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Determining the routes of transmission of ergot alkaloids in cereal grains

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1. Abstract

The fungus *Claviceps purpurea* infects cereals and grasses at anthesis, producing an ergot sclerotia (the overwintering structure of the pathogen) in place of a grain. Ergot sclerotia contain a cocktail of ergot alkaloids that are highly toxic to humans and animals. Evidence has emerged that ergot alkaloids could potentially find their way into the human food chain, presenting a potential risk to human health. This report addresses a number of questions regarding the potential risk for contamination of clean cereal grains with *C. purpurea* ergot alkaloids.

Significant differences were seen in the size and weight of mature ergot sclerotia produced by seven different *C. purpurea* isolates grown on wheat. Ergot sclerotia produced by isolate 04-97.1 varied in size and weight when grown on wheat, barley and rye. The ergot sclerotia produced on the spring wheat variety Mulika were significantly heavier than those produced on barley or rye, although this did not directly associate with larger sclerotia, as the sclerotia produced on wheat were not significantly different in size to those produced on barley.

Very low levels of ergot alkaloids were found in honeydew with all *C. purpurea* isolates tested. Levels increased in sphacelia tissues, reaching levels as high as 3 million parts per billion (3 million µg of ergot alkaloids per kg of sclerotia) in mature ergot sclerotia. While significant differences were seen in the profiles of the 12 ergot alkaloids screened, in all three fungal tissues, significant differences in the total levels of ergot alkaloids were only apparent in mature sclerotia. The cereal host had no significant effect on either the total ergot alkaloid levels or the alkaloid profiles of isolate 04-97.1.

In wheat and barley, and to a lesser extent in rye, ergot alkaloids were found to transfer to healthy grain that developed above and below flowers infected with the *C. purpurea* isolate 04-97.1. No significant differences in ergot alkaloid levels or profiles were seen between grain collected from above and below *C. purpurea* infected flowers. However, the profile of ergot alkaloids found on grain was very different from the profile found in mature ergot sclerotia of isolate 04-97.1.

Significantly more ergot alkaloids were transferred to clean grain of wheat and barley from direct physical contact with broken pieces of sclerotia, compared to intact sclerotia. Significant differences were seen between wheat and barley. At the lower concentration of ergot sclerotia (i.e. 0.5g) more ergot alkaloid was transferred to grain of wheat than barley, while at the higher sclerotia concentration (i.e. 5g) more alkaloid was transferred to barley grain.

C. purpurea has a very broad host range. Isolate 04-97.1 was originally collected from black-grass, a common grass weed found in standing cereal crops. The ability of one isolate to infect and produce ergot alkaloids in so many cereal crops, indicates the scale of the problem *C. purpurea* presents to cereal production and contamination of the human food chain with ergot alkaloids.

2. Introduction

Ergot is a fungal disease (causal agent *Claviceps purpurea*) of cereals and grasses (including black-grass) that infects the flowers (Figure 1). The fungus gains entry at the time of anthesis, infecting the flower's female tissues and replacing grain with an ergot sclerotia (Figure 1C) (Haarmann *et al.,* 2009). Sclerotia are the overwintering structure of the fungus, and are highly toxic to humans and animals (Beuerle *et al*., 2012; Shelby, 1999). Toxicity is due to a range of toxic alkaloids, commonly known as ergot alkaloids, stored in the sclerotia. Ergot alkaloids have been deemed responsible for ergotism and the Middle Ages the condition became known as St Anthony's Fire, where symptoms include gangrenous extremities, convulsions, psychosis and death. Outbreaks were especially prevalent in the Middle Ages due to the consumption of a diet high in rye and other cereals (de Costa, 2002).

C. purpurea spores germinate on mature stigma hairs and grow down the style towards the female, ovule tissue. Microscopic studies suggest that the fungus does not grow beyond the rachis at the base of the ovary, but proliferates in the ovule tissue where a seed would normally develop (Haarmann *et al*., 2009). This mass of fungal hyphae is referred to as the sphacelial stage (Figure 1A). During the sphacelial stage the fungus produces millions of asexual conidia suspended in a sugary sap that is exuded from the infected flower as honeydew (Figure 1B). These conidia can be transported to new, uninfected flowers by rain splash and insects, resulting in new infections. Finally, at about 4–6 weeks after infection, an ergot sclerotium (Figure 1C) is formed in the flower.

Figure 1 *Claviceps purpurea* infection stages on wheat.
(A) sphacelium, (B) honeydew and (C) mature ergot sclerotia

During this infection process *C. purpurea* produces a range of toxic ergot alkaloids. However, very little is known about when and where in the *C. purpurea* infection process these alkaloids are

produced, and whether the profile of alkaloids differs between infected tissues (e.g. sphacelial, honeydew and sclerotia), between *C. purpurea* isolates and in different *C. purpurea*-host infections (e.g. wheat, barley and rye). Both honeydew and sphacelial tissue (which surrounds the phloem and xylem tissues that enter the ovule) could be routes for alkaloid transfer within the ear, resulting in contamination of healthy grain. It is known that mycotoxins, including deoxynivalenol (DON), produced by *Fusarium culmorum* are able to travel in the ear via the xylem vessels and phloem sieve tubes (Kang, 1999). Although a previous study in three wheat varieties, using a mixture of five UK and three Canadian *C. purpurea* isolates, indicated that alkaloid levels were low in honeydew (16 to 5,459 µg/kg; Tittlemier *et al*., 2016), we cannot discount the possibility of differences between individual *C. purpurea* isolates.

Despite post-harvest removal of sclerotia by standard cleaning methods: colour sorting and gravity tables, ergot alkaloids have been detected in 'clean' grain samples (Beuerle *et al*., 2012; Byrd *et al.,* 2017; MacDonald *et al*., 2017). Recent findings have suggested that alkaloids are localised towards the edge of the sclerotia and within the sclerotia groove (Nielen *et al.,* 2014), meaning that damaged or abraded sclerotia may pose a contamination risk to adjacent grain. This project sets out to determine to what extent ergot alkaloids may contaminate otherwise clean lots of grain, pre-harvest - within the ear, and post-harvest - during processing and transportation of grain.

The European Commission Contaminant Working Group is proposing to bring in changes to the limits of sclerotia found in cereal grain and, for the first time, to impose a threshold of total ergot alkaloids in processed grain, including milling products. For cereal milling products from wheat, spelt, barley and oats, it is expected that a limit of 75–200 ppb will be set for alkaloids. For rye products, the limit will be higher c.250–500 ppb, while for cereal-based food for infants and young children it will be lower, < 50 ppb. It is likely that the proposal will reduce the minimum levels of ergot sclerotia in unprocessed grain lots to 0.02% (0.2g/kg), instead of the current 0.05% (0.5g/kg).

The project results presented here had sought to address four objectives –
Objective 1*: To determine whether ergot alkaloids are transferred from C. purpurea infected flowers to healthy grain within the same ear.*
Objective 2: *To determine whether the potential transfer of ergot alkaloids from C. purpurea infected flowers to healthy grain differs between cereal species, comparing wheat, barley and rye.*
Objective 3: *To determine whether C. purpurea produces differing levels of ergot alkaloids in different fungal structures, comparing honeydew, sclerotia and sphacelia.*
Objective 4: *To determine to what extent ergot alkaloids are transferred to healthy grain during direct, physical contact with whole, partial and sclerotia dust.*

3. Materials and methods

3.1. *Claviceps purpurea* inoculations and sample collection

3.1.1 Plant material

C. purpurea infection and alkaloid production was examined in three cereal species; wheat, barley and rye. The 2017 AHDB Recommended List wheat variety Mulika (Blackman Agriculture via Senova, spring wheat, nabim Group 1) and barley variety Concerto (Limagrain, spring barley, malting), and the descriptive list rye variety Mephisto (Saaten-Union, hybrid) were used. Plants were grown in a John Innes compost, with additional fertiliser, in 11cm pots in the glasshouse (16 hour day/8 hour night cycle, 10,000 lux sodium lights). Mephisto seedlings were vernalised for 8 weeks at 4ºC before transferring to the glasshouse.

3.1.2 *Claviceps purpurea* isolates

At NIAB we hold a collection of single-spored *C. purpurea* isolates collected from a range of cereal and grass species, from the UK, Germany and Canada. Seven single spore isolates of *C. purpurea* were used in this study (Table 1). Spores of each isolate were revived from -80ºC storage and honeydew bulked on the spring wheat variety Mulika. The honeydew containing conidia was collected (10 to 14 days after inoculation) and diluted in sterile water to a concentration of 1 x 10^5 spores/ml. The conidia were inoculated into flowers just before anthesis using a hypodermic syringe as described in Gordon *et al*., (2015).

Table 1 *Claviceps purpurea* isolates

***C. purpurea* isolates**	**Original host and location of origin**	**Year of collection**	**Additional info**
04-97.1	Black-grass (*Alopecurus myosuroides)*; UK	2004	Strain used to identify QTL for sclerotia size (Gordon *et al* ., 2015)
04-41.1	Black-grass (*A. myosuroides)*; UK	2004	UK isolate used in LINK study to identify resistance in UK winter wheat, 2008
03-20.1	Wheat (*Triticum aestivum*); UK	2003	UK isolate used in LINK study to identify resistance in UK winter wheat, 2008
03-48.1	Wheat (*T. aestivum*); UK	2003	UK isolate used in LINK study to identify resistance in UK winter wheat, 2008
Rye 20.1	Rye (*Secale cereale*); Germany	unknown	The strain of *Claviceps* that has had its' genome sequenced (Ensembl fungi)
EL2	Wheat (*T. aestivum*); Manitoba, Canada	1996	Canadian isolate used to identify resistance in durum wheat (Menzies, 2004)
EL4	Ergot from seed-cleaning plant; Manitoba, Canada	1996	Canadian isolate used to identify resistance in durum wheat (Menzies, 2004)

3.1.3 Pathogen inoculations and sampling

Expt. 1: To determine whether alkaloids produced by *C. purpurea* are able to transfer into "healthy" grain the middle flowers on ears of the spring wheat variety Mulika, the barley variety Concerto and the rye variety Mephisto were inoculated with the *C. purpurea* isolate 04-97.1 (Figure 2). Isolate 04-97.1 is highly aggressive and results in high infection rates (Gordon *et al*., 2015). The central flowers of the 1st and 2nd ears of approximately 10 plants of each variety were inoculated just before anthesis where the stigmas were fluffy but no pollen had been released (Zadoks Growth Stage 59) as described in (Gordon *et al*., 2015). For wheat and rye, eight central flowers were inoculated, while for barley only four central flowers were inoculated. Honeydew was collected into an Eppendorf tube from all inoculated flowers as soon as it appeared (approx. 10-14 days after inoculation) and stored at 4^{o}C. Fourteen days after inoculation, whole ears were harvested, and sphacelia extracted and stored at -20^{o}C. Further ears were left to allow ergot sclerotia and healthy gain to mature (approx. 6 weeks after inoculation). Each replicate honeydew, sphacelia and ergot sclerotia sample was made up of infected flowers from approximately 10 ears.

Mature grain was carefully dissected away from the ergot sclerotia that had formed in the middle flowers. Mature grain that had formed above and below the *C. purpurea* inoculated flowers was first

removed from the ear, either by hand or using tweezers sterilised in 70% ethanol. 12-14 wheat grains were harvested above and below the ergot per ear and for barley 8-10 grains were harvested above and below the ergot per ear. 4-8 rye grains were harvested above and below as there were more blind florets due to lower pollination efficiency. Great care was taken not to allow the grain to come into physical contact with sclerotia that had formed in the middle, inoculated flowers. The grain that had develop below and above the *C. purpurea* inoculated flowers was collected and kept as separate samples for alkaloid testing. Grain from uninoculated plants was used as a control. Once all grain had been removed from ears, the sclerotia, where formed, were collected from the middle, *C. purpurea* inoculated flowers. All sampling was carried out by one person to minimise variation introduced by human error.

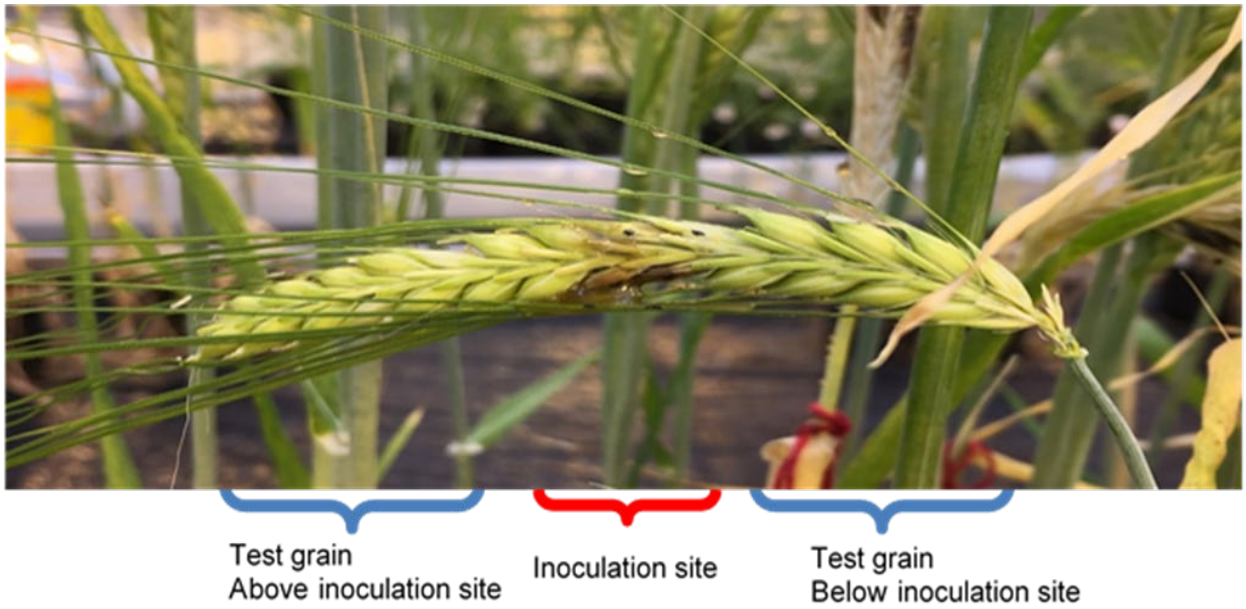
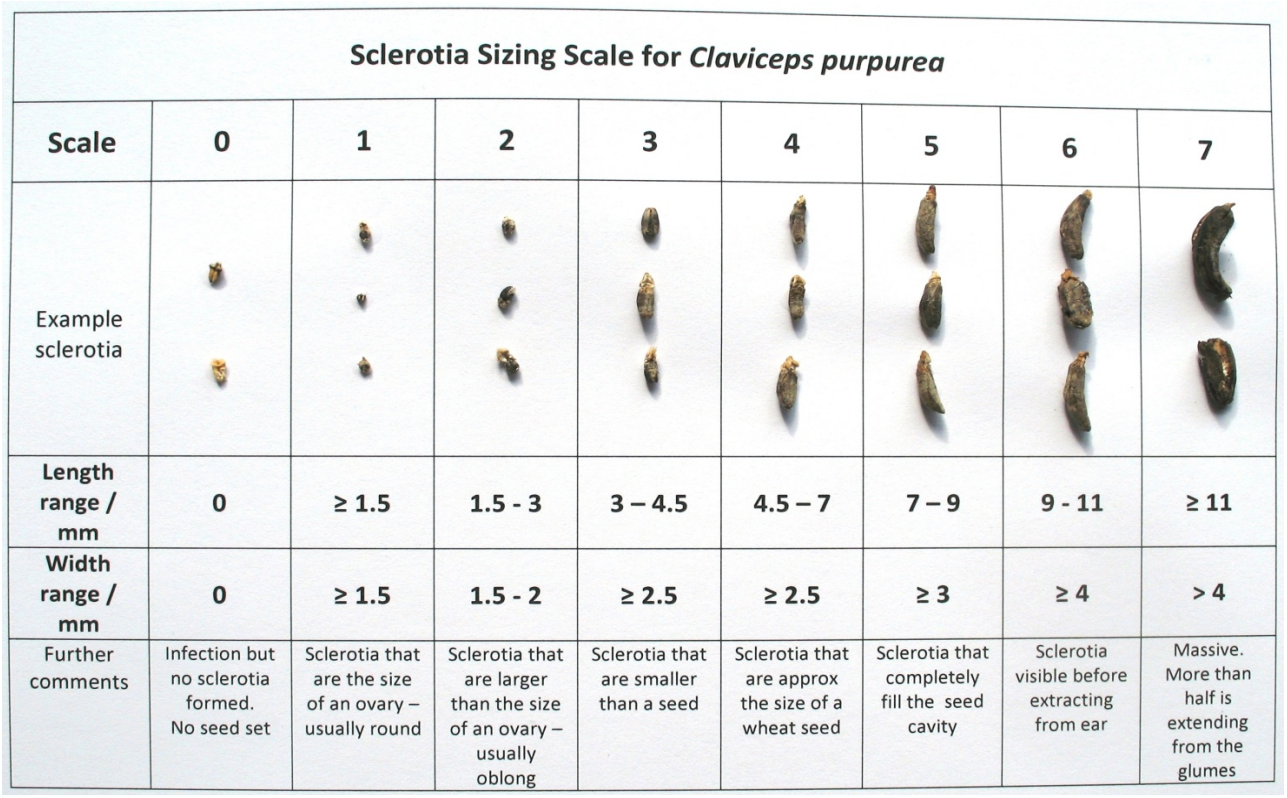


Figure 2 Experimental *Claviceps purpurea* inoculation procedure.

Expt. 2: To assess whether different isolates of *C. purpurea* produced different ergot alkaloid profiles we inoculated the wheat variety Mulika with one of seven different single spore isolates (Table 1). Inoculation of middle flowers was carried out as for Expt.1. Honeydew, sphacelia, sclerotia were all collected for ergot alkaloid analysis.

The size of ergot sclerotia were analysed using the NIAB ergot size 0–7 scale (Figure 3). Sclerotia weight was determined using a MARVIN seed analyser. Ergot sclerotia mean weight and mean size were calculated per ear.



Sclerotia Sizing Scale for *Claviceps purpurea*								
Scale	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Example sclerotia								
Length range / mm	**0**	**≥ 1.5**	**1.5 - 3**	**3 – 4.5**	**4.5 – 7**	**7 – 9**	**9 - 11**	**≥ 11**
Width range / mm	**0**	**≥ 1.5**	**1.5 - 2**	**≥ 2.5**	**≥ 2.5**	**≥ 3**	**≥ 4**	**> 4**
Further comments	Infection but no sclerotia formed. No seed set	Sclerotia that are the size of an ovary – usually round	Sclerotia that are larger than the size of an ovary – usually oblong	Sclerotia that are smaller than a seed	Sclerotia that are approx the size of a wheat seed	Sclerotia that completely fill the seed cavity	Sclerotia visible before extracting from ear	Massive. More than half is extending from the glumes

Figure 3 The NIAB ergot sclerotia sizing scale.

3.2 Physical transfer of ergot alkaloids to healthy grain

To determine to what extent ergot alkaloids could be transferred to clean grain during transportation and processing, when either whole or fragments of ergot sclerotia can come into physical contact with grain, we undertook “tumbling” experiments. Ergot sclerotia from the *C. purpurea* isolate 04-97.1 were produced as described above. Sclerotia were used whole, or broken into fragments of approximately 0.5 to 5 mm in diameter, in tumbling experiments with either wheat or barley grain. Two dosage rates were used, one rate to match the current EU guidelines for allowable levels of ergot in grain (Regulation (EC) No 1881/2006: 0.5 g/kg in unprocessed cereals) and a higher rate (5 g/kg) to represent a worst-case scenario. Whole ergot sclerotia or sclerotia particles were added to 200g of Mulika spring wheat and Concerto spring barley grain. The samples were tumbled for 20min in a Kek-Gardner blender, then spread onto a tray and visually screened for the presence of ergot sclerotia particles. Sclerotia were removed and weighed to ensure full recovery. The grains were ground into wholegrain flour using a laboratory mill (LM 3100, Perten, Sweden) and the ergot alkaloid levels and profiles determined. The pipeline for these tumbling experiments is shown in Figure 4.

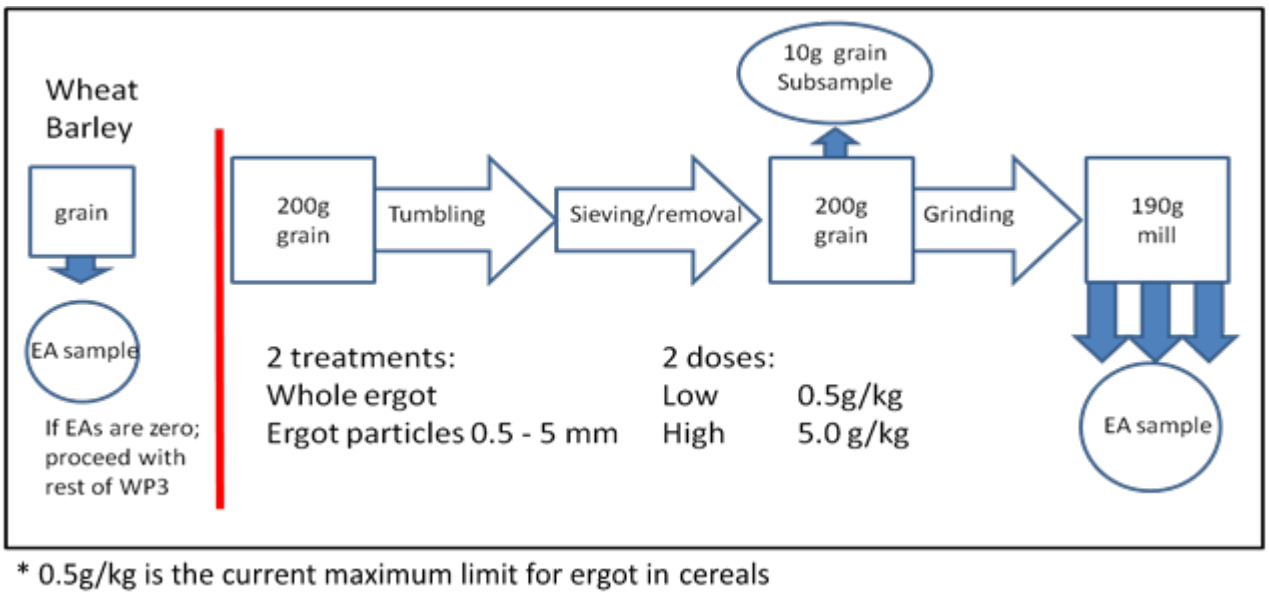


Figure 4 The experimental outline of the tumbling experiments.

3.3 Ergot alkaloid assays

Sclerotia used in these assays were selected in the size range 5–6 NIAB scale (7–11 mm in length), with sclerotia from approximately 2–3 ears being pooled to make one replicate sample of 1g. To provide 1g of replicate sphacelial tissue, approximately three infected ears were required. Honeydew was pooled from 8 to 12 ears to provide over 1ml per replicate. All *C. purpurea* samples were transported to Campden BRI on dry ice. On average, 2 to 3 inoculated ears were required to obtain the 1g of above or below grain required for each replicate ergot alkaloid analysis. Three replicate samples of all test tissues were sent to Campden BRI for ergot alkaloid analyses.

The six major ergot alkaloids; ergocornine, ergocristine, ergocryptine, ergometrine, ergosine, ergotamine, and their respective –inine epimers (Figure 5) were analysed by a LC-ECI-MS/MS procedure based on a published method (Krska and Crews, 2007). The ergot sclerotia and grain samples (0.5g) were ground and extracted into acetonitrile/ammonium carbonate buffer. The sphacelium samples were directly blended into the solvent/buffer mixture, while honeydew samples were resuspended in the buffer prior to extraction in the solvent/buffer solution. The extracted samples were finally cleaned-up by dispersive solid phase extraction (SPE), prior to LC-ESI-MS/MS determination with a limit of quantification for each ergot alkaloid and epimer of 1 µg/kg.

3.4 Statistical analyses

Significant variation between data sets was examined used a modified ANOVA approach, General Linear Regression, in Genstat v.16. The model applied was replicates + treatments. Significant differences, expressed as t-test values, were calculated for ergot sclerotia size, ergot sclerotia weight, and for total and individual ergot alkaloid content.

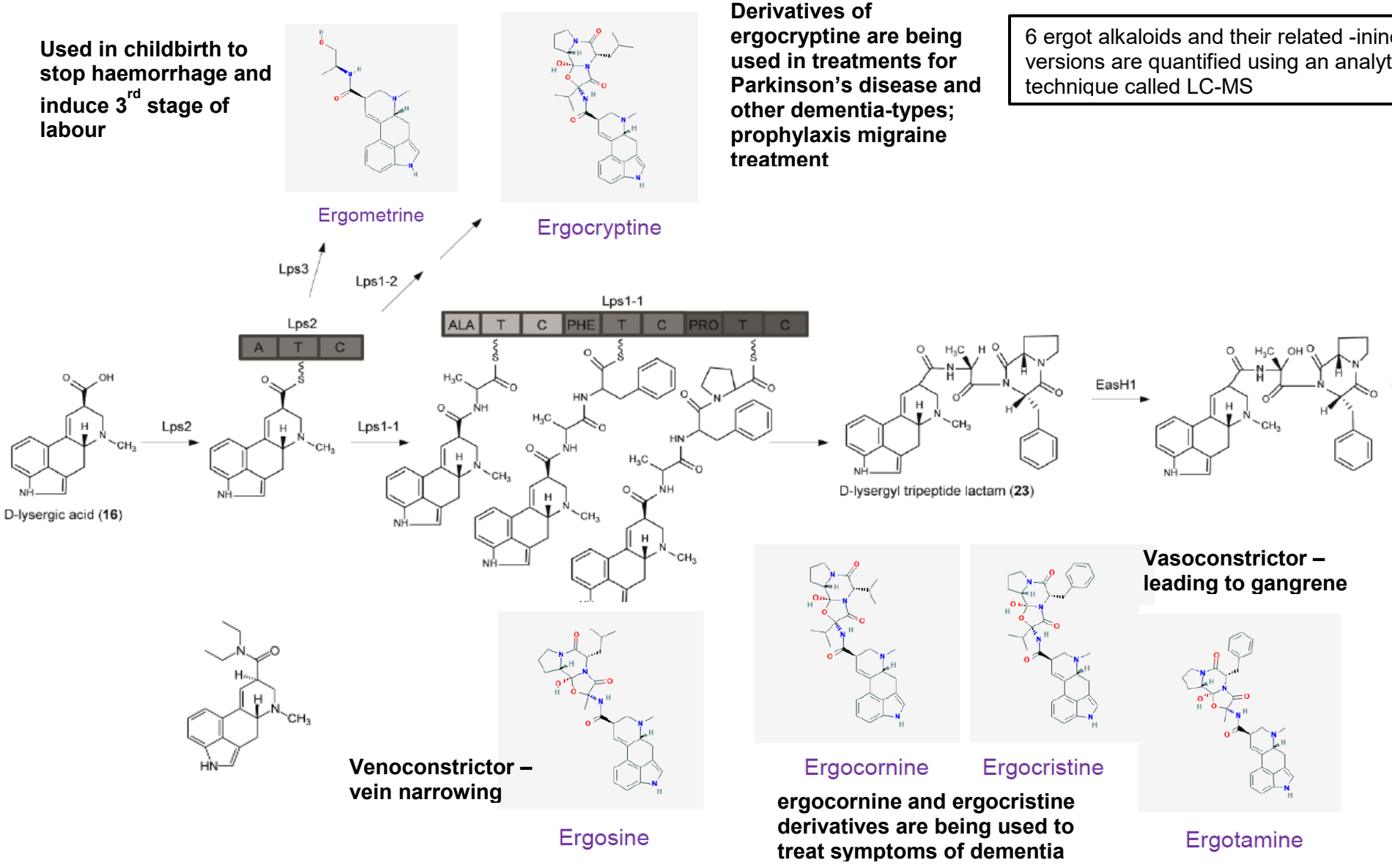


Figure 5 Ergot alkaloids analysed in cereal *Claviceps purpurea* infections

4. Results

4.1 Comparisons between seven *Claviceps purpurea* isolates inoculated on wheat

The wheat variety Mulika was inoculated individually with one of seven isolates of *C. purpurea* (Table 1). Honeydew, sphacelia and mature ergot sclerotia (Figure 1) were collected from three replicate experiments. The ergot alkaloid profiles found in each of the three fungal tissues was measured using LC-ECI-MS/MS for 12 different alkaloids (Figure 5). Mature ergot sclerotia collected from each ear were weighed and the size determined using the NIAB ergot size 0-7 scale.

4.1.1 Variation in ergot sclerotia size and weight between seven isolates of *Claviceps purpurea*

The mean sclerotia weights and size (length) per ear are shown in Figure 6. Analysis of variation showed significant differences between the mean size ($F = 4.55$, $p < 0.001$) and mean weight ($F = 13.20$, $p < 0.001$) of mature ergot sclerotia produced by the seven different isolates of *C. purpurea* grown on the wheat variety Mulika. Isolate 04-97.1 produced the largest sclerotia, but these were only significantly larger ($t < 0.001$) than sclerotia produced by isolates EL4 and Rye 20.1. More variation was seen in the weight of sclerotia. The heaviest sclerotia were produced by isolates 04-97.1 and 03-20.1. Both isolates produced significantly heavier sclerotia ($t < 0.001$) compared to the other five isolates. Significant differences ($F = 4.39$, $p < 0.001$) in the total number of sclerotia produced per ear was only seen at t-values of 0.01, isolates 03-48.1, 04-41.1, EL2 and EL4 producing fewer sclerotia than the other three isolates.

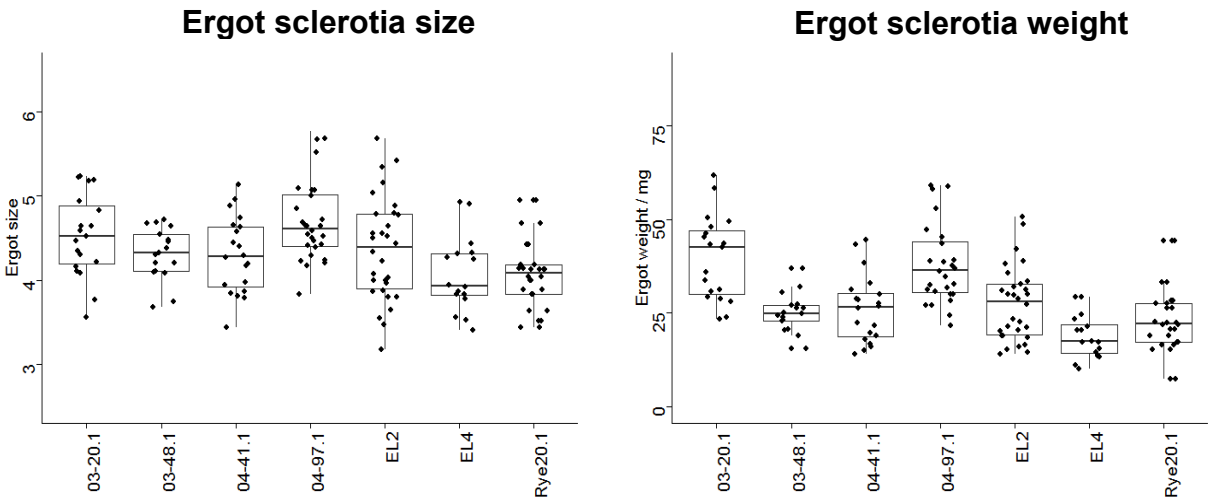
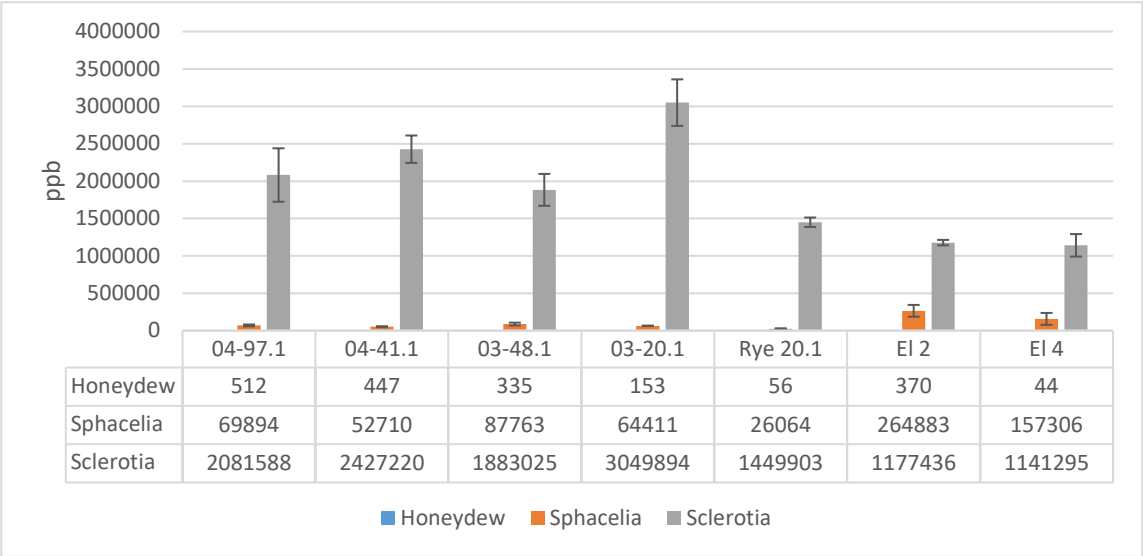


Figure 6. Box and whisker plots of mature ergot sclerotia size and weight data. Each data point represents the mean size or weight of sclerotia collected from a single ear. The box defines the upper and lower quartile and shows the median values.

4.1.2 Variation in ergot alkaloids in fungal tissues from seven isolates of *Claviceps purpurea*

Total ergot alkaloid levels (the sum of the 12 individual ergot alkaloids measured) for honeydew, sphacelial and mature ergot sclerotia produced by the seven *C. purpurea* isolates on the wheat variety Mulika are presented in Figure 7, (with the proportions of the 12 individual ergot alkaloids measured are presented in Figures 8 and 9). Significant differences in total ergot alkaloid levels were seen between the three fungal tissues. Very low levels of alkaloids were seen in honeydew. Ergot alkaloid levels were observed to accumulate in sphacelia, but were the highest in the mature sclerotia.



	04-97.1	04-41.1	03-48.1	03-20.1	Rye 20.1	El 2	El 4
Honeydew	512	447	335	153	56	370	44
Sphacelia	69894	52710	87763	64411	26064	264883	157306
Sclerotia	2081588	2427220	1883025	3049894	1449903	1177436	1141295

Figure 7 Levels of total ergot alkaloids in *Claviceps purpurea* tissues (mean values). The total ergot alkaloid levels (parts per billion) in honeydew, sphacelia and sclerotia of seven *C. purpurea* isolates grown on the wheat variety Mulika. The error bars show standard errors.

No significant differences were found between the isolates with regards to total ergot alkaloid levels found in honeydew ($F = 4.99$, $p = 0.009$). However, isolates did differ with respect to the proportions of each of the 12 individual ergot alkaloids, the levels of ergocornine ($F = 20.29$, $p < 0.001$), ergocorninine ($F = 12.00$, $p < 0.001$), ergocristine ($F = 7.68$, $p = 0.001$), ergometrine ($F = 10.49$, $p < 0.001$), ergometrinine ($F = 8.15$, $p = 0.001$) and ergosine ($F = 77.34$, $p < 0.001$) being significantly different between the seven isolates in honeydew.

In sphacelia no significant difference in total ergot alkaloid levels was found between the seven isolates ($F = 3.82$, $p = 0.023$). However, examination of the 12 individual ergot alkaloids found significant difference between the seven isolates for ergocornine ($F = 32.43$, $p < 0.001$), ergocorninine ($F = 12.04$, $p < 0.001$), ergocristine ($F = 38.08$, $p < 0.001$), ergocristinine ($F = 21.47$, $p < 0.001$), ergocryptinine ($F = 15.37$, $p < 0.001$), ergometrine ($F = 38.65$, $p < 0.001$), ergometrinine

(F = 58.07, p < 0.001), ergosine (F = 18.73, p < 0.001), ergotamine (F = 9.01, p < 0.001) and ergotaminine (F = 7.99, p = 0.001).

The ergot alkaloid levels in mature ergot sclerotia were very high, reaching levels of 3 million ppb in isolate 03-20.1. In sclerotia, significant differences were seen between isolates for total ergot alkaloid levels (F = 11.49, p < 0.001). The difference in ergot alkaloid profiles between the seven isolates were also significant for all 12 alkaloids, at a p < 0.001 and the F values ranged from 19.39 (ergocristinine) to 278.77 (ergocryptinine) (Figure 8 and 9). While significant differences in ergot alkaloid profiles were seen between the isolates, the ergot alkaloid profiles in the three fungal tissues from any one isolate were generally similar (Figure 8).

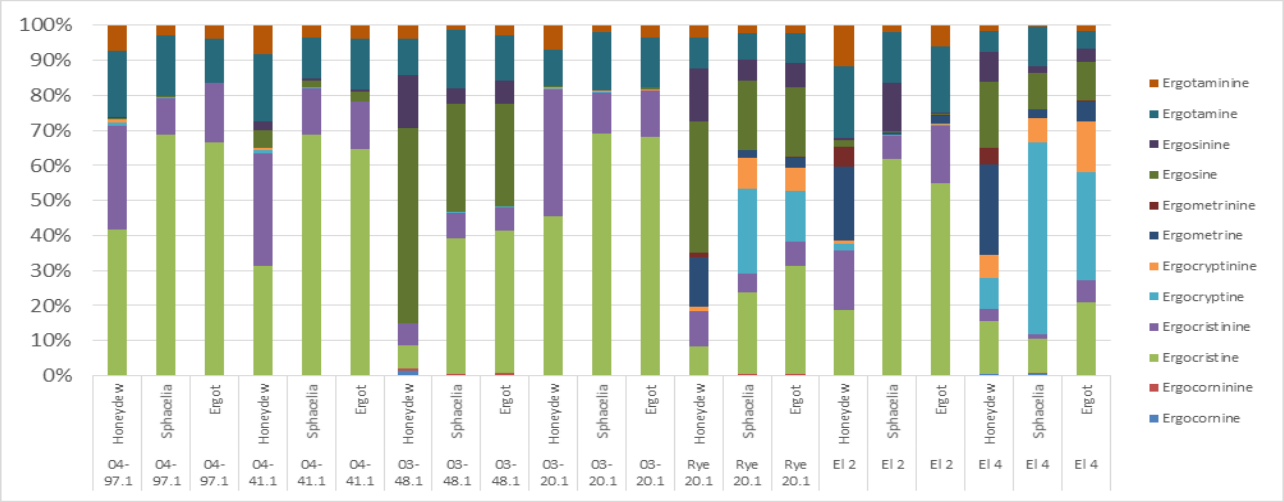


Figure 8. Proportions of ergot alkaloids in different fungal tissues from seven Claviceps purpurea isolates. The proportion of 12 ergot alkaloid levels (%) found in honeydew, sphacelia and sclerotia of seven *C. purpurea* isolates grown on the wheat variety Mulika, arranged by isolate.

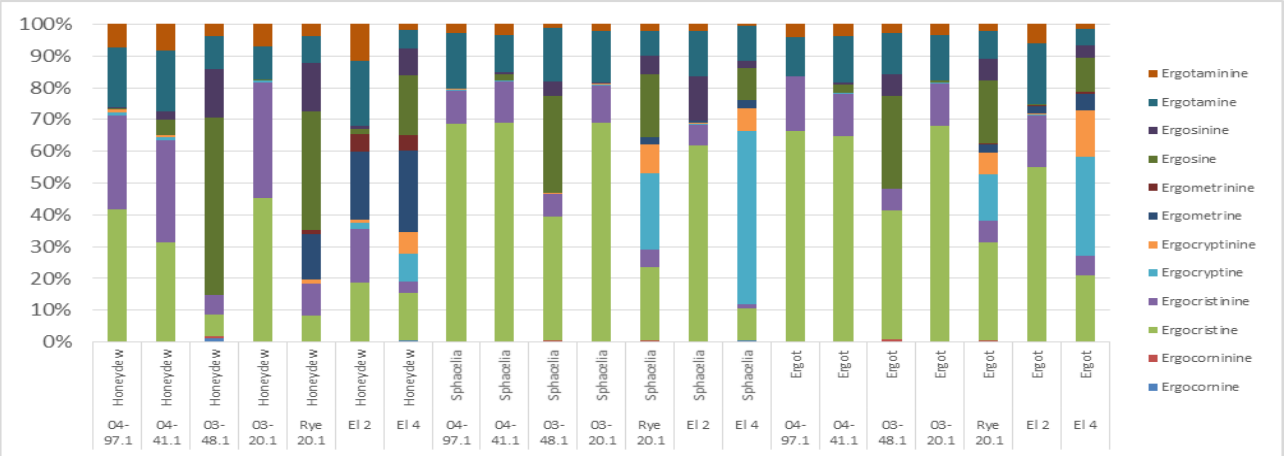


Figure 9. Proportions of ergot alkaloids in different fungal tissues from seven *Claviceps purpurea* isolates. The proportion of 12 ergot alkaloid levels (%) found in honeydew, sphacelia and sclerotia of seven *C. purpurea* isolates grown on the wheat variety Mulika, arranged by fungal tissue.

4.2 Comparison of *Claviceps purpurea* inoculations on wheat, barley and rye

The wheat variety Mulika, the barley variety Concerto and the rye variety Mephisto were all inoculated this the *C. purpurea* isolate 04-97.1. The middle flowers were inoculated (Figure 2) and honeydew, sphacelia and mature ergot sclerotia collected from the inoculated flowers. Grain was allowed to develop above and below the *C. purpurea* inoculated flowers.

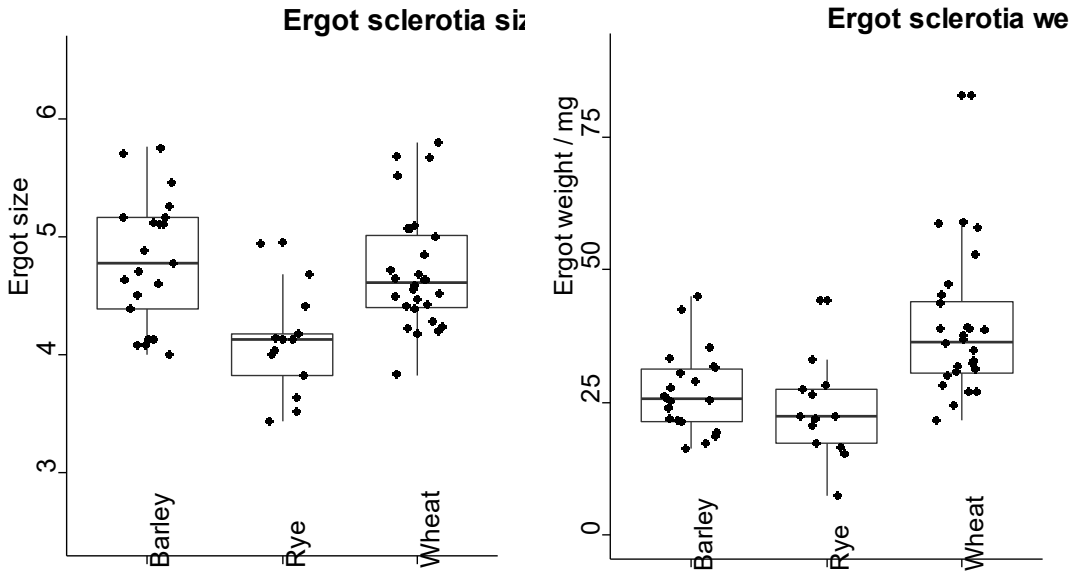


Figure 10. Box and whisker plots of mature ergot sclerotia size and weight data from *C. purpurea* isolate 04-97.1 grown on three cereal hosts. Each data point represents the mean size (length) or weight of sclerotia collected from a single ear. The box defines the upper and lower quartile and shows the median values.

4.2.1 Variation in ergot sclerotia size and weight of *Claviceps purpurea* grown on different cereal species

Figure 10 shows that the mature ergot sclerotia produced on the wheat variety Mulika were significantly heavier (recorded as a mean weight per ear) than those produced on barley or rye ($F = 12.83$, $p < 0.001$). However, the mean size per ear ($F = 9.08$, $p < 0.001$) indicated that the sclerotia formed on wheat were only significantly different in size to those formed on rye ($t = 0.001$), and did not differ in size from the sclerotia formed on barley. This lack of correlation between sclerotia size and weight suggests that the sclerotia produced on the barley variety Concerto were less dense than those produced on wheat or rye, with a potential lower fungal biomass.

4.2.2 Variation in ergot alkaloid profiles in *C. purpurea* isolate 04-97.1 grown on different cereal species

Honeydew and sphacelia of *C. purpurea* isolate 04-97.1 grown on wheat and barley were collected, while mature ergot sclerotia were collected from wheat, barley and rye. Total ergot alkaloids (Figure 11) and alkaloid profiles (Figure 12) were examined in each fungal tissue. Significant differences in total ergot alkaloid levels were seen between the three fungal tissues, with very low levels of alkaloids in honeydew. The levels of ergot alkaloids in sphacelia tissue (the developing ergot) were much higher than honeydew, confirming the hypothesis that ergot alkaloids are being produced well before final ergot maturation. Ergot alkaloid levels were observed to accumulate the highest in the mature sclerotia. When examining the mean values of total ergot alkaloids it appears that ergots that develop on rye contain a higher ergot alkaloid content by concentration compared to barley (and then wheat). However no actual significant differences were found between the different cereal species for total ergot alkaloid levels in honeydew ($F = 3.83$, $p = 0.190$), sphacelia ($F = 13.44$, $p = 0.067$), or mature ergot sclerotia ($F = 2.36$, $p = 0.210$). The three replicate values that were used to calculate the mean values in ergot sclerotia were very variable which is why the differences between the cereals were not significant (despite the standard error bars in Figure 11 suggesting they may be so). The addition of further replicates in future studies would be essential to give higher resolution.

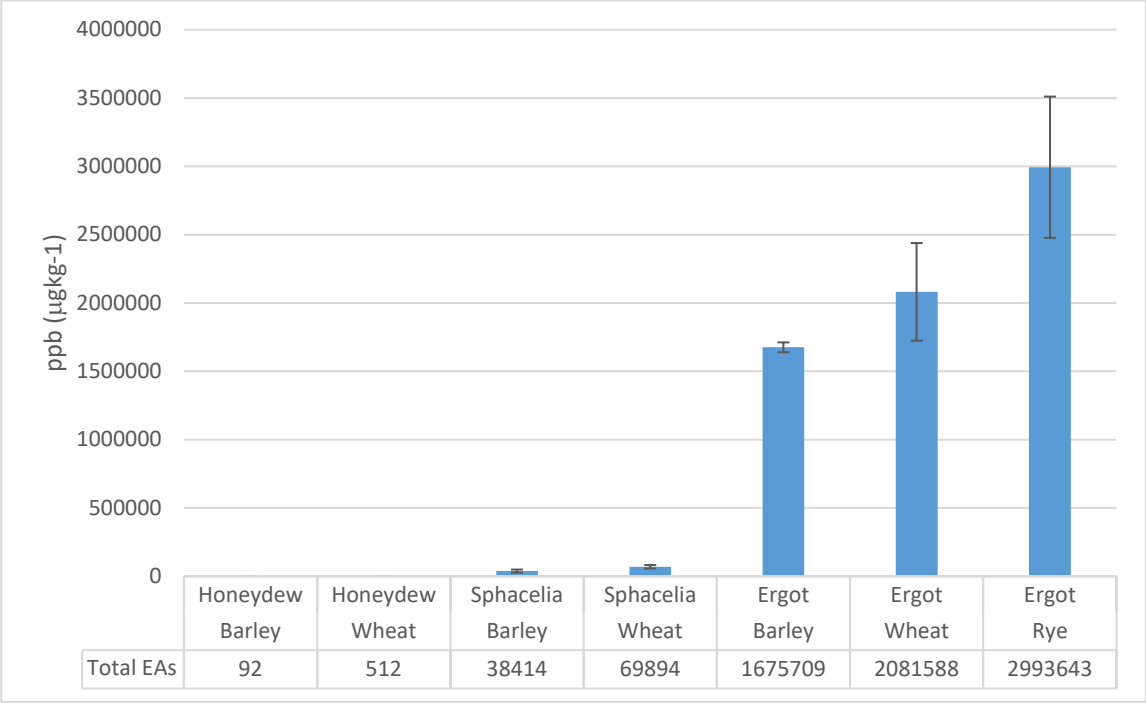


Figure 11 Mean levels of total ergot alkaloids in *Claviceps purpurea* tissues. The total ergot alkaloid levels (parts per billion) in honeydew, sphacelia and sclerotia of the *C. purpurea* isolate 04-97.1 grown on the wheat variety Mulika, barley variety Concerto and rye variety Mephisto. The error bars show standard errors.

The profiles of the 12 ergot alkaloids in honeydew, sphacelia or mature ergot sclerotia produced by *C. purpurea* isolate 04-97.1 (Figure 12) showed no significant differences between wheat, barley and rye at a F-probability of < 0.001. The differing profiles observed in honeydew between wheat and barley in Figure 12 for the two most prevalent ergot alkaloids ergocristinine and ergocristine is likely to be because these two compounds are epimers of each other and have the ability to interconvert under differing environmental conditions such as light, and are unlikely to be as a result of any underlying biological differences.

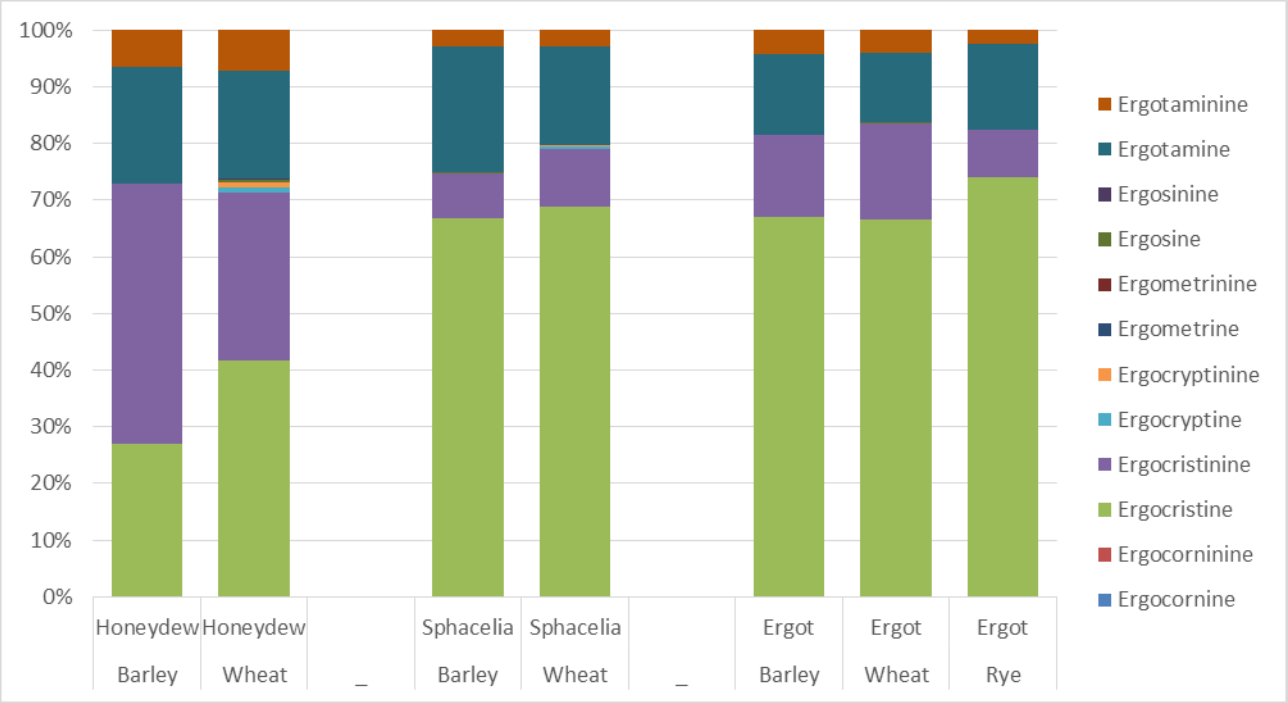
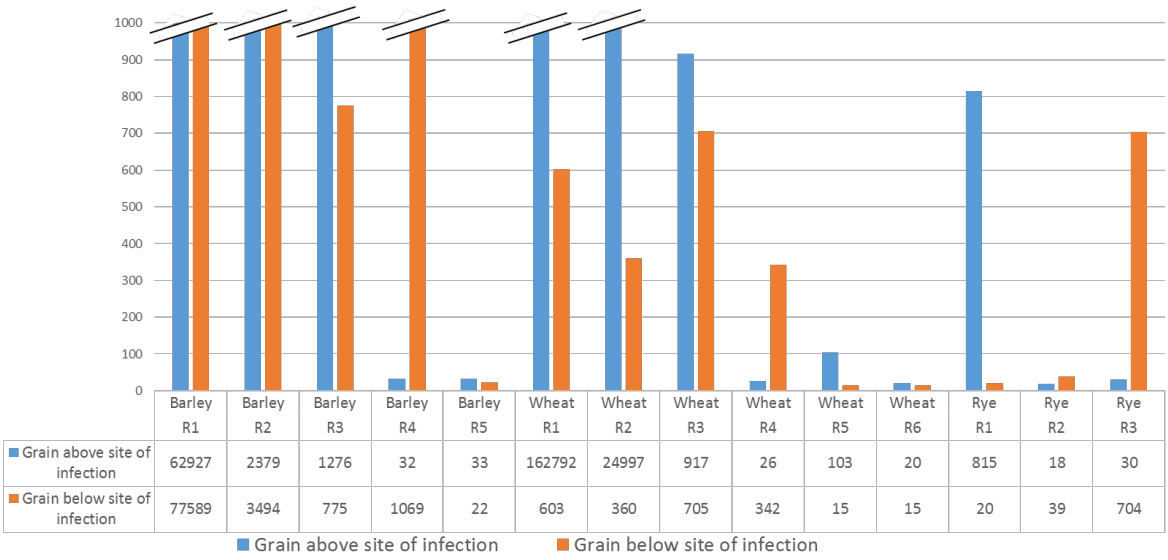


Figure 12 Proportions of ergot alkaloids in *Claviceps purpurea* fungal tissues grown on different cereal hosts. The proportion of 12 ergot alkaloids (%) found in honeydew, sphacelia and sclerotia of *C. purpurea* isolate 04-97.1 grown on the wheat variety Mulika, the barley variety Concerto and the rye variety Mephisto.

4.2.3 Ergot alkaloid profiles on healthy grain formed above and below flowers infected with *Claviceps purpurea*

Healthy grain that developed above and below the wheat, barley and rye flowers inoculated with *C. purpurea* isolate 04-97.1 was harvested and tested for the presence of ergot alkaloids in the grain (Figure 13). Despite large variation seen between some replicates, no significant differences were reported between replicates (F-probability = 0.065), cereal species (F-probability = 0.197) or grain collected from above and below *C. purpurea* infected flowers (F-probability = 0.298). When looking at the individual ergot alkaloids, again no significant differences between hosts or position of grain (above or below site of *C. purpurea* infection) were found.

The profile of ergot alkaloids produced by isolate 04-97.1 in mature ergot sclerotia consisted primarily of ergotamine and ergotaminine, and ergocristine and ergocristinine (Figure 12). However, when looking at the profile of ergot alkaloids found on healthy grain that had developed above and below flowers infected with isolate 04-97.1, the ergot alkaloid profiles were far more diverse (Figure 14 and 15). This would indicate the ability of *C. purpurea* isolate 04-97.1 to produce a wide range of ergot alkaloids, but a preference to accumulate a subset of these alkaloids in sclerotia.



	Barley R1	Barley R2	Barley R3	Barley R4	Barley R5	Wheat R1	Wheat R2	Wheat R3	Wheat R4	Wheat R5	Wheat R6	Rye R1	Rye R2	Rye R3
Grain above site of infection	62927	2379	1276	32	33	162792	24997	917	26	103	20	815	18	30
Grain below site of infection	77589	3494	775	1069	22	603	360	705	342	15	15	20	39	704

Figure 13 Levels of total ergot alkaloids in healthy grain collected above and below *Claviceps purpurea* infected flowers. The total ergot alkaloid levels (parts per billion) in grain that developed above and below flowers inoculated with the *C. purpurea* isolate 04-97.1 grown on the wheat variety Mulika, barley variety Concerto and rye variety Mephisto. The total ergot alkaloid levels of individual replicate tests are shown. (Axis capped at 1000 ppb)

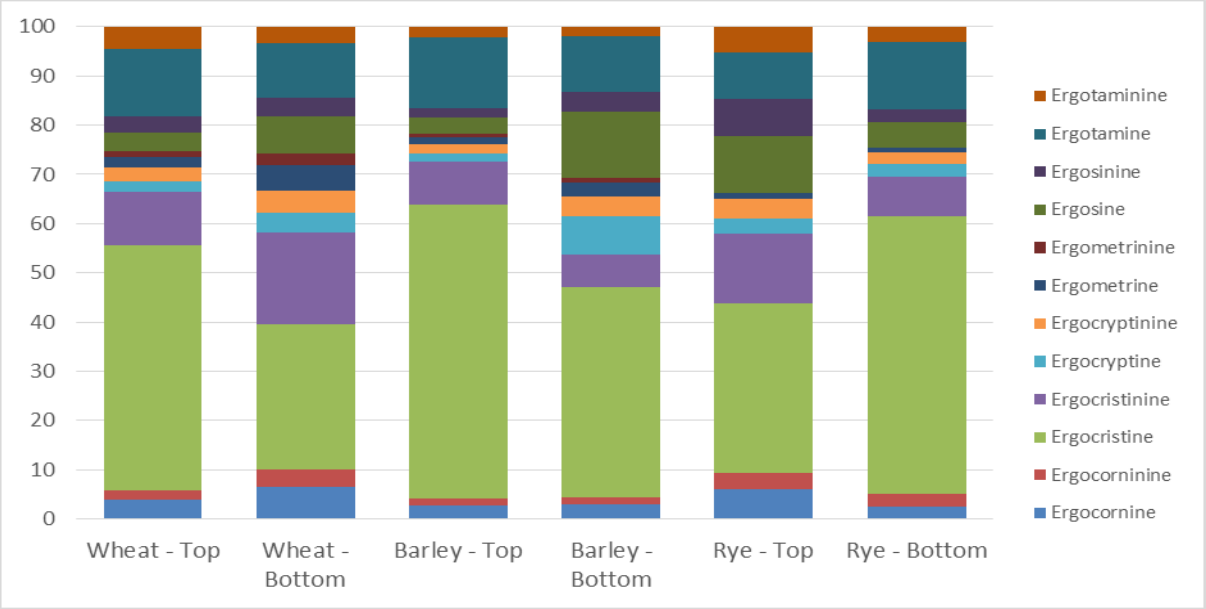


Figure 14 Proportions of ergot alkaloids in healthy grain collected above and below *Claviceps purpurea* infected flowers. The proportion of 12 ergot alkaloids (%) found in grain that developed above and below flowers inoculated with the *C. purpurea* isolate 04-97.1 grown on the wheat variety Mulika, barley variety Concerto and rye variety Mephisto. The proportions of replicate means are shown.

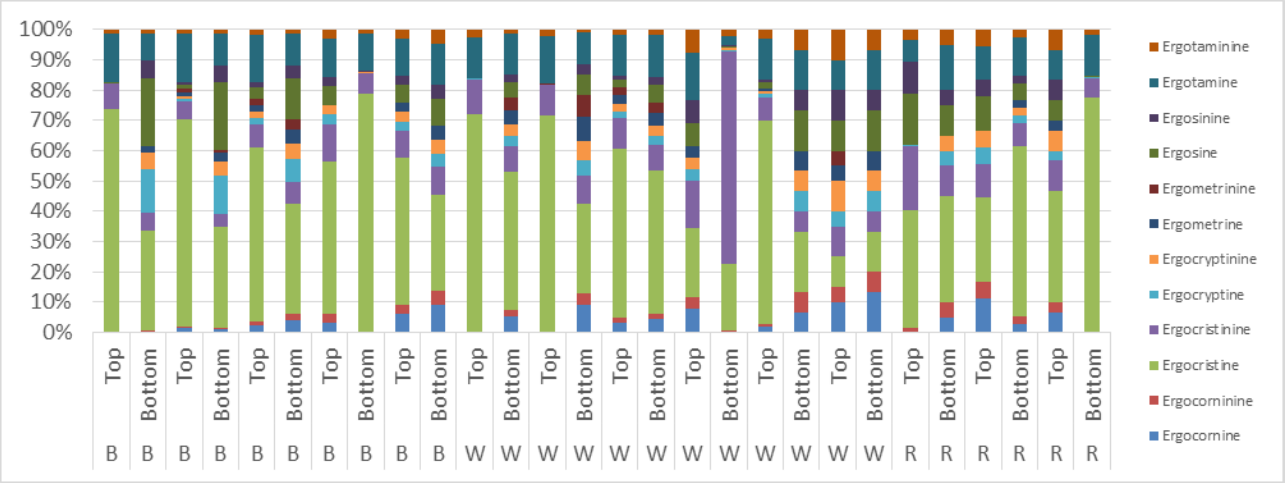


Figure 15 Proportions of ergot alkaloids in healthy grain collected above and below *Claviceps purpurea* infected flowers. The proportion of 12 ergot alkaloids (%) found in grain that developed above and below flowers inoculated with the *C. purpurea* isolate 04-97.1 grown on the wheat variety Mulika, barley variety Concerto and rye variety Mephisto. The proportions of individual replicate tests are shown.

4.4 Physical transfer of ergot alkaloids to clean grain

Clean grain of wheat and barley was subjected to physical contact with whole ergot sclerotia and broken sclerotia. Two ratios of grain to sclerotia were tested, 0.5g and 5g of sclerotia per kilogramme of grain. After tumbling the ergot sclerotia were removed and the grain tested for the presence of ergot alkaloids.

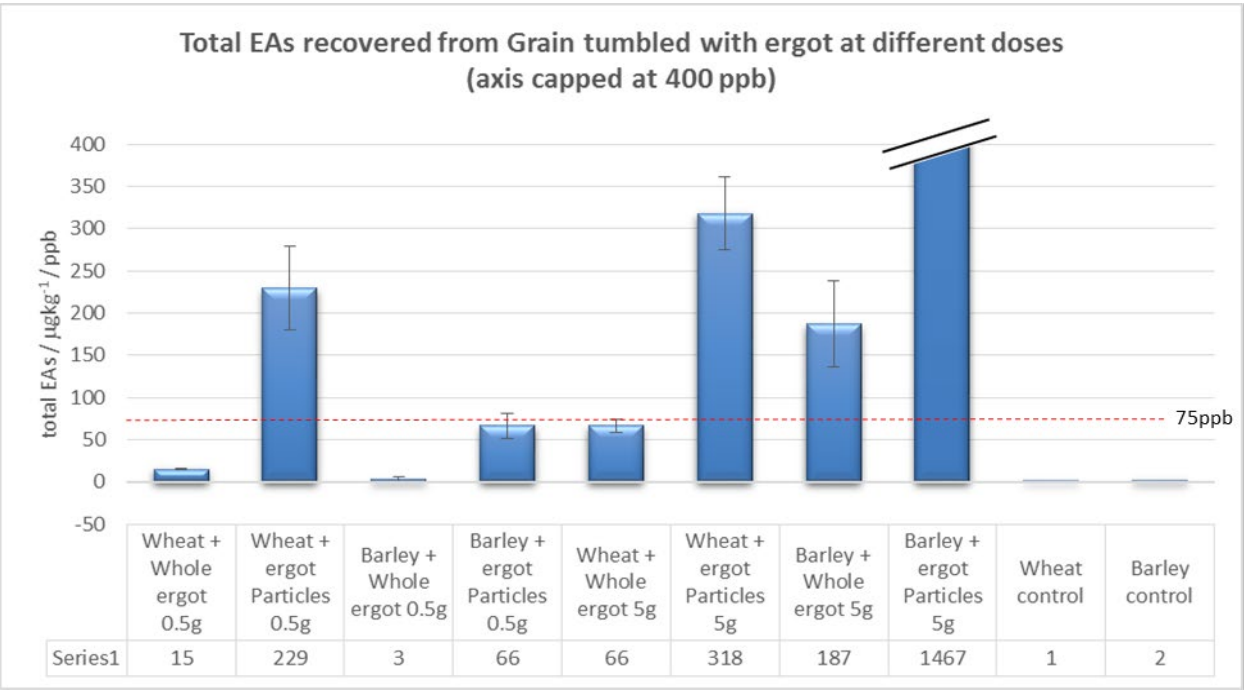


Figure 16 Total ergot alkaloid levels found on clean wheat and barley grain that had been in direct physical contact with whole mature ergot sclerotia, or broken particles of sclerotia. Two ratios of grain to sclerotia were tested, 0.5g of sclerotia and 5g of sclerotia per kg of grain. (Axis capped at 400 ppb).

Significantly more ergot alkaloids were transferred to clean grain of both wheat and barley by broken pieces of sclerotia compared to intact sclerotia ($F = 595.04$, $p < 0.001$), with greater levels of ergot alkaloids being found on previously clean grain at the higher concentration of ergot sclerotia (i.e. 5g per kg of grain). Significant differences were seen between wheat and barley ($F = 271.51$, $p < 0.001$), as well as a significant interaction between cereal grain and the nature (whole or broken) and concentration of ergot sclerotia ($F = 315.23$, $p < 0.001$). At the lower concentration of ergot sclerotia (i.e. 0.5g per kg), more ergot alkaloid was transferred to wheat grain, but at the higher sclerotia concentration (i.e. 5g per kg) more alkaloid was transferred to barley grain (Figure 16).

The profile of ergot alkaloids seen on wheat and barley grain (Figure 17) reflected the profile of alkaloids seen in mature ergot sclerotia of isolate 04-97.1, namely ergocristine, ergocristinine, ergotamine and ergotaminine (Figure 12). An analysis of these four ergot alkaloids showed significant differences between wheat and barley grain for ergocristine ($F = 150.20$, $p < 0.001$), ergocristinine ($F = 514.04$, $p < 0.001$) and ergotamine ($F = 377.79$, $p < 0.001$) levels, but not for ergotaminine ($F = 6.30$, $p = 0.023$) levels.

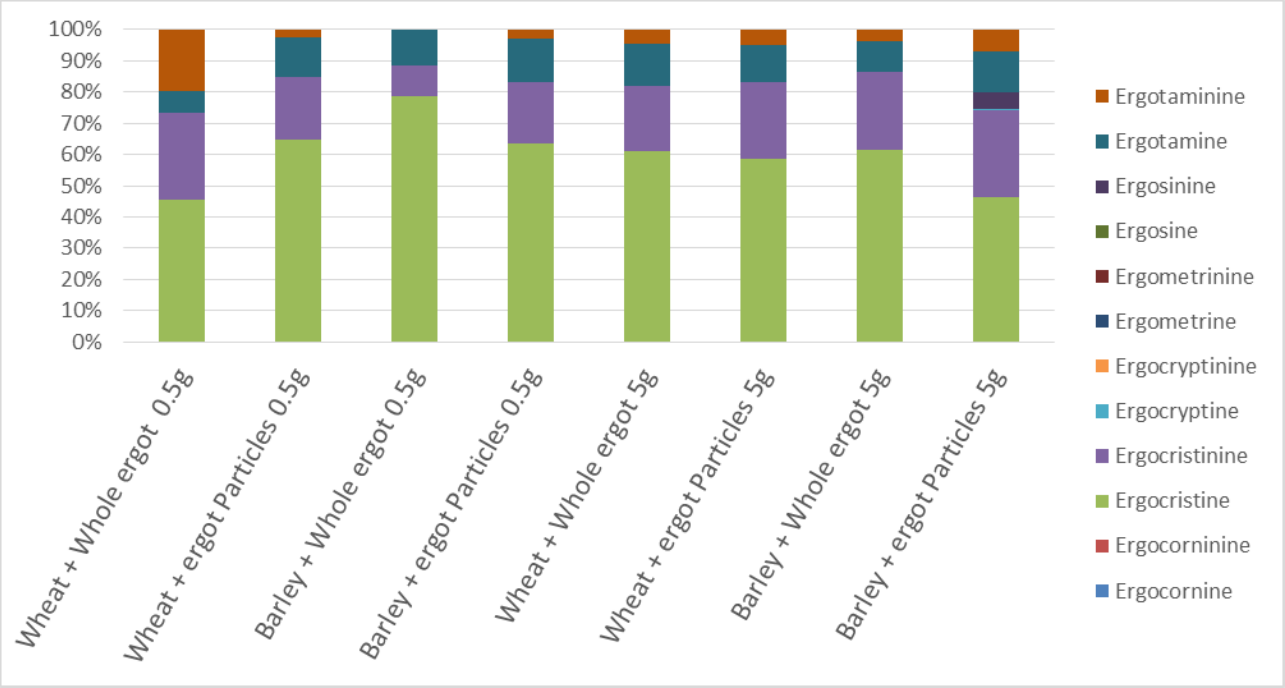


Figure 17 Proportions of ergot alkaloids on clean wheat and barley grain that had been in direct physical contact with whole or broken particles of ergot sclerotia of isolate 04-97.1.

5. Discussion

The fungal pathogen *Claviceps purpurea* infects the flowers of cereals and grasses, producing an ergot sclerotia in place of a grain. Ergot sclerotia contain a cocktail of ergot alkaloids that are highly toxic to humans and animals, and despite post-harvest removal of sclerotia by standard cleaning methods, ergot alkaloids have been detected in 'clean' grain samples (Beuerle *et al*., 2012; Byrd *et al*., 2017; MacDonald *et al*., 2017). The European Commission Contaminant Working Group is proposing to bring in changes to the limits of sclerotia found in cereal grain and, for the first time, to impose a threshold of total ergot alkaloids in milling products. For cereal milling products from wheat, spelt, barley and oats, it is expected that a limit of 75–200 ppb (75–200 ug ergot alkaloid per kg of grain) will be set for alkaloids. For rye products, the limit will be higher *c.*250–500 ppb, while for cereal-based food for infants and young children it will be lower (< 50 ppb). It is likely that the proposal will also reduce the minimum levels of ergot sclerotia in unprocessed grain lots to 0.02% (0.2g of ergot sclerotia per kg of grain), instead of the current 0.05% (0.5g per kg).

This project set out to determine to what extent ergot alkaloids may contaminate otherwise clean lots of grain. There are currently two routes by which this may happen, pre-harvest – within the ear, and post-harvest – during processing and transportation of grain. Ergot alkaloids may be transferred to healthy grain within cereal ears from flowers that have become infected with *C. purpurea*. While there is no evidence that the fungus can grow past the base of the ovary, and enter the rachis, allowing the fungus to move between flowers, it is not known whether the ergot alkaloids produced by the fungus can move between flowers. The second route of potential alkaloid transfer onto clean

grain is during grain transportation, physical contact between whole and/or broken ergot sclerotia and grain allowing transfer of alkaloids to the outside of grain.

During the *C. purpurea* infection cycle the fungus produces asexual conidia which are discharged from the flowers in a sugary substance called honeydew. Very low levels of ergot alkaloid were found in honeydew of all seven *C. purpurea* isolates examined, the highest level recorded being 800 ppb. However, on wheat and barley grain total ergot alkaloid levels of 162,792 ppb and 77,589 ppb, respectively were reported (Figure 13). Therefore, honeydew is unlikely to be the source of ergot alkaloid contamination found on healthy grain produced above and below infected flowers.

As *C. purpurea* develops, increasing levels of ergot alkaloids are laid down within fungal tissues. Ergot alkaloid levels were observed to accumulate in sphacelia, reaching the highest levels in the mature sclerotia, with isolate 03-20.1 having the highest levels of ergot alkloids (at over 3 million ppb in wheat). It has been shown in other flower-infecting fungal pathogens, namely *F. culmorum*, that mycotoxins produced by the fungus can move within the ear via the xylem vessels and phloem sieve tubes (Kang 1999). The sphacelial tissue produced by *C. purpurea* grows to fill the ovary cavity, surrounding the phloem and xylem tissues that enter the ovule. Ergot alkaloids that are synthesised in the sphacelial tissue could, therefore, find their way into the xylem and phloem tissue and be routes for transport of ergot alkaloids into otherwise healthy grain.

Significant differences in ergot alkaloid levels and profiles were observed between the seven *C. purpurea* isolates, with each isolate having its own distinct ergot alkaloid profile. These ergot alkaloid profiles were, however (at least for isolate 04-97.1), not altered by the cereal host on which *C. purpurea* was grown. The broader range of ergot alkaloids seen on initially healthy grain (all 12 ergot alkaloids found) produced above and below flowers infected with *C. purpurea* isolate 04-97.1, compared to the limited profile seen in mature ergot sclerotia (four ergot alkaloids dominated – ergoamine, ergotaminine, ergocristine and ergocristinine), would support a broad synthesis of alkaloids by this isolate, but a specific selection of alkaloids for storage in mature ergot sclerotia. The broader range of ergot alkaloids found on grain would also indicate a transfer of alkaloids within the ear, between flowers, and not due to accidental contamination of grain during removal of mature sclerotia from ears.

The function of ergot alkaloids in *C. purpurea* is unknown. They could have a role in pathogenicity, host range and/ or act as a defence against predation. Previous studies have shown isolate 04-97.1 to be highly aggressive and result in high infection rates (Gordon *et al*., 2015), but although isolate 04-97.1 produced the heaviest sclerotia, it did not produce the highest levels of total ergot alkaloids in sclerotia. While *C. purpurea* isolates can infect a wide range of cereal and grass species, the ergot alkaloid profile of isolate 04-97.1 did not differ when grown on wheat, barley and rye, as would be

expected if ergot alkaloids had a function in determining host range. In the related fungal genus *Neotyphodium*, a symbiont of grasses, ergot alkaloids and related compounds are transferred to the grass host, through the vascular system. Here they act as protectants against insect and animal predation, in a process known as 'defensive mutualism' (Panaccione *et al*., 2014). The role of ergot alkaloids is, therefore, most probably one of defence against predation, although further work would be needed to substantiate this hypothesis.

The physical contact (tumbling) experiments indicate that ergot alkaloids can be readily transferred to clean seed from both whole, intact ergot scelrotia, as well as broken, particles of sclerotia, although the latter resulted in more alkaloid transfer. However, significant differences were seen between wheat and barley. At the lower concentration of ergot sclerotia (0.5g/kg), more ergot alkaloid was transferred to grain of wheat than barley, while at the higher sclerotia concentration (5g/kg), more alkaloid was transferred to barley grain.

It is evident from these studies that a significant risk of transfer of *C. purpurea* ergot alkaloids to clean cereal grain exists within the cereal production chain, both at the stage of crop production and during transportation of harvested grain. Total ergot alkaloid levels in sclerotia reached levels as high as 3 million ppb (3 million µg of ergot alkaloids per kg of sclerotia). The average weight of a single sclerotia was 38 mg. Therefore, a single sclerotia could contain 114 µg of ergot alkaloids. If undetected, a single ergot sclerotia in a kg of grain would result in an ergot alkaloid contamination of 114 µg/kg (114 ppb), which falls within the limits proposed by the European Commission Contaminant Working Group of 75 – 200 ppb (75 – 200 ug ergot alkaloid per kg of grain).

Further R&D recommended

The findings of these studies raise a number of important questions that need clarification and/or further study.

1) Levels of ergot alkaloids transferred to rye grain within the ear were substantially lower than found on wheat and barley grain. Due to the extensive variation between replicate experiments it cannot be asserted as to whether this is a genuine feature of rye infections, in that ergot alkaloids are unable to move from *C. purpurea* infected flowers onto healthy grain within a rye ear, or just due to experimental variation. Therefore, we would recommend repeating these experiments, including a larger number of replicates.
2) The route of transfer of ergot alkaloids from infected flowers to healthy grain is hypothesised to be via the plant vascular system (xylem and/ or phloem). It is important to determine how the ergot alkaloids move from the infected flowers to healthy grain, where in the healthy flowers and grain the alkaloids are deposited, when this transfer occurs during the *C. purpurea* infection life cycle and whether there is a difference in the transferability of different ergot alkaloids.

3) This study focused on a single variety of three cereal crops, spring wheat, spring barley and a winter rye using a single isolate of *C. purpurea*. Further studies should be carried out with more isolates, increased replication and could be widened to include more varieties including winter wheat, winter barley, and other cereal crops including oats, spelt etc.
4) Preliminary discussions with cereal breeding groups has identified an interest to collaborate with NIAB in the identification of new sources of resistance to *C. purpurea*. KWS, Syngenta and RAGT have all expressed an interest in wheat resistance in relation to their hybrid wheat breeding programmes.
5) While the preliminary results suggest that ergot alkaloids are not transferred to healthy rye grain within the ear to any significant levels, we cannot rule out the possibility of physical contact transfer to clean rye grain during transportation. Therefore, the physical contact (tumbling) experiments should be repeated, and rye included to determine the ability of ergot alkaloids to adhere to the surface of rye grains.
6) The broad host range of *C. purpurea* has serious implications for ergot alkaloid contamination of cereal grains. Black-grass is a major weed problem in cereal fields. As an annual grass species, practices such as no-till farming and continuous cereal cropping support black-grass seed survival. It is worth mentioning that ergot sclerotia on grass species tend to be smaller and more easily breakable. Therefore, contamination with black-grass ergot sclerotia during harvest presents a greater risk of physical transfer of ergot alkaloids during transportation of the grain. Further studies on the effects of cropping systems, grass weed management and *C. purpurea* population epidemiology on the levels of ergot alkaloids found on grain would, therefore, be advisable.

Recommended measures to reduce the ergot alkaloid contamination of grain

- Transporting grain with high levels of ergot sclerotia contamination would increase the risk of transfer of ergot alkaloids to otherwise clean grain; this is especially true if the sclerotia are broken, and should be avoided.
- Removing ergot sclerotia on-farm would prevent contamination of clean grain with ergot alkaloids during storage and/or transportation.
- Appropriate weed management and cropping system rotations should be practised that reduce the risk of grass weeds reaching flowering within a standing cereal crop.
- Control black-grass (and other grass hosts of *C. purpurea*) in field margins. Mow grass margins to prevent flowering before the cereal crop has reached anthesis.
- If land is contaminated with ergot sclerotia, consider tilling to bury them to > 5 cm, thereby reducing the potential for sclerotia to germinate and spread ascospore in the following season.
- Avoid planting a cereal crop on land that had a high infestation of *C. purpurea* in the previous growing season.

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7. Acknowledgements

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