Barley disease screening: a multiplex digital droplet PCR approach for the detection of *Ramularia collo-cygni, Rhynchosporium graminicola* and *Pyrenophora teres*

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ABSTRACT

Barley is the most widely grown cereal crop in Ireland and faces substantial yield loss annually due to airborne, seed-borne, and soil-borne pathogens. The most prevalent and destructive of these include Ramularia leaf spot, Barley leaf scald and Net blotch, caused by *Ramularia collo-cygni, Rhynchosporium graminicola* and *Pyrenophora teres* respectively. Infected seeds are considered as the major source of inoculum for these diseases. The absence of varietal resistance for these fungal pathogens suggests the need for better disease management strategies. Accurate pathogen detection is one of the most widely accepted disease management strategies. Here we demonstrate two digital droplet PCR (ddPCR) assays, based on TaqMan probe chemistry, for the detection of the above pathogens. ddPCR is a third generation of PCR, relying on sample partition into thousands of droplets in which each droplet acts as a reaction unit, enabling pathogen detection at extremely low levels. We developed a multiplex assay capable of detecting *R. graminicola*, *R.collo-cygni* and *P. teres*. For *P. teres* samples, a further assay was designed to distinguish between the two economically significant forms of *P. teres* i.e. *P. teres* f. maculata (Ptm; spot form of Net blotch) and *P. teres* f. teres (Ptt; net form of Net blotch). The assay offers sensitive and selective pathogen detection from barley seeds to pico moles/µl level. Finally, the efficacy of the assay was demonstrated by screening a collection of historical barley seeds.

1 | INTRODUCTION

After wheat and corn, barley is the 3rd most widely produced cereal crop in Europe, making up almost 20% of the total cereals produced (European Union Crop Production, 2024). Due to the combination of agronomic and climatic factors, the potential for high yields and favourable end-markets, barley accounts for approximately 70% of all cereals produced in Ireland (CSO, 2023). Unfortunately, as with all crops, barley can succumb to a spectrum of diseases that if left unchecked can seriously impact potential yields, both quantitatively and qualitatively. Most notable amongst these are the fungal foliar diseases ramularia leaf spot (RLS) caused by *Ramularia collo-cygni,* barley scald caused by *Rhynchosporium graminicola,* and the spot and net forms of net blotch (SFNB and NFNB), caused by *Pyrenophora teres* f. *maculata* (Ptm) and *P*. *teres* f. *teres* (Ptt) respectively (Huss et al. 1992; Jalli et al. 2020; Zhang et al. 2020).

All three diseases impact yield by reducing the healthy leaf area of the crop available for photosynthesis, and as barley is typically more sink limited than source the earlier disease develops in the crop the greater the potential impact on yield (Bingham et al. 2007). As is the case with all plant diseases and pests, limiting initial inoculum/pest sources is critical to achieving sustainable control and is the principal tactic of integrated pest management (Barzman et al. 2015). For *R. collo-cygni*, *R. graminicola* and *P. teres* trash and stubble from the previous season's crops, volunteers and infected seed, all act as potential sources of initial inoculum. For *R. graminciola* and *P. teres* it has been previously demonstrated that seed-borne infection represents a significant means of disease transmission and can directly impact disease levels and subsequent impacts on barley yields (Fountaine et al. 2010; Hysing & Wiik, 2013). For *R. collo-cygni*, whilst the pathogen can be readily detected in seed, and it has been

24 demonstrated that it can grow from the seed into the developing seedling, the importance of
25 seed-borne infection in the aetiology of RLS remains to be determined (Havis et al. 2006).

26 Traditionally seed has been tested for the presence of pathogens using culture dependent
27 techniques, whereby the test seeds are cultured on a selective growth media and subsequent
28 microbial growth is examined morphologically and/or microscopically to confirm the
29 contaminating species (Hiddink et al. 2023). However, there is a major disadvantage of
30 culture-dependent assays which is that they are restricted to those pathogens that can be
31 easily grown in culture and have a relatively fast growth rate. Of the three major barley
32 pathogens identified above, it is only possible to routinely identify *P. teres* through culture-
33 dependent assays as both *R. graminicola* and *R. collo-cygni* are relatively slow growing and
34 are outcompeted by other fungi (pathogenic or non-pathogenic) present in barley seed.
35 Identification of *P. teres* in culture can also be difficult as it can be mistaken for *P. graminea*,
36 which exhibits a similar phenology in culture. Similarly, it is not possible to distinguish
37 between Ptt and Ptm when grown in culture or by using osmotic or blotter tests (Brodal,
38 1997). More recently developed detection methods that rely on detection of nucleic acids
39 (either DNA or RNA) have improved specificity compared to culture-based detection
40 methods. They also allow a wider range of pathogens to be detected, whilst simultaneously
41 reducing costs and run time (Elnifro Elfath et al. 2000).

42 Seed samples are unfortunately often complex matrixes, which can inhibit or impair DNA
43 based reactions and assay sensitivities (Lu et al. 2020), in particular those such as qPCR on
44 which quantification is based on comparable efficiencies of reactions required between the
45 test sample and samples/standards of a known DNA concentration (Hindson et al. 2013).
46 Digital droplet PCR (ddPCR, also referred to as digital PCR (dCPR) or crystal digital PCR (cdPCR)

depending on the platform used) is a third generation of PCR in which the starting DNA sample is partitioned through the use of microfluidics into thousands of samples which subsequently undergo PCR reactions. By coupling partitioning and PCR with fluorescent dye based DNA probes it is possible to use endpoint analysis to confirm the presence or absence of the targeted DNA partitioned samples. Through the use of Poisson's Law it is then possible to determine the total copies of the target DNA in the starting sample. As quantification is based on an end point reaction it is not as sensitive to PCR inhibitors etc which can impact methods such as qPCR which can be strongly influenced by the efficiencies of the PCR reaction (Basu, 2017). Therefore, ddPCR allows for the accurate and sensitive quantification of specific DNA targets, even in the most complex of matrices, making it an ideal platform for the detection of pathogens in cereal seeds.

Different PCR based methods have been developed for each of the commonly occurring barley pathogens, with qPCR assays available for both *P. teres* and *R. graminicola* (Bates et al. 2001; Fountaine et al. 2010), and a dPCR based method for detection of *R. collo-cygni* recently published by Knight et al. (2022). To exploit the multi-spectral capacity of the Stilla 3-colour Nacia® ddPCR system here we combine the *R. graminicola* and *R. collo-cygni* assays of Fountaine et al. (2010) and Knight et al. (2022), with a *P. teres* probe based assay to provide a multiplex ddPCR assay to simultaneously detect and quantify the presence of the three pathogens. A second multiplex ddPCR is also described to further differentiate those samples which were positive for *P. teres* in order to determine whether form i.e. Ptm or Ptt.

2 | MATERIALS AND METHODS

A multistage process of assay design, optimisation and application was developed and is outlined in Figure 1.

70 **2.1 | Fungal strains and DNA extraction**

71 All fungal cultures used in the study (Supplementary Table 1) were acquired from the
72 phytopathological collection in Teagasc, Oak Park, Co. Carlow. DNA was extracted from these
73 cultures following lyophilisation and homogenisation using a Qiagen DNeasy Plant Mini kit
74 (Qiagen, Germany), following the manufacturer's instructions. Extracted DNA was quantified
75 using Invitrogen™ Qubit™ Fluorometer with a Qubit™ 1X dsDNA BR Assay (Invitrogen, US).

76 **2.1 | Assay designs and initial optimization**

77 For the initial ddPCR multiplex assay to detect and distinguish between the three target fungal
78 pathogens, the published fluorescence probe based assays for the detection of *R. collo-cygni*
79 (Knight et al. 2022) and *R. secalis* (Fountaine et al. 2010) were selected. A separate set of
80 primers and probe to detect *P. teres* was designed using Primer 3Plus
81 (https://www.primer3plus.com/index.html) based on the Internal Transcribed Spacer (ITS)
82 region of *P. teres* (OM471876.1). To further distinguish between Ptt and Ptm a set of primers
83 and probe was designed using PrimerQuest™ (Integrated DNA Technologies) based on
84 differences in their mRNA region as described by Poudel et al. (2017) (accession numbers Ptt:
85 KX909556.1 and Ptm: KX909562.1). Details of all primers and probes are summarized in Table
86 1. Initial primer and probe optimisation, including identifying annealing temperatures, was
87 conducted using routine PCR and visualised using agarose gel electrophoresis (see
88 Supplementary Figure S1 for further details).

89 **2.2 | ddPCR optimisation**

90 Following these initial validation steps, the assays were transferred to ddPCR system (naica®
91 Crystal Digital PCR 3 colour system, Stilla Technologies, France). ddPCR using the naica®

92 Crystal Digital PCR 3 colour is separated into four stages: 1) the reaction sample (primers,
93 probes, DNA, etc) is partitioned into nano-sized droplets on a chip (up to 17k on a Ruby chip
94 or 30k on a Sapphire chip), 2) partitioned samples undergo up to 50 PCR cycles, 3) once the
95 PCR is complete a scan of the chip is taken under different wavelengths (corresponding to the
96 excitation of the FAM, HEX and Cy5 dyes) is undertaken, and 4) scanned chips are analysed to
97 identify droplets with the target DNA amplified based on presence or absence of the specified
98 molecular probes and associated dyes. In the present study all reactions were conducted
99 using the Ruby Chip (up-to 17k droplets per chamber) and 5X naica® multiplex PCR MIX (Stilla
100 technologies, France) and only reactions with >10k analysable droplets were included.
101 An initial series of reactions were performed to optimize concentrations of primers and
102 probes required to differentiate between positive and negative droplets. Reaction mixtures
103 were prepared in accordance with the guidelines provided by Stilla Technologies, consisting
104 of 1 µl of Buffer A, 0.2 µl of Buffer B, 0.25 µl (both supplied as part of the 5X naica® multiplex
105 PCR MIX each of the primers (0.05 µM final concentration), 0.15 µl of each probe (0.06 µM
106 final concentration), 0.85 µl of Barley DNA, 1 µl of template DNA (0.1 ng/µl) and nuclease free
107 water (Fisher Scientific, UK) was added to reach a final volume of 5 µl. All PCR reactions were
108 performed in the presence of pathogen-free barley DNA as a matrix, extracted from two-week
109 barley seedlings (variety: RGT Planet, Goldcrop Ltd, Ireland) grown under controlled
110 conditions (16 h of light, 8 h of dark, at 20 and 16 °C) Thermal cycling conditions for both
111 assays are as follows; partition at 25°C followed by 95°C for 3 minutes, then 5 cycles of 95°C
112 for 10 seconds followed by 66°C for 30 seconds, and then 35 cycles at 95°C for 10 second and
113 64°C for 15 second. A compensation matrix was designed according to the guidelines provided
114 by Stilla Technologies, to normalize the fluorescence overlap and spill over.

115 **2.2 | Determining Limits of Blank (LoB) and Limits of Detection (LoD)**

116 Limit of blank (LoB) and Limit of detection (LoD) for the assays were calculated in multiplex
117 fashion and in accordance with the guidelines provided by Stilla Technologies. LoB can be
118 defined as the maximum target concentration expected in the blank sample with a probability
119 PLoB = 1 – α (typically 1 – α = 95% where α = 5% of false positives), where α is the probability
120 of making a false positive decision in a real blank sample (no DNA of any target except the
121 background DNA). Whereas, LoD is defined as the minimum concentration, a non-zero,
122 statistically significant value, that can be detected with a probability of 1 – β (typically 1 – β =
123 95% where β = 5% of false negatives), β is the probability making a false negative decision in
124 a real positive sample.
125 To determine the LoB, 30 blank (negative for the target for which LoB is being performed)
126 reaction replications were performed for each target. To calculate LoD, ten-fold serial
127 dilutions of a known target DNA concentration was performed and a non-zero, statistically
128 significant concentration detected by the assay, designated as low-level sample was
129 calculated. Afterwards, five independent low-level samples were prepared and six reaction
130 replications of each were performed. Based on the copy per microliter (cp/µl) measured by
131 the fluorescence detector, LoD and LoB was calculated by using the online tool provided by
132 Stilla Technologies (https://www.stillatechnologies.com/digital-pcr/statistical-tools/limit-
133 detection/).
134 **2.3 | Linearity of the assay**
135 To calculate the relationship between the expected and measured quantities of each of the
136 target pathogen DNA, linearity of the assay was calculated. To achieve this, a 10-fold dilution
137 series (0.01-0.00001 ng/µl) of each pathogen DNA (*R. collo-cygni*, *R. graminicola* and *P. teres*)
138 was prepared and tested individually whilst keeping a constant DNA concentration of the

139 other two pathogens (0.1 ng/µl) in the reaction mixture. The log10 values of concentration
140 against copy number/µl (cp/ µl) were plotted and correlation was calculated.

141 **2.4 | Specificity of the assays**

142 Specificity of both multiplex assays was performed by challenging the system with a number
143 of barley pathogens including *Botrytis cinerea (Bc), Parastagonospora nodorum (Pn),*
144 *(Alternaria alternate (Aa), Fusarium graminearum (Fg), Microdochium nivale (Mn)* and
145 *Fusarium culmorum (Fc)* accessions that are stored as part of the Phytopathological collection
146 in Teagasc, Oak Park, Co. Carlow. Additionally, specificity of the Ptt and Ptm assay was also
147 verified against *R. collo-cygni* and *R. graminicola* in a singleplex reaction. Each of the
148 pathogens was tested in an individual ddPCR reaction.

149 **2.5 | Detection of pathogens in leaf and seed samples**

150 To validate the reliability of the assay for naturally infected barley samples, historical winter
151 and spring barley seed DNA from 2016–2018, with variable susceptibility to leaf blotches,
152 were evaluated using the assays. Any droplet population above LoD of the respective assay
153 was considered positive. Samples positive for *P. teres* were further tested by the form specific
154 assay to confirm *P. teres* form. Volunteers are one of the sources of inoculum for RLS, barley
155 leaf scald and net blotch, efficacy of the assay was also assessed in wheat volunteers from
156 barley field with visual symptoms of RLS (Teagasc wheat trials in Knockbeg, Ireland (2023).
157 Leaves were freeze dried and DNA was extracted using kit (Qiagen, Germany). Extracted DNA
158 (1 µl) was used as a template in the reaction mixture described in section 2.2.

159 **3 | Results**

160 **3.1 | Assay design and optimization**

161 For the detection of *R. collo-cygni* and *R. graminicola*, primers and probes were deployed
162 from published assays (Fountaine et al. 2010; Knight et al. 2022). However, for *P. teres*
163 detection and to distinguish between both Ptt and Ptm it was necessary to design novel
164 fluorescent probes. The initial multiplex detection assay was specific for the three pathogens,
165 with no fluorescence overlap observed between the different probes-pathogen combinations
166 (Figure 2A). Similarly, for the *P. teres* form specific assay there was no fluorescence overlap
167 between the probe-pathogen combination, demonstrating specificity of the assay (Figure 2B).
168 For *R. graminicola, R. collo-cygni and P. teres* the LoB were calculated to be 0.68, 0 and 1.34
169 cp/µl respectively. For the *P. teres* form specific assay and LoB of 0 cp/µl was calculated for
170 both Ptt and Ptt. Limits of detection (LoD) for *R. graminicola, R. collo-cygni and P. teres* in the
171 multiplex assay were calculated as 1.67 cp/µl, 1.06 cp/µl and 4.21 cp/µl respectively.
172 Additionally, LoD was calculated as 1.44 for Ptt assay and 0.82 for Ptm assay. No positive
173 droplets, irrespective of probe, were detected in negative control (barley DNA only) (Figure
174 2A & B).

175 **3.2 | Linearity of the assay**

176 To determine the accuracy of the assay, the linearity of the assay was ascertained by
177 correlating the projected DNA copies of each target in a sample and copy numbers detected.
178 For each probe-pathogen combination R^2 values of >0.99 were calculated. Linearity of the
179 assay also indicated the lower limit of detection of the assay, which is 0.00001 ng/µl (Figure
180 3). The linearity of the *P. teres* form specific assay was not determined as it was specifically
181 designed to distinguish between forms following initial detection of *P. teres* using the
182 multiplex assay.

183 **3.3 | Selectivity of the assay**

184 Considering the potential application of our assays, selectivity of both the assays was
185 investigated against range of fungi that may be found on or in barley seeds and leaves. No
186 positive droplets were detected in any of the fluorescent channels for any of the fungi tested,
187 indicating that the assays are highly specific (Figure 4A & B).

188 **3.4 | Detection of barley pathogens in wheat and rye**

189 The sequence similarity between the wheat pathogen *R. graminicola* and the rye pathogen *R.*
190 *secalis,* suggested that the developed assay could be used for detection of the pathogen from
191 rye seed. Rye seed and leaf DNA was tested against the assay. Rye seed samples that did not
192 receive the standard fungicide regime were found to be positive for the presence of the
193 pathogen *R. secalis* (Figure 5). Wheat leaf DNA was also tested for the presence of *R. collo-*
194 *cygni.* The assay showed a considerable droplet population for *R. collo-cygni*. However, wheat
195 samples displayed positive droplets for the presence of *R. graminicola and P. teres* DNA in the
196 samples (Figure 6).

197 **3.5 | Detection of *R. collo-cygni*, *R. graminicola* and *P. teres* in barley seed samples**

198 To determine the application of assays to test the presence of pathogens in naturally infected
199 barley samples, DNA previously extracted from seeds of winter and spring barley from 2016
200 (Kildea et al. 2024) were tested. Only samples exhibiting cp/µl equal to or above the limits of
201 detection, as identified above, were deemed positive for the respective pathogens. Of the 35
202 winter barley samples tested, 16 samples were positive for *R. collo-cygni*, two samples were
203 positive for *R. graminicola,* while 19 were positive for *P. teres.* For the 32 spring barley
204 samples, 15 were positive for *R. graminicola*, 25 samples were positive for *R. collo-cygni,* and
205 25 positive for *P. teres*. Nine of the spring and winter barley samples that were found to be
206 positive for *P. teres* were tested to determine the form determination. None of the samples
207 tested positive for the presence of *P. teres* f. maculata. In the case of winter barley, eight out

of nine samples were positive for *P. teres* f. maculata and two samples were positive for *P. teres* f. teres as well as *P. teres* f. maculata (Supplementary information).

4 | Discussion

Clean seeds are undeniably important for healthy crops and this is reliant on early detection of specific disease agents, which can further facilitate the execution of IPM based disease control strategies (Dussart et al. 2020; Tsedaley, 2015). The ddPCR assays described, offer a selective, reliable and highly sensitive means of detecting and quantifying three of the most economically important barley pathogens in a single reaction. As the assay is based on the digital PCR concept and is therefore end-point in nature which makes it ideal for detecting the different pathogens in complex plant materials, such as seeds/grains (Liu et al. 2011).

To validate the efficacy of our assays and investigate the prevalence of *R. collo-cygni, R. graminicola* and *P. teres* in real samples we screened a selection of historical barley seed samples from 2016 to 2018, rye grains harvested in 2023 and wheat leaves sampled from volunteer wheat plants in a winter barley field in 2023. Unsurprisingly, the barley seed samples were contaminated with variable levels of the three pathogens, with a proportion coinfected with all three pathogens. Only *R. graminicola* was detected in the rye grains, whilst all three pathogens were detected in the winter wheat leaves, the latter result is unsurprising given the fact that the leaves tested were collected from wheat volunteers collected from a winter barley field. Whilst *R. collo-cygni* has previously been reported in wheat in Ireland (Zia et al. unpublished), the detection of *R. gramincola* and *P. teres* in the sample may simply reflect the presence of both pathogens on the wheat leaf surface rather than colonisation of the leaf tissue. This highlights a potential limitation of the assays. As they are based on the detection of DNA of the respective pathogens, they cannot determine if the detected

231 pathogen was actively colonising the plant tissue, or if the pathogen detected was even viable.
232 However, it is important to note that these limitations are not restricted to the assays
233 developed and described in this paper, but are a drawback of all DNA-based detection
234 methods (Ficetola et al. 2015). It is therefore critical that any positive detections are assessed
235 in the context of detection (host, etc.) and the potential risk(s) posed by the pathogen. As
236 mentioned previously it is more likely that the positive detections for *R. graminciola* and *P.*
237 *teres* from the wheat volunteers are consequential to levels of both diseases in the
238 surrounding barley crop. However, it is worth highlighting that in addition to *R. collo-cygni*
239 being noted as able to colonise wheat, Ptt has been reported as causing disease on wheat in
240 Hungary (Tóth et al. 2008), Russia (Mikhailova et al. 2010) and most recently in Brazil (Garozi
241 et al. 2020). Further analysis of wheat grains and/or a wider wheat disease survey is
242 warranted to determine if either pathogen is more widespread in wheat crops.

243 Those barley seed samples that presented positive for *P. teres* were subsequently tested to
244 determine what form of *P. teres* was present. Amongst the spring barley samples tested only
245 Ptt was present. For the winter barley seed samples both Ptt and Ptm was detected, with a
246 number of samples positive for both. All samples with detectable levels of Ptm, irrespective
247 of the presence of Ptt, were from the cultivar KWS Tower, which had by the late 2010s
248 become highly susceptible to *P. teres*, and in particular Ptm (Recommendation list in
249 supplementary information). Positive detection of both Ptt and Ptm could indicate of a
250 potential Ptt/Ptm hybrid, however based upon the number of droplets positive for both in
251 the test samples this appears unlikely. However, such detections do suggest such hybrids may
252 arise, with recent reductions in fungicide sensitivity and efficacy in Western Australia
253 associated with such a hybrid (Mair et al. 2020; Turo et al. 2021).

254 It is worth noting that within the multi-pathogen assay the region targeted for *P. teres* was
255 ITS region, whilst in the form specific assay we targeted the Ptt form-specific region PttQ5
256 mRNA sequence and the Ptm form-specific region PtmQ11 mRNA sequence. The copy
257 number for ITS in different fungi can vary considerably (Lofgren et al. 2019) and the copy
258 number of PtmQ11 and PttQ5 are unknown in *P. teres.* As a number of samples that tested
259 positive for *P. teres* in the multi-pathogen assay, were neither positive for either Ptt or Ptm
260 suggests PtmQ11 and PttQ5 have a lower copy number compared to ITS region in *P. teres.*
261 Therefore, ~ 15 cp/µl of *P. teres* sequence are required to confirm the presence of Ptt or Ptm
262 from the samples.

263 Infected seed is the most likely source of introduction of new pathogens and resistance
264 mechanisms across the globe for all of the three pathogens discussed (Stam et al. 2019).
265 Therefore, seed screening for pathogen levels and presence of resistance alleles should be
266 regular practice prior to seed transportation. Furthermore, establishment of pathogen
267 threshold levels and rejection of seeds with high pathogen levels can minimize the risk of
268 future outbreaks. Seed is only one of the sources of these diseases however prevention is the
269 key to suppression.

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275 **Conflict of interest declaration**

None declared.

Data availability statement

The data that support the findings of this study are available on reasonable request from the corresponding author.

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404

405 **List of Figures**

406 **Figure 1**

407 Summarized workflow of the digital droplet PCR assay

408

409 **Figure 2**

410 Specificity of the multiplex ddPCR assays, pathogen detected are indicated on x-axis (A)
411 Multiplex assay for the detection of *R. graminicola* , *R. collo-cygni* and *P. teres* (B) Multiplex
412 assay for the detection of forms of *P. teres*, FAM channel detects *P. teres* f. teres while HEX
413 channel is to detect *P. teres* f. maculata. Cy5 was used as an internal control.

414

415 **Figure 3**

416 Linearity of the multiplex assay for the detection of (A) *P. teres,* (B) *R. graminicola* and (C) *R.*
417 *collo-cygni*.

418

419 **Figure 4**

420 Selectivity of the multiplex assays (A) Multiplex assay for the detection of *R. collo-cygni*, R.
421 *graminicola* and *P. teres* challenged with various barley pathogens (B) *P. teres* form specific
422 assay exposed to various barley pathogens.

423

424

425 **Figure 5**

426 Detection of *R. secalis* (FAM channel) and *R. collo-cygni* (HEX channel) in Rye seed DNA (3
427 and 5); sample 15 and 17 indicate positive and negative controls for the multiplex assay
428 respectively.

429

430 **Figure 6**

431

432 Detection of *R. collo-cygni* from wheat leaf DNA (sample 9, 11 and 13) collected from wheat
433 volunteers in the barley field, sample 16 and 32 indicate positive and negative controls for

434 the multiplex assay. Droplet population in FAM and Cy5 channel indicate the possible cross
435 contamination of the wheat leaves with *R. graminicola* and *P. teres* spores from the barley.

436