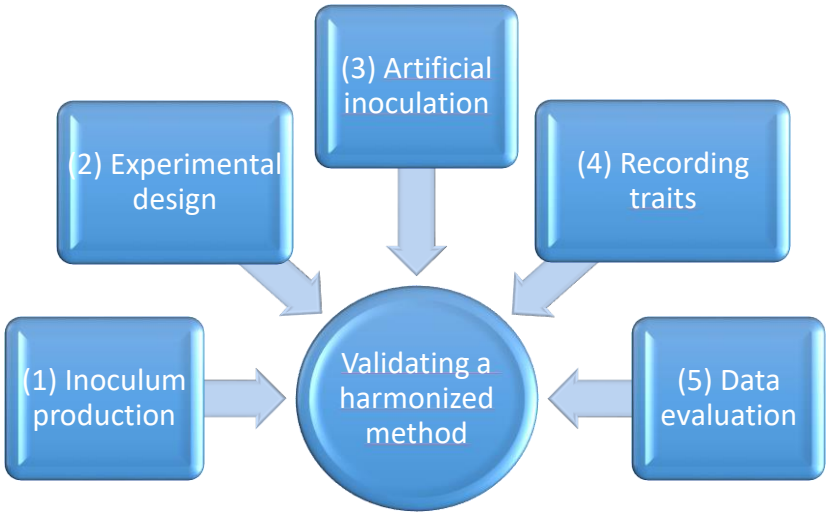

Fig. 1 Adjusting screws for validating a harmonized method for testing ergot-related traits

A standardized protocol for producing high quality ergot inoculum with high germination rates was successfully applied in all experiments (Publication 1-4) as previously described (Miedaner et al., 2010b) because the germination rate is one of the key quality parameters of the inoculum (Tenberge, 1999). Here, problems could arise when inoculation is outside the narrow time frame of flowering due to delays, technical or delivery problems. For this, germination rates of spores were analysed on water agar (WA) after freezing (data not published). A reason for the low initial values (55%) might be that the experiment started after trial inoculations and the inoculum was already stored 2 week at 4°C. Freezing lowers the germination rate of the spores after 6 weeks with minimal differences between -20°C (41%) and -80°C (39%). Thus, inoculum should be freshly prepared prior to inoculation although the inoculum could be stored at 4°C for a short time until usage. Consequently, germination rates should be always analysed prior to inoculation to guarantee sufficiently high infection levels.

Secondly, a field design with minimized neighbouring effects due to border plots in a completely randomized chessboard-like design revealed moderate to high repeatabilities and heritabilites and good differentiation among genotypes (Publication 1-4). Thus, the testing system worked very well for analysing ergot reaction in winter rye as previously shown (Miedaner et al., 2010b). Reasonable genotypic variation could be observed for ergot severity in years with high natural disease pressure (Publication 1). Nevertheless, a reliable artificial inoculation procedure was of huge importance in most environments to guarantee a considerable differentiation of genotypes especially in years with unfavourable infection conditions (Miedaner et al., 2010a; Publication 1,2). High correlation between both inoculation methods (Publication 1) indicated that results obtained under artificial inoculation represent the practical situation under natural infection conditions very well. Spray inoculation during flowering via tractor was suitable for field trials when evaluating a large number of genotypes simultaneously because ergot infections could be observed in almost all locations (Publication 1,2,4). In greenhouse and polyethylene tunnel trials spray inoculation could be used to generate solid infection levels (Publication 3). Here, the spraying could be handled routinely, location-independent and is, therefore, less prone to error making ergot experiments in the greenhouse promising also in the winter season with less environmental risks due to drought or storm (Duarte-Galvan et al., 2012). Another inoculation possibility is the needle inoculation. At this, the principal is exemplified by the motorised “golden hamster” (in German “Goldhamster”, Putoma AG Luzern, Switzerland) as professional inoculation and harvesting machine formerly used by the pharmaceutical industry (Universität Hohenheim, 2021). In this context, a needle inoculation approach by hand resulted in considerable ergot infections (Publication 3). Obviously, the clear advantage of this method was that all entries, also male-fertile rye, could be examined without pollen because the inoculation was done before flowering when the heads were still in the leaf sheath (BBCH 45-49) (Meier, 2018). Additionally, this method gave the advantage to analyse partially or fully fertile material in the field without complex and laborious isolation constructions or distances. At this, field approaches are better related to reality than greenhouse experiments (Publication 3). However, a further improvement and optimization of the needle method has to be done to conduct also large-scale studies which decrease possible error factors. Nevertheless, field and greenhouse experiments were correlated to a certain extent (Publication 3) illustrating that spray as well as needle inoculation can be used for evaluating ergot without pollen.

The traits ergot severity (Publication 1,2,4) and ergot incidence (Publication 3) were recorded as resistance trait with high repeatablities as shown in previous studies (Miedaner et al., 2010a, 2010b; Schwake-Anduschus et al., 2020). For high-throughput analysis of ergot severity and EA content, a flow scheme was developed (Figure 4). Here, harvesting was done at BBCH 87-89 (Publication 2,4) because EAs were produced rather during later stages of sclerotial development (Loo and Lewis, 1955). Additionally, samples were stored until milling at < 37°C and afterwards at -20°C (Publication 2,4) since EAs are highly temperature-sensitive (Bryła et al., 2019). The restorer index was a key parameter for the ergot reaction and can be calculated from anther rating (Publication 1,2,4) for estimating the pollen amount in a more accessible and comprehensible way (Geiger et al., 1995). When working with ergot-related traits, attention should be payed to checking the prerequisite of analyses of variance (ANOVA) and transformation should be done in case of need because the residuals were often not normally distributed in the environments (Publication 1, 2, 4) as also demonstrated in other studies (Miedaner et al., 2010a; Mirdita et al., 2008). An explanation was that much more low values appeared in comparison to high values for biological reasons such as limited source-sink-relationships leading to a distribution that was strongly skewed to the left.

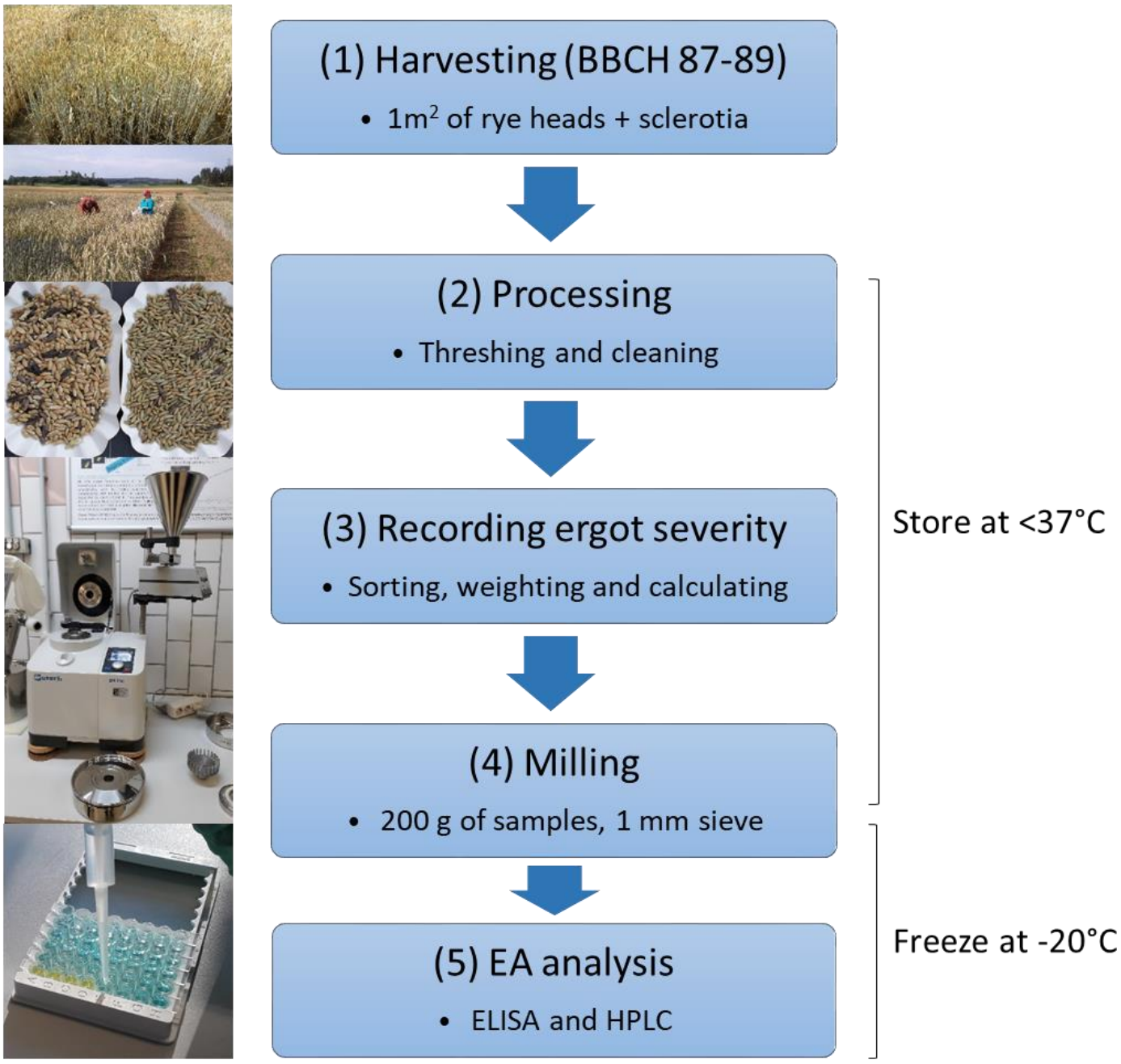

Fig. 4 Flow scheme for high-throughput analysis of ergot severity and EA content

7.2 Factors influencing ergot reaction

7.2.1 Environment

The environment was of upmost importance for ergot severity (Publication 1, 2, 4) also without pollen in field and greenhouse trials (Publication 3) and for EA content (Publication 2,4). This was in agreement for *Claviceps purpurea* in rye (Dhillon et al., 2010; Mainka et al., 2007; Miedaner et al., 2010b; Miedaner and Geiger, 2015; Mirdita and Miedaner, 2009) and *Claviceps africana* in sorghum (Wang et al., 2000; Workneh and Rush, 2006, 2002). For all experiments, locations as well as years were relevant (Publication 1-4). An explanation of the high environmental impact was that ergot severity and restorer index were tightly correlated (Publication 1,2,4). In this context, pollen shedding is strongly affected by weather while favourable infection conditions (wet, cool) negatively influence pollen production, viability, and flight (Miedaner and Geiger, 2015). Additionally, viable pollen depends on the severity of environmental conditions since moving in the air is drying the pollen (Fritz and Lukaszewski, 1989). But also without any pollen different infection levels and a large variation regarding their ergot reaction could be detected for individual locations and years (Publication 3). Although uniform handling and maintenance of environmental conditions seem to be easier in the greenhouse (Publication 3). In addition to that, averaged results for ergot severity and ergot incidence and also the differentiation among genotypes were higher in the greenhouse (spray inoculation) than in the field (needle inoculation) in almost all cases (Publication 3). Thus, also without pollen testing across several locations and years was definitely necessary as previously reported for other ergot-related traits (Publication 1,2,4). In conclusion, weather or climate conditions remain to be one of the main driving factors for ergot infection (Mainka et al., 2007; Orlando et al., 2017, Publication 1-4).

The same location could display a completely different ergot severity (Publication 1,2,4), ergot incidence (Publication 3) or EA content in different years (Publication 2,4). In literature it was shown that the locations with the highest ergot development were not always the best selection environment with the highest breeding success (Dhillon et al., 2010). Possible inequalities of soil or micro-climate were minimized by conducting multi-locational trials (Publication 1-4). But surrounding plant species could also contributed to a higher ergot incidence caused by generating a favourable micro-climate for ascospore discharge probably by shading as shown for ergot in a ryegrass - wheat complex (Mantle and Shaw, 1976).

Furthermore, genotype by environment interaction was of huge importance for all ergot-related trials (Publication 1-4) what was in agreement with the literature (Dhillon et al., 2010; Mainka et al., 2007; Miedaner et al., 2010b; Miedaner and Geiger, 2015; Mirdita and Miedaner, 2009). In the greenhouse without pollen, genotype by environment interaction was the most important source of variation (Publication 3). This illustrated that prediction of locations regarding ergot even in greenhouse trials without pollen was difficult or even impossible. So, plant breeders and scientific researches have to perform multi-environmental trials at locations with different agro-climatically conditions ideally with high annual precipitation.

Another aspect was the interaction between fungal isolate and environment because development and infection process of the pathogen itself was highly influenced by environmental conditions (Mainka et al., 2007). Here, moist and cool weather were optimal for spore germination (Menzies and Turkington, 2015). In addition, environmental conditions could affected poikilothermic insects contributing to *Claviceps purpurea* proliferation whereas ergot occurred in clusters under natural conditions for Kentucky bluegrass (*Poa pratensis*) (Dung et al., 2019). Some *Claviceps purpurea* strains could be more encouraged by environmental conditions than others (Jungehülsing and Tudzynski, 1997; Meinicke, 1956; Schulze, 1953). This might be an explanation for varying ergot severities found for different countries (Publication 1) although isolate-by-environment interaction variance was not significant (Publication 1,2,4). Thus, the origin of the isolate must not be taken in consideration with a great effort but rather determining the aggressiveness of the inoculum as key factor for achieving a good genotypic differentiation. The EA profile was demonstrated to be rather stable across different years for the individual isolates (Publication 2) and for an isolate mix (Publication 2,4). Nevertheless, changes of the EA profile seem to occur frequently caused by environmental conditions (EFSA, 2012; Schummer et al., 2020) and even seasonal-dependent variations of the EA formation appeared for the systemic endophytic fungus *Epichloë* when infecting *Festuca sinensis* (Lin et al., 2019). An explanation of the stable EA profile (Publication 2, 4) could be that main driving factors supporting EA profile changes such as experimental design, processing, detection technique, analysed species, respective cultivar, and product type were handled similar.

7.2.2 Genotype

High variations regarding ergot severity could be observed for commercially available population and hybrid cultivars (Publication 1). So, the choice of variety was relevant for ergot contamination (Appelt and Ellner, 2009; Krska and Crews, 2008; Mainka et al., 2007; Orlando et al., 2017; Pažoutová, 2002) and is an effective tool for farmers in practice. Genotypic variation regarding ergot severity could be found in all studies (Publication 1,2,4) also without pollen (Publication 3) as already mentioned in other rye studies (Miedaner et al., 2010b, 2010b; Mirdita et al., 2008). For other cereals such as spring and durum wheat, triticale, or barley, the genotype was an important factor influencing the reaction to *Claviceps purpurea* in one of the three main components of the disease reaction: frequency of sclerotia, size of sclerotia, and amount of honeydew (Mainka et al., 2007; Menzies, 2004; Platford and Bernier, 1976, 1970). In durum wheat, the genotype was a significant factor influencing EA concentrations (Tittlemier et al., 2016). Plant breeding requires genetic variation (Brown and Caligari, 2011). Thus, developing new superior cultivars that are less susceptible to ergot is possible and less susceptible genotypes can be utilized directly as resistance donors in existing breeding cycles (Publication 1-4) confirming the literature (Dhillon et al., 2010; Miedaner et al., 2010a, 2010b; Miedaner and Geiger, 2015; Mirdita and Miedaner, 2009). Additionally, it was demonstrated that values of ergot severity and incidence in the greenhouse were positively correlated (Publication 3). Consequently, highly contaminated entries comprised a large number of rather small ergot bodies because the sclerotial size is limited by the sink-source relationship. A large number of small sclerotia were even more difficult for mechanical cleaning processes because size was then similar to kernels (Miedaner and Geiger, 2015). Therefore, the greatest threat would arise from genotypes producing many small sclerotia.

For reducing ergot, breeders concentrate currently on creating cultivars with a high and environmentally stable pollen production to outperform the competitive situation of pollen and fungal spores (Engelke, 2002; Geiger and Miedaner, 2009; Miedaner and Geiger, 2015). For balancing a smaller pollen shedding out, blending of 5-10% population rye in commercial hybrid cultivars is done by some companies (Engelke, 2002; Miedaner et al., 2008). But mixing population rye was not always successful for all tested hybrid cultivars showing a high ergot contamination (Publication 1). Although blending lowered ergot severity and incidence considerably for entries that are more susceptible, the effect could be neglected for cultivars

with low ergot reaction and was often not enough to reduce ergot in practice (Miedaner et al., 2010b). So, blending seems to be only a supplementary measures against ergot.

As for now, full restoration of male fertility by introgression of effective restorer genes was the most favourable way to minimize ergot infection in hybrid rye varieties (Miedaner and Geiger, 2015; Publication 1,4). The restorer index was clearly increased by a raising proportion of the restorer gene what led to a considerable reduction of ergot at artificial inoculation as well as under natural infection conditions (Publication 1). This strong relationship between pollen and ergot could be found in all experiments (Publication 1,2,4). Interestingly, introgression of the IRAN IX *Rfp1* gene, even in small proportions, could reduce ergot considerably (Publication 1). For example introgression of 25% was followed by an 70% decrease of ergot severity (Klotz, 2002; Publication 1). That was of particular interest for breeding purposes because significant yield reduction was ascertained for hybrids with a high proportion of a non-adapted restorer gene in earlier stages of backcrossing (Miedaner et al., 2017). Obviously, ergot contamination was substantially higher when hybrids were only partially restored or effective restorer genes were completely missing (Miedaner and Geiger, 2015; Publication 1,4). Additionally, the origin of the restorer gene was of crucial importance for the fertility restoration ability of the pollinator (Publication 1,4). This was even more conspicuous when comparing ergot severity of pollinators with restorer genes of European origin (minus) and those with non-adapted restorer genes (plus; Publication 1). The first one was often restoring in an inadequate way resulting in low quantities of pollen and, thus, high ergot contamination especially under adverse environmental conditions. A clear difference between European and exotic restorer genes could not be found for all cases (Publication 1) what might be probably associated with problems in the introgression process, when European restorer genes having already a high amount of effective restoration or the female seed parent was quite easy to restore.

The close relationship of anther rating and ergot severity clearly showed that cultivars with a high pollen amount possess also lower ergot contamination (Publication 1,2,4) but there was the question if breeding for cultivars with a higher and environmental stable pollen shedding also came together with a reduced EA content and how the EA content and profile was influenced by the genotype. For the pathosystem rye – *Claviceps purpurea*, the EA content was clearly influenced by the genotype (Publication 2,4) and it was shown that the EA content

can be clearly and considerably reduced by breeding (Publication 4). For this, cultivars with a high pollen shedding caused by introgression of non-adapted *Rf* genes (Miedaner et al., 2008, Publication 1,4) or exploiting variation in cytoplasm and maintainer genes of female CMS lines that are easier to restore (Publication 1,4; Vendelbo et al., 2021) showed a considerable less ergot contamination and simultaneously a reduced EA content (Publication 4). So, hybrid breeding for a lower EA contamination was promising when selecting male and female components carefully although intra-pool diversity and the number of potential crosses for new hybrids was reduced (Hallauer et al., 2010). Interestingly, the female line in the same cytoplasm contributed a much higher proportion on the total genetic variance for the EA content as for ergot severity although the male component was of higher importance for both traits (Publication 4). So, for the reduction of the EA content, attention should be paid to the male fertility restoration ability of the pollinator as well as the female restorability of the seed parent.

The female restorability of the seed parent was important for ergot-related traits although the male component was of higher importance (Publication 1,3,4). When focusing on the maternal side in a special testing system without any pollen small but significant genotypic differences regarding ergot severity and ergot incidence could be detected for unfertilized CMS-single crosses inoculated by needle and spray method (Publication 3) as previously described (Miedaner et al., 2010a). In addition, quantitative differences for susceptibility were reported among artificially inoculated CMS-inbred lines when excluding the factor pollen (Geiger and Bausback, 1979). A reason might be variable responses of the ovaries during the infection process or a resistance mechanism. Therefore, it could be a determining factor in future breeding programs to further reduce ergot when a high pollen amount was already reached. Here, a more ergot-resilient CMS single cross could be identified showing lower ergot contamination for all years and inoculation methods (needle, spray; Publication 3) what might be a promising candidate for future research or breeding programs. Large differences could be detected regarding the ease of restoration for the CMS female lines which affected also ergot contamination levels (Publication 1). At this, CMS female lines that were easier to restore showed also less ergot severity and *vice versa* (Publication 1). So, selecting appropriate CMS female lines in hybrid breeding is crucial for lowering ergot reaction. Additionally, moderate correlation between inbred lines and their testcrosses for ergot severity showed that pre-selection among lines per se was possible and only a smaller number of carefully

selected testcrosses had to be analysed (Miedaner et al., 2010a). Nevertheless, differences for ergot severity could be found for CMS female lines although the restoration ability was similar (Publication 1). A reason here could be different heading stages (Publication 1) and, thus, a disease escape. Another explanation might be physiological characteristics of the ovary although a real resistance against ergot based on physiological processes had not been detected so far in rye (Menzies and Turkington, 2015; Miedaner and Geiger, 2015; Publication 3). Nevertheless, QTL contributing to a partial resistance have been documented in bread wheat (Gordon et al., 2015) or durum wheat (Gordon et al., 2020) and resistance-related genes found for transcriptome analysis in wheat supporting the concept of a resistance against ergot. So, it might be exploited by breeders in future for creating new ergot resistant or at least distinctly less susceptible cultivars.

7.2.3 Fungal characteristics and isolate

The isolate was observed as significant factor influencing ergot severity as well as EA content in a genotype-unspecific way (Publication 1, 2,4). This confirmed findings in the literature because EA content and EA profile was dependent on *Claviceps* isolates and population dynamic of different strains (Appelt and Ellner, 2009; Mainka et al., 2007). The high impact of the isolates could be also observed for other cereals like wheat where large pathogenic variation and considerable differences among isolates were reported in accordance to the geographic origin (Menzies et al., 2017). The same could be observed in wheat sclerotia since EA concentrations were highly influenced by the isolate (Tittlemier et al., 2016). Furthermore, endophyte strains showed quantitative and qualitative variation regarding ability and concentration of EAs (Easton et al., 2002; Hill et al., 1991; Latch, 1994).

The country-specific inocula showed in the respective country higher ergot contamination than the foreign inocula in nearly all cases (Publication 1). Additionally, large differences regarding the EA quantity could be observed for the country-specific isolates although the ergot contamination was similar (Publication 2). This might be explained again by the fact that some *Claviceps purpurea* isolates could be more supported by specific environmental conditions (Jungehülsing and Tudzynski, 1997; Meinicke, 1956; Schulze, 1953). Isolates of *Claviceps purpurea* from diverse collection sites in different countries varied in their aggressiveness (Publication 1, 2) resulting in a different degree of pathogenicity and, thus,

occurrence of ergot sclerotia. Nevertheless, EAs seemed to act not as major virulence factors contributing to a more promising infection in the pathosystem *Claviceps purpurea* and rye (Publication 2). Here, a higher ergot severity level was not closely associated with an increased EA content (Publication 2) although it was indicated that EAs were associated with virulence in literature (Panaccione and Arnold, 2017). It should be noted here that EA biosynthesis genes and underlying functions were already studied (Gerhards et al., 2014; Young et al., 2015) but the function of EAs and the relevance for the infection process was not truly explored yet. So, further studies, probably with EA defective strains, will help to clarify the function of EAs and their impact on the infection process.

For *Claviceps purpurea,* large differences regarding the EA content could be detected for the isolates (Publication 2,4) what was already mentioned in literature (Taber and Vining, 1958). Since always a mix of different isolates is appearing in practice, farmers and breeders cannot influence or predict the isolate in the practical situation without artificial inoculation. So, although the concept of cross-protection was not really proven yet for ergot in rye, it might be possible in future like it is reported for ergot in tall fescue toxicosis (Bouton et al., 2002) or aflatoxin and “aflasafe” (Senghor et al., 2021). In this context, all tested isolates produced EAs in all experiments (Publication 2,4) but further screening should reveal naturally occurring isolates producing no EAs in the host plant as already mentioned for various *Epichloë* isolates (Young et al., 2015). At this, a sequence-related amplified polymorphism (SRAP) marker system revealed high genetic intraspecific variation for isolates originated from rye plots in plant breeding nurseries and naturally rye fields in Poland (Irzykowska et al., 2012). Another possibility could be the usage of transformation-mediated biotechnology as described for pharmaceutical production of EAs (Panaccione et al., 2012) when legislative regulations ever approve genetically modified organism (GMO) in practice. Furthermore, it should be noted here that although EA spectrum shifts seem to be very common (EFSA, 2012) and were also affected by the isolates (Battilani et al., 2009; Krska and Crews, 2008) all three country-specific isolates had a very similar and stable EA profile (Publication 2). It is known that different EA profiles were mostly the result of functional mutations of pathway genes (Gerhards et al., 2014; Young et al., 2015). The reason for the stable EA profile here might be again that the flow scheme and important parameters like handling or detection method was rather similar for all experiments (Publication 2,4).

7.3 Covariation of ergot severity and EA content measured by HPLC and ELISA

The covariation of ergot severity and EA content determined by HPLC showed an inconsistent picture (Publication 2,4) what was already mentioned in previous studies varying from low (Babič et al., 2020; Mainka et al., 2007; Schummer et al., 2018) to moderate (Bryła et al., 2018; Kenyon et al., 2018; Mulder et al., 2012; Publication 2; Schwake-Anduschus et al., 2020) to high (Grusie et al., 2017; Orlando et al., 2017; Publication 4; Tittlemier et al., 2015). A reason for the high correlations outlined in some studies might be the large genetic variation of the analyzed samples. For Publication 4, the focus was set on rye material with large genotypic variation from a breeding company. Additionally, the study was performed in a location that was in the light of past experience excellently suited for ergot research (Dhillon et al., 2010; Miedaner and Geiger, 2015; Publication 1-4). Additionally, it was known that for *Claviceps purpurea* the genetic variation of fungal populations was considerably higher in experimental plots of plant breeding stations under artificial infection than in naturally infected rye fields caused by the high genotype variety of host plants (Irzykowska et al., 2012). However, the correlation was often to be found not linear (Publication 2). This might be because of huge variations of the total EA amount of sclerotia of similar weight (Bürk et al., 2006; Frach and Blaschke, 1998; Grusie et al., 2017; Lauber et al., 2005; Lombaert, 2001; Lorenz and Hoseney, 1979; Schoch and Schlatter, 1985; Scott, 2009; Wolff and Richter, 1989). Furthermore, the correlation decreased considerably with major deviations from the regression for low EA concentrations (Publication 2). This was in agreement to previous findings (Grusie et al., 2017) and represented even more the real situation at practical fields (Byrd and Slaiding, 2017; Malysheva et al., 2014; Meister and Batt, 2014; Mulder et al., 2012; Müller et al., 2009; Ruhland and Tischler, 2008; van Dongen and de Groot, 1995; Wegulo and Carlson, 2011). An explanation could be the rising coefficient of error variation when EA concentrations were decreasing (Grusie et al., 2017). But also for high infection levels (ergot severity ≥0.05%), the correlation was not getting considerably better (Publication 2). Although it was previously well demonstrated that the EA content is highly influenced by environment and isolate (Publication 2) grouping of samples according to these parameters, *e.g.* separating locations or country-specific isolates, did not improve the covariation in a consistent way. Nevertheless, some locations and isolates showed higher correlations but this was often not stable and predictable across multiple years (Publication 2) where typically a variety of isolates occurs. This illustrated that this was not useful for breeders or farmers to effectively reduce ergot.

The tested ELISA kit showed considerably higher EA contents and distribution patterns when comparing to HPLC (Publication 2,4). A reason here was probably the fundamental differences of the underlying methodology of HPLC (Beuerle et al., 2012) and ELISA (LCTech, 2021). The HPLC is an internationally validated tool analyzing twelve specific EAs in a quantitative way (EFSA, 2012; Schardl, 2015; Shi and Yu, 2018). In contrast, the ELISA kit determines lysergic acid as progenitor in the EA pathway (LCTech, 2021). Since a wide variety of individual EAs were known (Křen and Cvak, 1999; Schiff, 2006) the ELISA could overestimated the HPLC results quite easy as demonstrated for three commercially available ELISA kits (Veršilovskis et al., 2019; Publication 2,4). Nevertheless, ELISA approaches were already used for examinating EAs (Shelby and Kelley, 1992; Tunali et al., 2000) especially for fescue toxicosis in livestock (Kenyon et al., 2018; Roberts et al., 2014; Schnitzius et al., 2001). Another problem here was that the working range of commercially available ELISA kits was in many cases not sufficiently large enough (Publication 2,4) also for naturally infected samples (Veršilovskis et al., 2019). At this, extremely high dilution seemed to be not appropriate to bring samples in the measuring area since an extra error must be added (Publication 2,4). Thus, the ELISA was not a good tool for untrained staff. Additionally, a varying consensus could be also an explanation because the group of EAs is highly diverse and similar in structure leading possibly to cross-reactivity (Florea et al., 2017; Gerhards et al., 2014; Kren et al., 1994; Schiff, 2006). Here, it was conceivable that cross-reactivity could vary for different EA groups (Shi and Yu, 2018).

This large discrepancy was also expressed by the fact that no relationship between ELISA and HPLC values was found illustrated by very low covariation values (Publication 2). The same could be observed for Kentucky blue grass (Schnitzius et al., 2001) even when concentrating on an individual EA such as ergovaline (Roberts et al., 2014). Although ergovaline showed high toxicity (Caradus et al., 2020) the compound is not covered by the HPLC protocol recommended by the EFSA in future regulations because it appeared to be present only in traces in *Claviceps purpurea* (EFSA, 2012). So, it was also not analyzed in this studies (Publication 2,4). No improvement of the correlation was detected by grouping the factors environment, genotype, isolate or infection level (ergot severity <0.05% and ≥0.05%; Publication 2). Thus, the ELISA was not the right tool for screening EAs in a large-scale for milling and food industry in their day-to-day routine in an appropriate way. Also separating of the samples into cultivars or individual locations will not be working. In contrast to this, when focusing on samples with large genetic variation the correlation between ELISA and HPLC

values was obviously raising from zero to moderate (Publication 4). So, further improvement would enable us to minimize the high number of samples that has to be analyzed in breeding programs and concentrate on the samples showing a low EA contamination.

Thus, the covariation between ergot contamination and EA content outlined in several studies remained to be volatile and highly variable depending on several factors such as genotype, isolate or environment. Here, the impact of these parameters were at the moment not fully understood. This pointed out that the sclerotia amount of a sample was not suitable to predict EA concentrations in the product in a reliable way (Publication 2) being in agreement to other researchers coming to the same conclusion for diverse cereals (Mainka et al., 2007; Mulder et al., 2012; Schummer et al., 2018; Schwake-Anduschus et al., 2020). For this reason, chemical analysis of EA content as part of the legislative regulation apart from ergot contamination is absolutely required to monitor and evaluate potential health risks for food and feed safety. In this context, HPLC – based approaches are recommended since the used ELISA was not an alternative due to missing coincidence and corresponding correlation.

7.4 Conclusion: what should breeders, farmers, food and milling industry do?

The major outcomes were related to clarify significant factors influencing ergot reaction in winter rye, to establish a harmonized method for testing ergot in field and greenhouse trials with/without pollen, to investigate the covariation between ergot severity and EA content determined by HPLC, and to test a commercial ELISA kit as suitable screening method. High heritabilities of almost all experiments and nearly perfect correlation between natural infection and artificial inoculation demonstrated that results of experimental trials represented the practical situation very well. Additionally, a harmonized method for testing ergot-related trials also without pollen could be validated. In figure 5, a complex picture can be drawn with a variety of significant factors, more precisely genotype, environment (location, year, country) and isolate, having an influence to a greater or lesser degree on the ergot reaction. Further, interaction of the individual factors, aspects for breeders and effective strategies for farmers, milling and food industry was demonstrated.

In detail, it was shown that the environment and environment × genotype interaction was of tremendous role for the ergot reaction. So, multi-environmental testing across several

locations and years with different agroclimatic conditions is absolutely necessary. Breeders and scientists should use locations with high annual precipitation resulting in a high infection pressure and ideal conditions for disease occurrence. Further, the environment is also important for farmers, milling and food industry because ergot contamination is increased in years with cool and rainy conditions. Thus, monitoring of the weather is important to estimate the potential disease risk and to take the respective safety and cleaning measures, if required.

The second significant factor with a major importance for ergot severity as well as EA content was the host genotype as illustrated in figure 5. The genotype can be strongly influenced by breeders in generating resistant cultivars. For ergot, less susceptible cultivars can be used right away in breeding cycles as resistance donors. Here, the male fertility restoration ability of the pollinator by introgression of effective and environmental-stable *Rf* genes was still one of the key factors for lowering ergot susceptibility in breeding programs. For large genotypic variation, it was also demonstrated that the EA content could be reduced simultaneously to a large extent by implementing a high pollen shedding. Further, the origin of the *Rf* gene was important while exotic *Rf* genes showed a better restoration compared to European *Rf* genes especially for unfavorable conditions. Here, low proportions of the *Rf* gene worked very well. Thus, significant yield reduction related to hybrids with a high proportion of a non-adapted restorer gene in earlier stages of backcrossing can be avoided. Blending of population rye in hybrid cultivars to overcome a lower pollen amount was only a complementary measures against ergot. Another way for breeders was the female restorability of the seed parent and using CMS cytoplasms that were quite easy to restore. Exploiting of maternal components in breeding cycles could reduce the EA content substantially. Here, the contribution of the female line on the total genetic variation was much higher for the EA content than for ergot severity although the male component was again of highest importance. A resistance mechanism could be not confirmed yet but significant maternal effects in a special test system without pollen could be observed. Therefore, exploring of a real physiological resistance will be worth the effort and will give us the potential to additionally reduce ergot especially when a high restorer index was already implemented.

EA content and ergot severity was strongly influenced by the isolate in a genotype-unspecific way although the EA profile was rather stable across the years and isolates. Huge differences could be observed for the EA content for three country-specific isolates despite a similar ergot

contamination. At this, ergot severity was not increased by a higher EA content illustrating that the EAs were not acting as major virulence factors in the infection process of *Claviceps purpurea*. Since the relevance of genotype-by-isolate interaction was low it could be concluded that the aggressiveness of the inoculum was not necessarily based on the origin of the isolate but had to be determined ahead of each inoculation.

For farmers, a “To do” list against ergot was mentioned (Figure 5). Here, the best strategy is to choose resistant cultivars that are less susceptible to ergot. In addition, a good disease management should be done with adequate agronomic measures like weed control, or deep plowing. It is necessary to monitor the ergot amount and in the case of need, milling and food industry could remove ergot by mechanical cleaning or optical colour-sorting machines to a large extent. The covariation between ergot severity and EA content measured by HPLC was fluctuating and often moderate for a diverse sampling. So, ergot contamination could not displayed the content of toxic EAs in an appropriate way making legislative changes and integration of EA content beneath ergot amount absolutely indispensable. Further, analyzing EAs is necessary for milling and food industry in their day-to-day routine to secure food and feed safety because a clear decrease of the correlation could be found for low ergot contaminations what is usually the case in practice. In contrast, the covariation was high for samples with large genotypic variation revealing a great potential in breeding cycles in future. Ergot contamination as well as EA content could be then simultaneously reduced to a large extent by implementing a high pollen amount via exotic *Rf* genes. The missing correlation between ELISA and HPLC data for a diverse set of samples leads to the conclusion that the tested ELISA kit was not a reliable screening tool for milling and food industry in their daily life. Problems could occur at the ELISA handling because the working range of commercially available ELISA kits was often too small creating the need of high and error-prone dilution series. So, just one clear recommendation could be made for the milling and food industry in their daily routine comprising a 3 step strategy: step one assessing the ergot amount based on the traditional approach, step two rejecting all samples that exceeded the visually visible limit as usual, and step 3 determining the EA content by HPLC for all remaining samples and rejecting again all samples that exceed the limit to guarantee that all food and feed samples are harmless, safe and will meet the European limits.

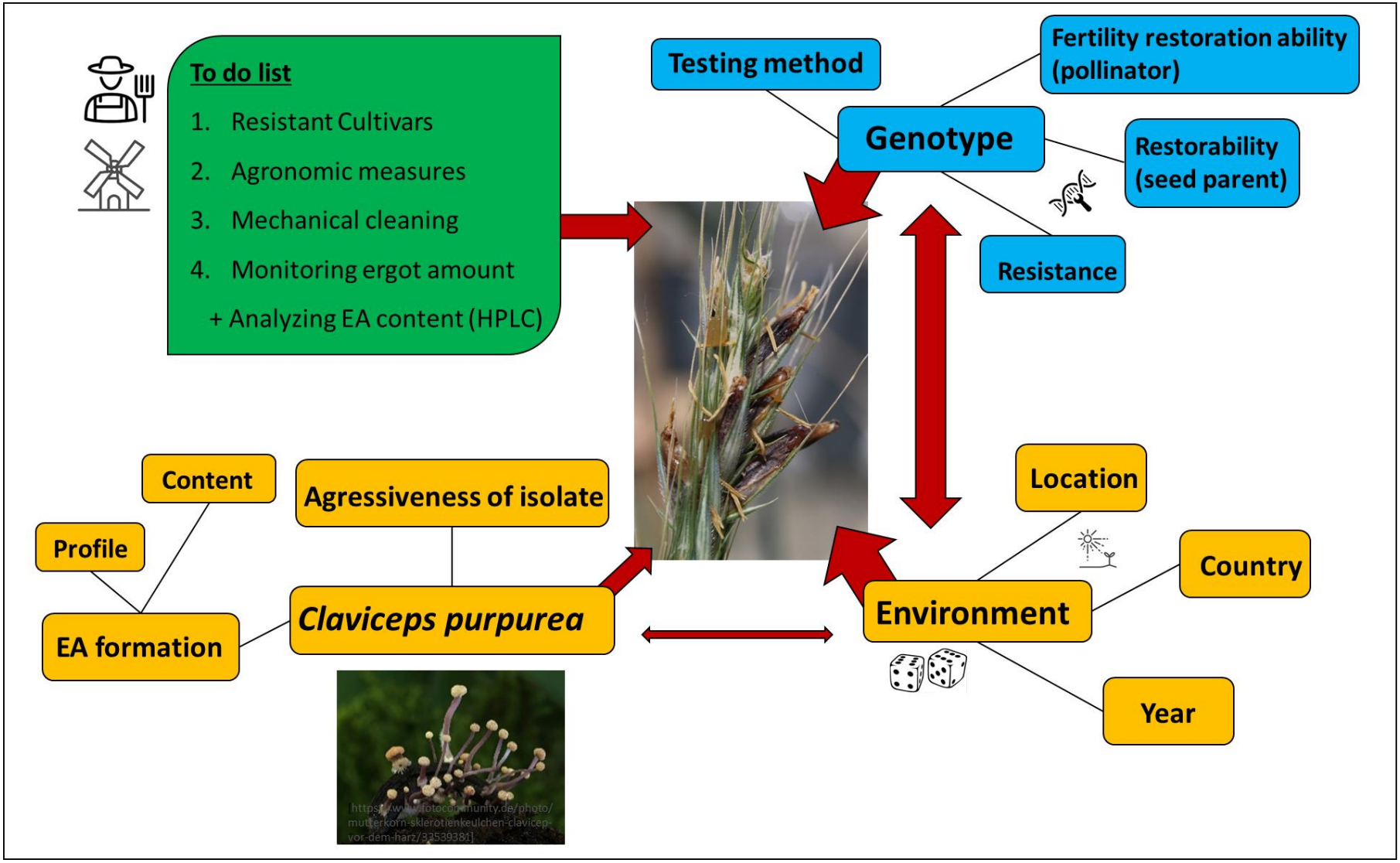

Fig. 5 Overview of significant factors and their interaction and relevance (symbolized by thickness of red arrows) for the ergot reaction: uncontrollable parameters (orange), aspects for breeders (blue), and strategies for farmers, milling and food industry (green)

8. Summary

Ergot caused by *Claviceps purpurea* [Fr.] Tul. is one of the oldest well-known plant diseases leading already in medieval times to severe epidemic outbreaks. After the occurrence of honeydew, the characteristic ergot bodies called sclerotia are formed on the ear. These are containing toxic ergot alkaloids (EAs). Strict limits are set within the European Union. Rye (*Secale cereale* L.) as cross-pollinating crop is particularly vulnerable to ergot since *Claviceps* cannot grow through intact glumes leading to a competitive situation of fungal spores and pollen during flowering. Resistance breeding against ergot focuses on generating cultivars with a high and environmental stable pollen shedding. Nevertheless, even today the threat is real as agricultural practice is changing such as skipping of deep plowing or maintaining flower strips including grasses that can serve as inoculum spreading areas. And even more serious screening studies revealed EAs in samples of the whole cereal value chain frequently. The overall aims were firstly to establish a harmonized method to test ergot resistance and EA contamination in winter rye and secondly to clarify major significant factors and their relevance, more precisely genotype, environment (location, year, country), and isolate. A third aim was created by the economic need due to prospective legislative changes. So, effort was paid to examine the covariation of ergot amount and EA content considering different factors. And finally to reveal the suitability of one commercial ELISA test as functional alternative by analysing 372 winter rye samples from different genotypes, locations from Germany, Austria, and Poland over two years, and three isolates.

In a first study, genotypes showed significant variation for ergot severity and pollen-fertility restoration after natural infection as well as artificial inoculation whereas a high positive correlation could be found between both inoculation treatments. Additionally, variances of environment, general combining ability (GCA), specific combining ability (SCA), and interactions were significant. Although male pollen-fertility restoration was of utmost importance, the female component was also significant. This illustrates that apart from promising selection of high restoration ability the maternal restorability could be exploited in future breeding programs especially when a high pollen amount is already reached.

In a second study, a large-scale calibration study was performed to clarify the covariation of ergot severity, EA content by HPLC and ELISA considering genotypes, locations, countries,

years, and isolates. EA profile was rather stable across country-specific isolates although large differences regarding the EA content were detected. The moderate covariation between ergot severity and EA content determined by HPLC indicates that a reliable prediction of the EA content based on ergot severity is not possible what can also not be explained by grouping effects of tested factors. Further, EAs seem not to act as virulence factor in the infection process since EA content showed no relationship to disease severity. Additionally, the missing correlation of ELISA and HPLC leads to the conclusion that the ELISA is not an appropriate tool what can be used safely to screen samples regarding ergot in the daily life.

In a third study, the genetic variation of male-sterile CMS-single crosses was analysed in a special design without pollen in field and greenhouse to identify resistance mechanisms and to clarify whether ergot can be reduced in the female flower. At this, comparison of needle (field) and spray (greenhouse) inoculation revealed medium to high correlations illustrating that both methods were suitable for this research. Significant environment and genotype by environment interaction variances were detected. So, testing across several environments (location, year) is necessary also without pollen. Further, small but significant genotypic variation and identification of one more ergot-resilient candidate revealed that selection of female lines could be promising to further reduce ergot contamination.

And in a fourth study, it was shown that the EA content of rye cultivars was lower for less susceptible genotypes. Thus, EA content can be considerably reduced by breeding. In contrast to study 2, a strong positive correlation could be found for ergot severity and EA content when analysing 15 factorial single crosses with artificial inoculation whereas also the correlation between ELISA and HPLC values was increased. The reason was that the trials were performed in empirically well suited locations for ergot research as well as the focus was set on samples with a large genotypic variation. The male pollen-fertility restoration was also here the most relevant component but the female component contributed an obviously higher proportion for the EA content than for ergot severity.

In conclusion, this thesis demonstrate that implementing of a high and environmental stable male fertility restoration ability via exotic *Rf* genes can effectively reduce ergot although also the female restorability enables great opportunities. The unpredictable covariation between ergot amount and EA content illustrates that both traits have to be assessed, in particular the EA content by a valid HPLC approach to guarantee food and feed safety.

9. Zusammenfassung

Mutterkorn wird durch *Claviceps purpurea* [Fr.] Tul. verursacht und ist eine der ältesten bekannten Pflanzenkrankheiten, welche bereits im Mittelalter zu schweren epidemischen Ausbrüchen geführt hat. Nach dem Auftreten vom Honigtau werden die charakteristischen Mutterkörner, auch Sklerotien genannt, an der Ähre gebildet. Diese enthalten toxische Mutterkornalkaloide (*engl.*: ergot alkaloids, EAs). Strenge gesetzliche Vorschriften gelten innerhalb der Europäischen Union. Roggen (*Secale cereale* L.) ist als Fremdbefruchter besonders anfällig gegenüber Mutterkorn, da *Claviceps* nicht durch geschlossene Hüllspelzen hindurchwachsen kann und sich somit eine Konkurrenzsituation von Pilzsporen und Pollen während der Blüte ergibt. Die Resistenzzüchtung gegen Mutterkorn konzentriert sich auf die Entwicklung von Sorten mit einer hohen und umweltstabilen Pollenschüttung. Dennoch ist die Gefahr auch heute vorhanden, da sich die landwirtschaftliche Praxis ändert, wie das Auslassen von tiefem Pflügen oder die Erhaltung von Blühstreifen inklusive Gräsern als mögliche Inokulumquelle. Und noch bedenklicher, zahlreiche Screening-Studien EAs wurden in Proben der gesamten Wertschöpfungskette von Getreide wiederholt nachgewiesen. Das oberste Ziel war es zum einen, eine harmonisierte Methode zu etablieren, um die Mutterkornresistenz und Akaloid-Kontamination von Winterroggen zu testen, und zweitens, wichtige Einflussfaktoren und deren Interkation, genauer Genotyp, Umwelt (Standort, Jahr, Land) und Isolat, aufzuklären. Ein drittes Ziel ergab sich hierbei durch eine zukünftige Gesetzesänderung und dem daraus folgenden ökonomischen Bedarf. Folglich lag ein besonderes Augenmerk auf der Untersuchung des Zusammenhangs zwischen Mutterkorngehalt und Alkaloid-Gehalt, ermittelt mit der HPLC, unter Berücksichtigung der Einflussfaktoren. Und schließlich die Eignung eines kommerziellen ELISA Tests als funktionale Alternative durch die Analyse von 372 Winterroggenproben von unterschiedlichen Genotypen, Standorten aus Deutschland, Österreich und Polen von 2 Jahren und 3 Isolaten zu prüfen.

In einer ersten Studie zeigten die Genotypen sowohl unter natürlicher Infektion als auch künstlichen Inokulation eine signifikante Variation hinsichtlich Mutterkornanteil und Pollenfertilitäts-Restauration, wobei die beiden Inokulationsverfahren eine starke, positive Korrelation zeigten. Zudem waren die Varianzen von Umwelt, generelle Kombinationseignung (GCA), spezifische Kombinationseignung und die Interaktionen signifikant. Obwohl die männliche Pollenfertilitäts-Restauration von höchster Wichtigkeit war, war auch die weibliche

Komponente signifikant. Dies verdeutlicht, dass neben der vielversprechenden Selektion auf hohe Restaurationsfähigkeit auch die weibliche Seite in zukünftigen Züchtungsprogrammen genutzt werden kann, vor allem, wenn bereits eine hohe Pollenmenge erreicht ist.

In einer zweiten Studie wurde eine groß angelegte Kalibrierungsstudie durchgeführt, um den Zusammenhang zwischen Mutterkornanteil, Alkaloid-Gehalt mit HPLC und ELISA unter Berücksichtigung von Genotypen, Standorten, Ländern, Jahren und Isolaten zu klären. Das Alkaloid-Profil war über die länder-spezifischen Isolate relativ stabil obwohl große Unterschiede hinsichtlich des Alkaloid-Gehalts auftraten. Der mittlere Zusammenhang zwischen Mutterkornanteil und Alkaloid-Gehalt, ermittelt mit HPLC, weist darauf hin, dass eine zuverlässige Vorhersage des Alkaloid-Gehalts auf Basis des Mutterkornanteils nicht möglich ist, was auch nicht durch Gruppierungs-Effekte der getesteten Faktoren erklärt werden. Des Weiteren scheinen die Mutterkorn-Alkaloide nicht als Virulenzfaktoren im Infektionsprozess zu agieren, da der Alkaloid-Gehalt keine Beziehung zum Schweregrad der Krankheit aufwies. Ergänzend führt die fehlende Korrelation von ELISA und HPLC zu der Schlussfolgerung, dass der ELISA keine zuverlässige Methode darstellt, um Proben im Alltag sicher hinsichtlich von Mutterkorn zu prüfen.

In einer dritten Studie wurde die genetische Variation von männlich sterilen CMS-Einfachkreuzungen in einem speziellen Versuchsaufbau für Pollenisolierung im Feld und Gewächshaus analysiert, um Resistenzmechanismen zu identifizieren und zu untersuchen, ob Mutterkorn durch die weibliche Blüte reduziert werden kann. Hierbei deckte ein Vergleich von Nadel- (Feld) und Sprüh- (Gewächshaus) Inokulation mittlere bis hohe Korrelationen auf, was veranschaulicht, dass beide Methoden für diese Forschung geeignet sind. Signifikante Umwelt- und Genotyp-Umwelt-Interaktions-Varianz wurden ermittelt. Es folgt, dass auch unter Pollenisolierung eine Prüfung über mehrere Umwelten (Standort, Jahr) notwendig ist. Außerdem zeigten kleine, aber signifikante genotypische Variation und das Auffinden eines widerstandsfähigeren Kandidaten gegenüber Mutterkorn, dass die Selektion von weiblichen Linien vielversprechend ist, um die Mutterkornkontamination weiter zu reduzieren.

Und in einer vierten Studie wurde gezeigt, dass der Alkaloid-Gehalt von Roggensorten für weniger anfällige Genotypen niedriger was. Dies bedeutet, dass der Alkaloid-Gehalt durch Züchtung deutlich reduziert werden kann. Im Gegensatz zu Studie 2 wurde bei der Analyse von 15 faktoriellen Einfachkreuzungen nach künstlicher Inokulation eine starke, positive

Korrelation für den Mutterkornanteil und Alkaloid-Gehalt gefunden, wobei auch die Korrelation zwischen ELISA und HPLC erhöht war. Der Grund hierbei war, dass die Versuche auf Standorten durchgeführt wurden, die erfahrungsgemäß hervorragend für die Untersuchung von Mutterkorn geeignet sind, und zum anderen, dass der Schwerpunkt auf Proben mit einer hohen genotypischen Variation lag. Die männliche Fertilitäts-Restauration war auch hier die maßgebliche Komponente, aber der Beitrag der signifikanten, weiblichen Seite hatte einen deutlich höheren Anteil für den Alkaloid-Gehalt als für den Mutterkornanteil.

Zusammenfassend zeigt diese Doktorarbeit, dass Mutterkorn effektiv durch die Einbringung einer hohen und umweltstabilen männlichen Fertilitätsrestauration durch exotische Restorergene reduziert werden kann, obwohl auch die weibliche Restaurierbarkeit große Chancen eröffnet. Der unberechenbare Zusammenhang zwischen Mutterkornanteil und Alkaloid-Gehalt zeigt, dass beide Merkmale erfasst werden müssen, insbesondere der Alkaloid-Gehalt durch eine valide HPLC-basierte Methode, um die Nahrungsmittel- und Futtermittelsicherheit zu gewährleisten.

10. References

Alderman, S., 2006. Ergot: biology and control. Available online: http://www.ars.usda.gov/SP2UserFiles/person/81/ErgotDVDtranscript.pdf (accessed on 4 September 2020)

Appelt, M., Ellner, F., 2009. Investigations into the occurrence of alkaloids in ergot and single sclerotia from the 2007 and 2008 harvests. Mycotoxin Res. 25, 95–101. https://doi.org/10.1007/s12550-009-0014-2

Babič, J., Tavčar-Kalcher, G., Celar, F., Kos, K., Červek, M., Jakovac-Strajn, B., 2020. Ergot and ergot alkaloids in cereal grains intended for animal feeding collected in Slovenia: occurrence, pattern and correlations. Toxins 12, 730. https://doi.org/10.3390/toxins12110730

Bandyopadhyay, R., Frederickson, D., McLaren, N., Odvody, G., Ryley, M., 1998. Ergot: a new disease threat to sorghum in the Americas and Australia. Plant Dis. 82. https://doi.org/10.1094/PDIS.1998.82.4.356

Battilani, P., Costa, L., Dossena, A., Gullino, M., Marchelli, R., Galaverna, G., Pietri, A., Dall´Asta, C., Giorni, P., Spadaro, D., Gualla, A., 2009. Scientific information on mycotoxins and natural plant toxicants. Scientific/Technical report submitted to EFSA. Available online: http://onlinelibrary.wiley.com/doi/10.2903/sp.efsa.2009.EN-24/pdf (accessed on 12 March 2020).

Bauer, J., Gross, M., Gottschalk, C., Usleber, E., 2016. Investigations on the occurrence of mycotoxins in beer. Food Control 63, 135–139. https://doi.org/10.1016/j.foodcont.2015.11.040

Beuerle, T., Benford, D., Brimer, L., Cottrill, B., Doerge, D., Dusemund, B., Farmer, P., Fürst, P., Humpf, H., Mulder, P., 2012. Scientific opinion on ergot alkaloids in food and feed. EFSA J. 10, 2798–2956. https://doi.org/10.2903/j.efsa.2012.2798

BMEL, 2019. Bundesministerium für Ernährung, Landwirtschaft und Verbraucherschutz. Besondere Ernte- und Qualitätsermittlung (BEE) Reihe: Daten-Analyse. Available online: https://www.bmel-statistik.de/fileadmin/daten/EQB-1002000-2019.pdf (accessed on 4 May 2021).

Bouton, J., Latch, G., Hill, N., Hoveland, C., McCann, M.A., Watson, R., Parish, J., Hawkins, L., Thompson, F., 2002. Reinfection of tall fescue cultivars with non-ergot alkaloid–producing endophytes. Agron. J. 94, 567–574. https://doi.org/10.2134/agronj2002.5670

Brown, J., Caligari, P., 2011. An introduction to plant breeding. John Wiley & Sons. ISBN: 1-4443-5770-0.

Bryła, M., Ksieniewicz-Woźniak, E., Podolska, G., Waśkiewicz, A., Szymczyk, K., Jędrzejczak, R., 2018. Occurrence of ergot and its alkaloids in winter rye harvested in Poland. World Mycotoxin J. 11, 635–646. https://doi.org/10.3920/WMJ2018.2322

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.

Bundessortenamt, 2020. Beschreibende Sortenliste für Getreide, Mais, Öl-und Faserpflanzen, Leguminosen, Rüben, Zwischenfrüchte [Descriptive variety list for cereals, maize, oil and fibreplants, pulse crops, beets, catch crops]. Available online: https://www.bundessortenamt.de/bsa/sorten/beschreibende-sortenlisten/download-bsl-im-pdf-format (accessed on 4 May 2021).

Bürk, G., Höbel, W., Richt, A., 2006. Ergot alkaloids in cereal products: results from the bavarian health and food safety authority. Mol. Nutr. Food Res. 50, 437–442. https://doi.org/10.1002/mnfr.200500192.

Byrd, N., Slaiding, I., 2017. Project report no. PR578 - RD-2012-3779: final project report: monitoring of mycotoxins and other contaminants in UK cereals used in malting, milling and animal feed.

Caporael, L.R., 1976. Ergotism: The satan loosed in Salem? Science 192, 21–26. 10.1126/science.769159.

Caradus, J.R., Card, S.D., Finch, S.C., Hume, D.E., Johnson, L.J., Mace, W.J., Popay, A.J., 2020. Ergot alkaloids in New Zealand pastures and their impact. N. Z. J. Agric. Res. 1–41. https://doi.org/10.1080/00288233.2020.1785514.

Cell Signaling Technology, 2021. Overview of enzyme-linked immunosorbent assay (ELISA). Available online: https://www.cellsignal.de/applications/elisa/elisa-overview (accessed on 13 May 2021).

Chaube, H., Singh, U., 1991. Plant disease management: principles and practices, 1st ed. CRC Press, Boca Raton, Florida. ISBN 9781315896625.

Cisneros-López, M., Mendoza-Onofre, L., González-Hernández, V., Zavaleta-Mancera, H., Mora-Aguilera, G., Hernández-Martínez, M., Córdova-Téllez, L., 2010. Synchronicity of pollination and inoculation with *Claviceps africana* and its effects on pollen–pistil compatibility and seed production in sorghum. Fungal Biol. 114, 285–292. https://doi.org/10.1016/j.funbio.2010.02.007.

Coufal-Majewski, S., Stanford, K., McAllister, T., Blakley, B., McKinnon, J., Chaves, A., Wang, Y., 2016. Impacts of cereal ergot in food animal production. Front. Vet. Sci. 3, 15. https://doi.org/10.3389/fvets.2016.00015.

Crews, C., 2015. Analysis of ergot alkaloids. Toxins 7, 2024–2050. https://doi.org/10.3390/toxins7062024.

Dahlberg, J., Bandyopadhyay, R., Rooney, W., Odvody, G., Madera-Torres, P., 2001. Evaluation of sorghum germplasm used in US breeding programmes for sources of sugary disease resistance. Plant Pathol. 50, 681–689. https://doi.org/10.1046/j.1365-3059.2001.00636.x.

De Costa, C., 2002. St anthony's fire and living ligatures: a short history of ergometrine. The Lancet 359, 1768–1770. https://doi.org/10.1016/S0140-6736(02)08658-0.

Debegnach, F., Patriarca, S., Brera, C., Gregori, E., Sonego, 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.

Demeke, T., Kidane, Y., Wuhib, E., 1979. Ergotism - a report on an epidemic, 1977-78. Ethiop. Med. J. 17, 107–113. https://doi.org/PMID: 546653.

DESTATIS, 2020. Land- und Forstwirtschaft, Fischerei: Wachstum und Ernte - Feldfrüchte. Avilable online: https://www.destatis.de/DE/Themen/Branchen-Unternehmen/Landwirtschaft-Forstwirtschaft-Fischerei/Feldfruechte-Gruenland/Publikationen/Downloads-Feldfruechte/feldfruechte-august-september-2030321202094.pdf?__blob=publicationFile (accessed on 29 April 2021).

Deutsche UNESCO-Kommission, 2019. Wissen. Können. Weitergeben. Bundesweites Verzeichnis Immaterielle Kulturerbe A-Z (German Inventory of Intangible Cultural Heritage), 3rd ed. ISBN 978-3-947675-06-7. Available online: https://www.unesco.de/publikationen?page=6#row-417 (accessed on 30 April 2021).

Deutsches Brotinstitut e.v., 2021. Die Deutsche Brotkultur. Available online: https://www.brotinstitut.de/brotkultur (accessed on 30 April 2021).

Dhillon, B., Mirdita, V., Miedaner, T., 2010. Preliminary evaluation of locations for conducting selection for resistance to ergot (*Claviceps purpurea*) in rye. Indian J. Plant Genet. Resour. 23, 265–268. https://www.indianjournals.com/ijor.aspx?target=ijor:ijpgr&volume=23&issue=3&article=002.

Diem, H., 1971. Effect of low humidity on the survival of germinated spores commonly found in the phyllosphere. Preece TF Ecol. Leaf Surf. Micro Org. 211–219.

Duarte-Galvan, C., Torres-Pacheco, I., Guevara-Gonzalez, R., Romero-Troncoso, R., Contreras-Medina, L., Rios-Alcaraz, M., Millan-Almaraz, J., 2012. Review. Advantages and disadvantages of control theories applied in greenhouse climate control systems. Span. J. Agric. Res. 10, 926–938. https://doi.org/10.5424/sjar/2012104-487-11.

Dung, J., Cheng, Q., Kaur, N., Walenta, D., Cating, R., Rondon, S., Frost, K., Alderman, S., Hamm, P., 2019. Population biology and epidemiology of *Claviceps purpurea* in cool-season grass seed crops, in: Anderson, N. (Ed.), . Presented at the Proceedings of the 10th International Seed

Conference, Corvallis, Oregon, USA, pp. 40–51. Available online: https://pure.au.dk/portal/files/173428846/ihsg_2019.pdf#page=86 (accessed on 6 May 2021).
Durrell, L., 1964. The composition and structure of walls of dark fungus spores. Mycopathol. Mycol. Appl. 23, 339–345. https://doi.org/10.1007/bf02049005.
Eadie, M., 2003. Convulsive ergotism: epidemics of the serotonin syndrome? Lancet Neurol. 2, 429–434. https://doi.org/10.1016/S1474-4422(03)00439-3.
Easton, H., Latch, G., Tapper, B., Ball, O., 2002. Ryegrass host genetic control of concentrations of endophyte-derived alkaloids. Crop Sci. 42, 51–57. https://doi.org/10.2135/cropsci2002.5100.
EFSA, 2012. S Scientific opinion on ergot alkaloids in food and feed. EFSA panel on contaminants in the food chain (CONTAM). EFSA 10, 2798. Available online: https://efsa.onlinelibrary.wiley.com/doi/pdf/10.2903/j.efsa.2012.2798 (accessed on 11 May 2021).
Engelke, T., 2002. Ansätze für eine integrierte Bekämpfung des Mutterkorns (*Claviceps purpurea* [Fr.] Tul.) im Roggen (Ph.D. Thesis). University of Göttingen, Göttingen. Cuvillier Verlag. ISBN: 3898734390.
European Communities, 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. L140, 10–22. Available online: https://eur-lex.europa.eu/legal-content/EN/ALL/?uri=celex:32002L0032 (accessed on 19 May 2021).
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. L283, 3. Available online: https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=celex%3A32015R1940 (accessed on 19 May 2021).
Eurostat, 2021. Roggen und Wintermenggetreide nach Fläche, Erntemenge und Feuchtigkeitsgehalt. Available online: https://ec.europa.eu/eurostat/databrowser/view/tag00049/default/bar?lang=de (accessed on 29 April).
Fehr, W., 1991. Principles of cultivar development: theory and technique. Macmillian: New York, NY, USA. ISBN: 0-9635989-0-2. ISBN 0029499208.
Flieger, M., Stodůlková, E., Wyka, S., Černý, J., Grobárová, V., Píchová, K., Novák, P., Man, P., Kuzma, M., Cvak, L., 2019. Ergochromes: heretofore neglected side of ergot toxicity. Toxins 11, 439. https://doi.org/10.3390/toxins11080439.
Flieger, M., Wurst, M., Shelby, R., 1997. Ergot alkaloids — sources, structures and analytical methods. Folia Microbiol. (Praha) 42, 3–30. https://link.springer.com/content/pdf/10.1007/BF02898641.pdf.
Florea, S., Panaccione, D., Schardl, C., 2017. Ergot alkaloids of the family Clavicipitaceae. Phytopathology 107, 504–518. http://dx.doi.org/10.1094/PHYTO-12-16-0435-RVW.
Frach, K., Blaschke, G., 1998. Separation of ergot alkaloids and their epimers and determination in sclerotia by capillary electrophoresis. J. Chromatogr. A 808, 247–252. https://doi.org/10.1016/S0021-9673(98)00099-5.
Freeman, B., Beattie, G., 2008. An overview of plant defenses against pathogens and herbivores. Plant Health Instr. https://doi.org/10.1094/PHI-I-2008-0226-01.
French, E., Kim, B.-S., Iyer-Pascuzzi, A.S., 2016. Mechanisms of quantitative disease resistance in plants. X Chromosome Inact. 56, 201–208. https://doi.org/10.1016/j.semcdb.2016.05.015.
Fritz, S., Lukaszewski, A., 1989. Pollen longevity in wheat, rye and triticale. Plant Breed. 102, 31–34. https://doi.org/10.1111/j.1439-0523.1989.tb00311.x.
Geiger, H., Bausback, G., 1979. Untersuchungen uber die Eignung pollensterilen Roggens zur parasitischen Mutterkornerzeugung. Z. Pflanzenzuchtung J. Plant Breed. 83, 163–175. ISSN : 0044-3298.

Geiger, H., Yuan, Y., Miedaner, T., Wilde, P., 1995. Environmental sensitivity of cytoplasmic genic male sterility (CMS) in *Secale cereale* L. Fortschritte Pflanzenzuechtung. ISSN: 0301-2727.

Geiger, H.H., Miedaner, T., 2009. Rye breeding, in: Carena, M.J. (Ed.), Cereals, Handbook of plant breeding. Springer-Verlag New York, pp. 157–181. ISBN: 978-0-387-72294-8.

Geiger, H.H., Schnell, F.W., 1970. Cytoplasmic Male Sterility in Rye (*Secale cereale* L.). Crop Sci. 10, 590–593. https://doi.org/10.2135/cropsci1970.0011183X001000050043x.

Gemperline, P., 2006. Practical guide to chemometrics, 2nd ed. CRC Press, Taylor and Francis Group, Boca Raton, Florida. ISBN: 978-0-429-11956-9.

Gerhards, N., Neubauer, L., Tudzynski, P., Li, S.-M., 2014. Biosynthetic pathways of ergot alkaloids. Toxins 6, 3281–3295. https://doi.org/10.3390/toxins6123281.

Gordon, A., Basler, R., Bansept-Basler, P., Fanstone, V., Harinarayan, L., Grant, P.K., Birchmore, R., Bayles, R.A., Boyd, L.A., O'Sullivan, D.M., 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., L, L., 2019. Project report no. 603: determining the routes of transmission of ergot alkaloids in cereal grains. AHDB Cereals & Oilseeds. Available online: https://ahdb.org.uk/determining-the-routes-of-transmission-of-ergot-alkaloids-in-cereal-grains (accessed on 19 May 2021).

Gordon, A., McCartney, C., Knox, R.E., Ereful, N., Hiebert, C.W., Konkin, D.J., Hsueh, Y.-C., Bhadauria, V., Sgroi, M., O'Sullivan, D.M., Hadley, C., Boyd, L.A., Menzies, J.G., 2020. Genetic and transcriptional dissection of resistance to *Claviceps purpurea* in the durum wheat cultivar Greenshank. Theor. Appl. Genet. 133, 1873–1886. https://doi.org/10.1007/s00122-020-03561-9.

Grusie, T., Cowan, V., Singh, J., McKinnon, J., Blakley, B., 2017. Correlation and variability between weighing, counting and analytical methods to determine ergot (*Claviceps purpurea*) contamination of grain. World Mycotoxin J. 10, 209–218. https://doi.org/10.3920/WMJ2016.2174.

Hallauer, A., Carena, M., Miranda Filho, J., 2010. Testers and combining ability, in: Quantitative genetics in maize breeding, Handbook of Plant Breeding. Springer, New York, NY, pp. 383–423. ISBN: 978-1-4419-0765-3.

Hill, N., Parrott, W., Pope, D., 1991. Ergopeptine alkaloid production by endophytes in a common tall fescue genotype. Crop Sci. 31, 1545–1547. https://doi.org/10.2135/cropsci1991.0011183X003100060033x.

Hulvová, H., Galuszka, P., Frébortová, J., Frébort, I., 2013. Parasitic fungus *Claviceps* as a source for biotechnological production of ergot alkaloids. Prague Symp. 2011 31, 79–89. https://doi.org/10.1016/j.biotechadv.2012.01.005.

Irzykowska, L., Weber, Z., Bocianowski, J., 2012. Comparison of *Claviceps purpurea* populations originated from experimental plots or fields of rye. Open Life Sci. 7, 839–849. https://doi.org/10.2478/s11535-012-0075-7.

Jakubczyk, D., Cheng, J., O'Connor, S., 2014. Biosynthesis of the ergot alkaloids. Nat Prod Rep 31, 1328–1338. https://doi.org/10.1039/C4NP00062E.

Jungehülsing, U., Tudzynski, P., 1997. Analysis of genetic diversity in *Claviceps purpurea* by RAPD markers. Mycol. Res. 101, 1–6. https://doi.org/10.1017/S0953756296001657.

Juroszek, P., Racca, P., Link, S., Farhumand, J., Kleinhenz, B., 2020. Overview on the review articles published during the past 30 years relating to the potential climate change effects on plant pathogens and crop disease risks. Plant Pathol. 69, 179–193. https://doi.org/10.1111/ppa.13119.

Kenyon, S., Roberts, C., Kallenbach, R., Lory, J., Kerley, M., Rottinghaus, G., Hill, N., Ellersieck, M., 2018. Vertical distribution of ergot alkaloids in the vegetative canopy of tall fescue. Crop Sci. 58, 925–931. https://doi.org/10.2135/cropsci2017.03.0202.

Kirchhoff, H., 1929. Beiträge zur Biologie und Physiologie des Mutterkornpilzes. Zentralblatt Für Bakteriol. Parasitenkd. 77, 310–369.

Klotz, K., 2002. Abhängigkeit des Befalls mit Mutterkorn (*Claviceps purpurea* [Fries] Tulasne) in Winterrogen (*Secale cereale* L.) bei unterschiedlicher Sortenstruktur und Prüfmethodik (Diploma thesis). University of Hohenheim, Stuttgart.

Kniel, B., Meißner, M., Koehler, P., Schwake-Anduschus, C., 2018. Studies on the applicability of HPLC-FLD and HPLC–MS/MS for the determination of ergot alkaloids in rye-containing breads. J. Consum. Prot. Food Saf. 13, 69–78. https://doi.org/10.1007/s00003-017-1142-9.

Komarova, E., Tolkachev, O., 2001. The chemistry of peptide ergot alkaloids. part 1. classification and chemistry of ergot peptides. Pharm. Chem. J. 35, 504–513. https://doi.org/10.1023/A:1014050926916.

Kombrink, E., Somssich, I., 1995. Defense responses of plants to pathogens, in: Callow, J., Andrews, J., Tommerup, I. (Eds.), Advances in Botanical Research. Academic Press, pp. 1–34. https://doi.org/10.1016/S0065-2296(08)60007-5.

Křen, V., Cvak, L., 1999. Ergot alkaloids and other metabolites of the genus *Claviceps*, in: Ergot - the genus *Claviceps*. Harwood Academic Publishers, Amsterdam, pp. 173–200. ISBN: 978-0-203-30419-8.

Kren, V., Harazim, P., Malinka, Z., 1994. *Claviceps purpurea* (ergot): culture and bioproduction of ergot alkaloids, in: Bajaj, Y. (Ed.), Medicinal and aromatic plants VII. biotechnology in agriculture and forestry. Springer, Berlin, Heidelberg, pp. 139–156. ISBN: 978-3-662-30371-9.

Krska, R., Crews, C., 2008. Significance, chemistry and determination of ergot alkaloids: a review. Food Addit. Contam. Part A 25, 722–731. https://doi.org/10.1080/02652030701765756.

Kushalappa, A.C., Yogendra, K.N., Karre, S., 2016. Plant innate immune response: qualitative and quantitative resistance. Crit. Rev. Plant Sci. 35, 38–55. https://doi.org/10.1080/07352689.2016.11489.

Last, F., Deighton, F., 1965. The non-parasitic microflora on the surfaces of living leaves. Trans. Br. Mycol. Soc. 48, 83-IN12. https://doi.org/10.1016/S0007-1536(65)80011-0.

Latch, G., 1994. Influence of *Acremonium* endophytes on perennial grass improvement. N. Z. J. Agric. Res. 37, 311–318. https://doi.org/10.1080/00288233.1994.9513069.

Lauber, U., Schnaufer, R., Gredziak, M., Kiesswetter, Y., 2005. Analysis of rye grains and rye meals for ergot alkaloids. Mycotoxin Res. 21, 258–262. https://doi.org/10.1007/BF02957588.

LCTech, 2021. ELISA rapid test ErgoREAD. Available online: https://www.lctech.de/en/products/elisa-rapid-test-ergoread.html (accessed on 13 May 2021).

Lee, M., 2010. The history of ergot of rye (*Claviceps purpurea*) III: 1940–80. J. R. Coll. Physicians Edinb. 40, 77–80. https://doi.org/doi:10.4997/JRCPE.2010.115.

Lee, M., 2009. The history of ergot of rye (*Claviceps purpurea*) I: from antiquity to 1900. J. R. Coll. Physicians Edinb. 39, 179–184. https://doi.org/PMID: 19847980.

Lin, W., Kuang, Y., Wang, J., Duan, D., Xu, W., Tian, P., Nzabanita, C., Wang, M., Li, M., Ma, B., 2019. Effects of seasonal variation on the alkaloids of different ecotypes of *Epichloë* endophyte-*Festuca sinensis* associations. Front. Microbiol. 10, 1695. https://doi.org/10.3389/fmicb.2019.01695.

Lombaert, G., 2001. Liquid chromatographic method for the determination of ergot alkaloids in cereal grains, in: Trucksess, M., Pohland, A. (Eds.), Mycotoxin Protocols, Methods in Molecular Biology™. Humana Press, pp. 215–224. ISBN: 978-0-89603-623-9.

Loo, Y., Lewis, R., 1955. Alkaloid formation in ergot sclerotia. Science 121, 367–368. https://doi.org/10.1126/science.121.3141.367.

Lorenz, K., Hoseney, R., 1979. Ergot on cereal grains. Crit. Rev. Food Sci. Nutr. 11, 311–354. https://doi.org/10.1080/10408397909527267.

Lundqvist, A., 1956. Self-incompatibility in rye I. genetic control in the diploid. Hereditas 42, 293–348. https://doi.org/10.1111/j.1601-5223.1956.tb03021.x.

MacDonald, S., Anderson, W., 2017. Research review no. PR575: a desk study to review current knowledge on ergot alkaloids and their potential for contamination to cereal grains. Available online: https://ahdb.org.uk/a-desk-study-to-review-current-knowledge-on-ergot-alkaloids-and-their-potential-for-contamination-to-cereal-grains (accessed on 19 May 2021).

Mainka, S., Danicke, S., Bohme, H., Ueberschar, K., Liebert, F., 2007. On the akaloid content of ergot (*Claviceps purpurea*). Landbauforsch. Volkenrode 57, 51. ISSN: 0458-6859.

Malysheva, S., Larionova, D., Diana Di Mavungu, J., De Saeger, S., 2014. Pattern and distribution of ergot alkaloids in cereals and cereal products from European countries. World Mycotoxin J. 7, 217–230. https://doi.org/10.3920/WMJ2013.1642.

Mantle, P., Shaw, S., 1976. Role of ascospore production by *Claviceps purpurea* in aetiology of ergot disease in male sterile wheat. Trans. Br. Mycol. Soc. 67, 17–22. https://doi.org/10.1016/S0007-1536(76)80002-2.

Mantle, P.G., Shaw, S., Doling, D.A., 1977. Role of weed grasses in the etiology of ergot disease in wheat. Ann. Appl. Biol. 86, 339–351. https://doi.org/10.1111/j.1744-7348.1977.tb01848.x.

Maríne Font, A., Moreno Martin, F., Costes, C., 1971. Study of the pigments of ergot. new method for studying ergot in flours. Ann. Falsif. Expert. Chim. Toxicol. 64, 80.

Maruo, V., Bracarense, A., Metayer, J.-P., Vilarino, M., Oswald, I., P, P., 2018. Ergot alkaloids at doses close to EU regulatory limits induce alterations of the liver and ntestine. Toxins 10, 183. https://doi.org/10.3390/toxins10050183.

McClymont Peace, D., Harwig, J., 1982. Screening for ergot particles in grain products by light microscopy. Can. Inst. Food Sci. Technol. J. 15, 147–149. https://doi.org/10.1016/S0315-5463(82)72381-8.

McCrea, A., 1931. The reactions of *Claviceps purpurea* to variations of environment. Am. J. Bot. 18, 50–78. https://doi.org/10.1002/j.1537-2197.1931.tb09571.x.

Meier, U., 2018. Growth stages of mono- and dicotyledonous plants: BBCH monograph. https://doi.org/10.5073/20180906-074619.

Meinicke, R., 1956. Die Bedeutung der Wirtspflanze für die Ausbildung des Alkaloidmerkmals der Sklerotien von *Claviceps purpurea* Tul. Flora Oder Allg. Bot. Ztg. 143, 395–427. https://doi.org/10.1016/S0367-1615(17)33124-5.

Meister, U., Batt, N., 2014. *Fusarium* toxins and ergot alkaloids in rye of the federal state Brandenburg harvested 2013. Presented at the Proceedings of the 36th Mycotoxin Workshop, Göttingen, p. 131. Available online: http://www.mycotoxin-workshop.de/36th_Mycotoxin_Workshop_2014_-_Proceedings.pdf (accessed on 12 May 2021).

Meleard, B., 2016. Degradation and epimerization of wheat ergot alkaloids during French baking test. Available online: https://www.english.arvalisinstitutduvegetal.fr/file/galleryelement/pj/84/76/8a/c1/meleard_alkaloids_and_bread_mytox739905815900991417.pdf (accessed on 1 June 2021).

Menzies, J., 2004. The reactions of Canadian spring wheat genotypes to inoculation with *Claviceps purpurea*, the causal agent of ergot. Can. J. Plant Sci. 84, 625–629. https://doi.org/10.4141/P03-086.

Menzies, J., Turkington, T., 2015. An overview of the ergot (*Claviceps purpurea*) issue in western Canada: challenges and solutions. Can. J. Plant Pathol. 37, 40–51. https://doi.org/10.1080/07060661.2014.986527.

Menzies, J.G., Klein-Gebbinck, H.W., Gordon, A., O'Sullivan, D.M., 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. Roggenanabau Eine erfolgreiche Alternative, 1st ed. DLG-Verlag. ISBN: 978-3-7690-2018-2.

Miedaner, T., Dänicke, S., Schmiedchen, B., Wilde, P., Wortmann, H., Dhillon, B., Geiger, H., 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., Geiger, H., 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., Herter, C.P., 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

Miedaner, T., Juroszek, P., 2021a. Climate change will influence disease resistance breeding in wheat in northwestern Europe. Theor. Appl. Genet. 134, 1771–1785. https://doi.org/10.1007/s00122-021-03807-0.

Miedaner, T., Juroszek, P., 2021b. Global warming and increasing maize cultivation demand comprehensive efforts in disease and insect resistance breeding in north-western Europe. Plant Pathol. 70, 1032–1046. https://doi.org/10.1111/ppa.13365.

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., Wilde, P., Wortmann, H., 2008. 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.

Mielke, H., 2000. Studien über den Pilz *Claviceps purpurea* (Fries) Tulasne unter Berücksichtigung der Anfälligkeit verschiedener Roggensorten. Mitteilungen Aus Biol. Bundesanst. Für Land-Forstwirtsch. Berl.-Dahl. 375. ISSN: 3826332598.

Mirdita, V., Dhillon, B., Geiger, H., Miedaner, T., 2008. Genetic variation for resistance to ergot (*Claviceps purpurea* [Fr.] Tul.) among full-sib families of five populations of winter rye (Secale cereale L.). Theor. Appl. Genet. 118, 85–90. 10.1007/s00122-008-0878-0.

Mirdita, V., Miedaner, T., 2009. Resistance to ergot in self-incompatible germplasm resources of winter rye. J. Plant Phytopathol. 157, 350–355. https://doi.org/10.1111/j.1439-0434.2008.01499.x.

Money, N., 2016. Chapter 12 - Fungi and biotechnology, in: Watkinson, S., Boddy, L., Money, N. (Eds.), The fungi (Third Edition). Academic Press, Boston, pp. 401–424. ISBN: 978-0-12-382034-1.

Mühle, E., Breuel, K., 1977. D Das Mutterkorn – Ein Gräserparasit als Gift- und Heilpflanze, 2nd ed. Westarp Wissenschaften (Die Neue Brehm-Bücherei), Hohenwarsleben. ISBN: 3-89432-576-3.

Mulder, P., van Raamsdonk, L., can Egmond, H., Voogt, J., van Brakel, M., van der Horst, G., de Jong, J., 2012. Dutch survey ergot alkaloids and sclerotia in animal feeds. Available online: http://edepot.wur.nl/234699 (accessed on 12 May 2021).

Müller, C., Kemmlein, S., Klaffke, H., Krauthause, W., Preiß-Weigert, A., Wittkowski, R., 2009. A basic tool for risk assessment: a new method for the analysis of ergot alkaloids in rye and selected rye products. Mol. Nutr. Food Res. 53, 500–507. https://doi.org/10.1002/mnfr.200800091.

Musée Unterlinden, 2021. The Isenheim altarpiece. Available online: https://www.musee-unterlinden.com/en/oeuvres/the-isenheim-altarpiece/ (accessed on 11 May 2021).

Orlando, B., Maumené, C., Piraux, F., 2017. Ergot and ergot alkaloids in french cereals: occurrence, pattern and agronomic practices for managing the risk. World Mycotoxin J. 10, 327–338. https://doi.org/10.3920/WMJ2017.2183.

Panaccione, D., Arnold, S., 2017. Ergot alkaloids contribute to virulence in an insect model of invasive aspergillosis. Sci. Rep. 7, 8930. https://doi.org/10.1038/s41598-017-09107-2.

Panaccione, D., Ryan, K., Schardl, C., Florea, S., 2012. Analysis and modification of ergot alkaloid profiles in fungi. Methods Enzymol. 515, 267–290. https://doi.org/10.1016/B978-0-12-394290-6.00012-4.

Panaccione, D.G., Cipoletti, J.R., Sedlock, A.B., Blemings, K.P., Schardl, C.L., Machado, C., Seidel, G.E., 2006. Effects of ergot alkaloids on food preference and satiety in rabbits, as assessed with gene-knockout endophytes in perennial ryegrass (*Lolium perenne*). J. Agric. Food Chem. 54, 4582–4587. https://doi.org/10.1021/jf060626u.

Pandey, B., 1992. A textbook of plant pathology: pathogen and plant disease. S. Chand and Company. https://books.google.de/books?id=x75tq4XPosoC.

Paré, P., Farag, M., Krishnamachari, V., Zhang, H., Ryu, C.-M., Kloepper, J., 2005. Elicitors and priming agents initiate plant defense responses. Photosynth. Res. 85, 149–159. https://doi.org/10.1007/s11120-005-1001-x.

Pažoutová, S., 2002. The evolutionary strategy of *Claviceps*, in: White, J., Bacon, C., Hywel-Jones, N. (Eds.), Clavicipitalean fungi: Evolutionary Biology, Chemistry, Biocontrol and Cultural Impacts. CRC Press, New York, Base, pp. 329–354. ISBN: 978-0-8247-4255-3.

Platford, R., Bernier, C., 1976. Reaction of cultivated cereals to *Claviceps purpurea*. Can. J. Plant Sci. 56, 51–58. https://doi.org/10.4141/cjps76-009.

Platford, R., Bernier, C., 1970. Resistance to *Claviceps purpurea* in spring and durum wheat. Nature 226. https://doi.org/10.1038/226770a0.

Potter, D., Stokes, J., Redmond, C., Schardl, C., Panaccione, D., 2008. Contribution of ergot alkaloids to suppression of a grass-feeding caterpillar assessed with gene knockout endophytes in perennial ryegrass. Entomol. Exp. Appl. 126, 138–147. https://doi.org/10.1111/j.1570-7458.2007.00650.x.

Rajamuthiah, R., Mylonakis, E., 2014. Effector triggered immunity. Virulence 5, 697–702. https://doi.org/10.4161/viru.29091.

Roberts, C., Davis, D., Looper, M., Kallenbach, R., Rottinghaus, G., Hill, N., 2014. Ergot alkaloid concentrations in high- and low-moisture Tall fescue silage. Crop Sci. 54, 1887–1892. https://doi.org/10.2135/cropsci2013.05.0318.

Ruhland, M., Tischler, J., 2008. Determination of ergot alkaloids in feed by HPLC. Mycotoxin Res. 24, 73–79. https://doi.org/10.1007/BF02985284.

Savary, S., Willocquet, L., Pethybridge, S., Esker, P., McRoberts, N., Nelson, A., 2019. The global burden of pathogens and pests on major food crops. Nat. Ecol. Evol. 3, 430–439. https://doi.org/10.1038/s41559-018-0793-y.

Schardl, C., Panaccione, D., Tudzynski, P., 2006. Chapter 2 Ergot alkaloids – biology and molecular biology, in: Cordell, G. (Ed.), The Alkaloids: Chemistry and Biology. Academic Press, pp. 45–86. https://doi.org/10.1016/S1099-4831(06)63002-2

Schardl, C.L., 2015. Introduction to the toxins special issue on ergot alkaloids. Toxins 7, 4232–4237. https://doi.org/10.3390/toxins7104232.

Schiff, P., 2006. Ergot and its alkaloids. Am. J. Pharm. Educ. 70, 98. https://doi.org/10.5688/aj700598.

Schmitz, N., 2003. Bioethanol in Deutschland, Schriftenreihe - Nachwachsende Rohstoffe. Landwirtschaftsverlag, Münster. ISBN: 3-7843-3217-X.

Schnitzius, J., Hill, N., Thompson, C., Craig, A., 2001. Semiquantitative determination of ergot alkaloids in seed, straw, and digesta samples using a competitive enzyme-linked immunosorbent assay. J. Vet. Diagn. Invest. 13, 230–237. https://doi.org/10.1177/104063870101300307.

Schoch, U., Schlatter, C., 1985. Gesundheitsrisiken durch Mutterkorn aus getreide. Mitteilungen Aus Leb. Hyg. 76, 631–644. ISSN: 0026-6841.

Schulze, T. von, 1953. Zur Variabilität des Alkaloidgehalts von "*Claviceps purpurea*" in der Oberlausitz. Pharmazie 8, 412–416. PMID: 13088284.

Schumann, G., Uppala, S., 2002. Ergot of rye. Available online: https://www.apsnet.org/edcenter/disandpath/fungalasco/pdlessons/Pages/Ergot.aspx (accessed on 5 May 2021).

Schummer, C., Brune, L., Moris, G., 2018. Development of a UHPLC-FLD method for the analysis of ergot alkaloids and application to different types of cereals from Luxembourg. Mycotoxin Res. 34, 279–287. https://doi.org/10.1007/s12550-018-0322-5.

Schummer, C., Zandonella, I., van Nieuwenhuyse, A., Moris, G., 2020. Epimerization of ergot alkaloids in feed. Heliyon 6, e04336. https://doi.org/10.1016/j.heliyon.2020.e04336.

Schwake-Anduschus, C., 2018. Mutterkorn und Ergotalkaloide — eine aktuelle sicherheitsrelevante Betrachtung. DAF e.V. Tagung Berlin. Available online: https://www.agrarforschung.de/fileadmin/download/2018/Schwake-Anduschus.pdf (accessed on 28 July 2021).

Schwake-Anduschus, C., Lorenz, N., Lahrssen-Wiederholt, M., Lauche, A., Dänicke, S., 2020. German monitoring 2012–2014: ergot of *Claviceps purpurea* and ergot alkaloids (EA) in feedingstuffs and their toxicological relevance for animal feeding. J. Consum. Prot. Food Saf. 15, 321–329. https://doi.org/10.1007/s00003-020-01298-7.

Scott, P., 2009. Ergot alkaloids: extent of human and animal exposure. World Mycotoxin J. 2, 141–149. https://doi.org/10.3920/WMJ2008.1109.

Scott, P., 2007. Analysis of ergot alkaloids — a review. Mycotoxin Res. 23, 113–121. https://doi.org/10.1007/BF02951506.

Senghor, A., Ortega-Beltran, A., Atehnkeng, J., Jarju, P., Cotty, P., Bandyopadhyay, R., 2021. Aflasafe SN01 is the first biocontrol product approved for aflatoxin mitigation in two nations, Senegal and The Gambia. Plant Dis. PDIS-09. https://doi.org/10.1094/PDIS-09-20-1899-RE.

Senthilkumar, T., Jayas, D., White, N., Fields, P., Gräfenhan, T., 2016. Near-Infrared (NIR) hyperspectral imaging: theory and applications to detect fungal infection and mycotoxin contamination in food products. Indian J. Entomol. 78, 91–99. https://doi.org/10.5958/0974-8172.2016.00029.8.

Sharma, N., Sharma, V., Manikyam, H., Krishna, A., 2016. Ergot alkaloids: a review on therapeutic applications. Eur. J. Med. Plants 1–17. https://doi.org/10.9734/EJMP/2016/25975.

Shelby, R., Kelley, V., 1992. Detection of ergot alkaloids from *Claviceps* species in agricultural products by competitive ELISA using a monoclonal antibody. J. Agric. Food Chem. 40, 1090–1092. https://doi.org/10.1021/jf00018a037.

Shi, H., Yu, P., 2018. Exploring the potential of applying infrared vibrational (micro)spectroscopy in ergot alkaloids determination: Techniques, current status, and challenges. Appl. Spectrosc. Rev. 53, 395–419. https://doi.org/10.1080/05704928.2017.1363771.

Spoel, S., Dong, X., 2012. How do plants achieve immunity? Defence without specialized immune cells. Nat. Rev. Immunol. 12, 89–100. https://doi.org/10.1038/nri3141.

Strickland, J., Looper, M., Matthews, J., Rosenkrans, C., Flythe, M., Brown, K., 2011. Board-invited review: St. Anthony's Fire in livestock: causes, mechanisms, and potential solutions. J. Anim. Sci. 89, 1603–1626. https://doi.org/10.2527/jas.2010-3478.

Sudisha, J., Sharathchandra, R., Amruthesh, K., Kumar, A., Shetty, H., 2012. Pathogenesis related proteins in plant defense response, in: Mérillon, J., Ramawat, K. (Eds.), Plant Defence: Biological Control. Springer, Dordrecht, pp. 379–403. https://doi.org/10.1007/978-94-007-1933-0_17.

Taber, W., Vining, L., 1958. The influence of certain factors on the in vitro production of ergot alkaloids by *Claviceps purpurea* (Fr.) Tul. Can. J. Microbiol. 4, 611–626. https://doi.org/10.1139/m58-067.

Tenberge, K., 1999. Biology and life strategy of the ergot fungi, in: Kren, V., Cvak, L. (Eds.), Ergot: the genus *Claviceps*. Harwood Academic Publishers, pp. 25–56. ISBN: 978-90-5702-375-0.

Thakur, R., Williams, R., 1980. Pollination effects on pearl millet ergot. Phytopathology 70, 80–84. http://dx.doi.org/10.1094/Phyto-70-80.

Tittlemier, S., Drul, D., Roscoe, M., McKendry, T., 2015. Occurrence of ergot and ergot alkaloids in western Canadian wheat and other cereals. J. Agric. Food Chem. 63, 6644–6650. https://doi.org/10.1021/acs.jafc.5b02977.

Tittlemier, S., Drul, D., Roscoe, M., Menzies, J., 2016. The effects of selected factors on measured ergot alkaloid content in *Claviceps purpurea*-infected hexaploid and durum wheat. World Mycotoxin J. 9, 555–564. https://doi.org/10.3920/WMJ2015.2019.

Tittlemier, S., Drul, D., Roscoe, M., Turnock, D., Taylor, D., Fu, B., 2019. Fate of ergot alkaloids during laboratory scale durum processing and pasta production. Toxins 11, 195. https://doi.org/10.3390/toxins11040195.

Topi, D., Jakovac-Strajn, B., Pavšič-Vrtač, K., Tavčar-Kalcher, G., 2017. Occurrence of ergot alkaloids in wheat from Albania. Food Addit. Contam. Part A 34, 1333–1343. https://doi.org/10.1080/19440049.2017.1307528.

Tudzynski, P., Tenberge, K., Oeser, B., 1995. *Claviceps purpurea*, n: Singh, U., Singh, R., Kohmoto, K. (Eds.), Pathogenesis and host specificity in plant diseases: Histopathological, Biochemical, Genetic and Molecular Bases. Pergamon, pp. 161–187. ISBN: 0-08-042273-X.

Tunali, B., Shelby, R., Morgan-Jones, G., Kodan, M., 2000. Endophytic fungi and ergot alkaloids in native Turkish grasses. Phytoparasitica 28, 375–377. https://doi.org/DOI:10.1007/BF02981832.

Universität Hohenheim, 2021. Die Mutterkorn Impf- und Erntemaschine „Goldhamster“. Available online: https://www.uni-hohenheim.de/alumni-news-artikel?tx_ttnews%5Btt_news%5D=36083&cHash=92017e0a93912de06343f30c3e53d8dc (accessed on 26 July 2021).

van Dongen, P., de Groot, A., 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.

Vendelbo, N., Mahmood, K., Sarup, P., Kristensen, P., Orabi, J., Jahoor, A., 2021. Genetic architecture of male fertility restoration in a hybrid breeding system of rye (Secale cereale L.). Res. Sq. https://doi.org/10.21203/rs.3.rs-275908/v1.

Venkatesh, N., Keller, N., 2019. Mycotoxins in conversation with bacteria and fungi. Front. Microbiol. 10. https://doi.org/10.3389/fmicb.2019.00403.

Veršilovskis, A., Pereboom-de Fauw, D., Smits, N., Mulder, P., Mol, H., de Nijs, M., 2019. EURL-MP-report_001. screening of ergot alkaloids by ELISA test kits available on the market. Available online: https://www.wur.nl/en/show/EURL-MP-report_001-Screening-of-ergot-alkaloids-using-ELISA-kits.htm (accessed on 12 May 2021).

Wang, E., Meinke, H., Ryley, M., Herde, D., Henzell, B., 2000. On the relation between weather variables and sorghum ergot infection. Aust. J. Agric. Res. 51, 313–324. https://doi.org/10.1071/AR99072.

Wegulo, S., Carlson, M., 2011. Ergot of small grain cereals and grasses and its health effects on humans and livestock. Available online: https://extensionpublications.unl.edu/assets/pdf/ec1880.pdf (accessed on 5 May 2021).

Willingale, J., Mantle, P., 1985. Stigma constriction in pearl millet, a factor influencing reproduction and disease. Ann. Bot. 56, 109–115. https://doi.org/10.1093/oxfordjournals.aob.a086978.

Willingale, J., Mantle, P., Thakur, R., 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.

Wolff, J., Richter, W., 1989. Chemische Untersuchungen an Mutterkorn. Getreide Mehl Brot 43, 103–107.

Workneh, F., Rush, C., 2006. Weather factors associated with development of sorghum ergot in the texas panhandle. Plant Dis. 90, 717–722. https://doi.org/10.1094/PD-90-0717.

Workneh, F., Rush, C., 2002. Evaluation of relationships between weather patterns and prevalence of sorghum ergot in the texas panhandle. Phytopathology 92, 659–666. https://doi.org/10.1094/PHYTO.2002.92.6.659.

Young, C., Schardl, C., Panaccione, D., Florea, S., Takach, J., Charlton, N., Moore, N., Webb, J., Jaromczyk, J., 2015. Genetics, genomics and evolution of ergot alkaloid diversity. Toxins 7, 1273–1302. https://doi.org/10.3390/toxins7041273.

Zheng, M., Richard, J., Binder, J., 2006. A review of rapid methods for the analysis of mycotoxins. Mycopathologia 161, 261–273. https://doi.org/10.1007/s11046-006-0215-6.

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- Final grade: 1,6

Work experience

as from 04.10.2021	**Research associate** PHARMAPLANT - Arznei- und Gewürzpflanzen Forschungs- und Saatzucht GmbH (Artern, Thuringia)
03/2012 – 03/2018	**Part-time job** Bäckerei Kodisch (Stadtsteinach, Bavaria)
04/2017 – 09/2017	**Student assistant** Leibniz-Institut für Pflanzengenetik und Kulturpflanzen-forschung (IPK), Genbank (Gatersleben, Saxony-Anhalt)
08/2016 – 09/2016	**Student assistant** Martin-Luther-Universität Halle-Wittenberg (Professur für Pflanzenzüchtung; Halle/Saale, Saxony-Anhalt)
12/2015 – 03/2016	**Internship intercultural knowledge** GBBR (Gesellschaft für Bildung und berufliche Rehabilitation, Halle/Saale, Saxony-Anhalt)
08/2014 – 12/2014	**Student assistant** Julius-Maximilians Universität Würzburg (Würzburg, Bavaria) Lehrstuhl für Botanik II – Ökophysiologie und Vegetationsökologie, Dr. Jana Leide

Memberships

- Society for Plant Breeding "Gesellschaft für Pflanzenzüchtung e.V." (GPZ), Germany

03/2019 – 05/2021: Official "PhD speaker" of State Plant Breeding Institute (University of Hohenheim)

Kupferberg, den 12.09.2021

Place, date

Signature

Total list of all publications:

Peer-review:

[1)] Kodisch A, Wilde P, Schmiedchen B, Fromme FJ, Rodemann B, Tratwal A, Oberforster M, Wieser F, Schiemann A, Jørgensen LN, Miedaner T (2020) Ergot infection in winter rye hybrids shows differential contribution of male and female genotypes and environment. Euphytica 216:65. https://doi.org/10.1007/s10681-020-02600-2

[2)] Kodisch A, Oberforster M, Raditschnig A, Rodemann B, Tratwal A, Danielewicz J, Korbas M, Schmiedchen B, Eifler J, Gordillo A, Siekmann D, Fromme FJ, Wuppermann FN, Wieser F, Zechner E, Niewińska M, Miedaner T (2020) Covariation of Ergot Severity and Alkaloid Content Measured by HPLC and One ELISA Method in Inoculated Winter Rye across Three Isolates and Three European Countries. Toxins. 12(11):676. https://doi.org/10.3390/toxins12110676

[3)] Kodisch A, Schmiedchen B, Eifler J, Gordillo A, Siekmann D, Fromme FJ, Oberforster M, Miedaner T (2022) Maternal differences for the reaction to ergot in unfertilized hybrid rye (Secale cereale). European Journal of Plant Pathology. European Journal of Plant Pathology. 163: 181–191. https://doi.org/10.1007/s10658-022-02467-0

[4] Miedaner T, Kodisch A, Raditschnig A, Eifler J (2021) Ergot alkaloid contents in hybrid rye are reduced by breeding. Agriculture. 11(6): 526. https://doi.org/10.3390/agriculture11060526

Article:

[1] Kodisch A, Oberforster M, Raditschnig A, Rodemann B, Eifler J, Schmiedchen B, Fromme FJ, Siekmann D, Krystofik R, Marciniak K, Wieser F, Zechner E, Tratwal A, Danielewicz J, Miedaner T. Influence of isolate, rye hybrid and environment on the ergot reaction of winter rye (extended abstract). 69. Jahrestagung der Vereinigung der Pflanzenzüchter und Saatgutkaufleute Österreichs, Raumberg-Gumpenstein, 19.-21. November 2018. ISBN-13: 978-3-900932-63-3

[2] Miedaner T, Kodisch A. Mutterkorn im Roggen – Alte Krankheit neu entdeckt. DLG-Mitteilungen 2020, 5: 12-13

[3] Kodisch A, Miedaner T. Mutterkornalkaloide in Winterroggen: Zusammensetzung, Einflussfaktoren und Zusammenhang mit dem Sklerotienanteil im Erntegut. Zeitschriftenbeitrag: Getreide, Mehl und Brot“ der Arbeitsgemeinschaft Getreideforschung e. V. (AGF), Juni 2021

[4] Kodisch A, Oberforster M, Raditschnig A, Rodemann B, Fromme FJ, Siekmann D, Krystofik R, Schmiedchen B, Eifler J, Gordillo A, Wieser F, Zechner E, Tratwal A, Danielewicz J, Niewińska M, Miedaner T. Reducing ergot susceptibility in rye for minimizing alkaloid contamination (extended abstract). International Symposium on Rye Breeding & Genetics, Wernigerode, 20.-23. Juni 2021

13. Declaration

Annex 3

Declaration in lieu of an oath on independent work

according to Sec. 18(3) sentence 5 of the University of Hohenheim's Doctoral Regulations for the Faculties of Agricultural Sciences, Natural Sciences, and Business, Economics and Social Sciences

1. The dissertation submitted on the topic
   "Analyzing resistance to ergot caused by *Claviceps purpurea* [Fr.] Tul. and alkaloid contamination in winter rye (*Secale cereale* L.)"

is work done independently by me.

2. I only used the sources and aids listed and did not make use of any impermissible assistance from third parties. In particular, I marked all content taken word-for-word or paraphrased from other works.

3. I did not use the assistance of a commercial doctoral placement or advising agency.

4. I am aware of the importance of the declaration in lieu of oath and the criminal consequences of false or incomplete declarations in lieu of oath.

I confirm that the declaration above is correct. I declare in lieu of oath that I have declared only the truth to the best of my knowledge and have not omitted anything.

Kupferberg, den 12.09.2021

Place, date

Signature