# The use of Real-Time PCR in the diagnosis and monitoring of *Rhizoctonia cerealis* in winter wheat grown in production fields

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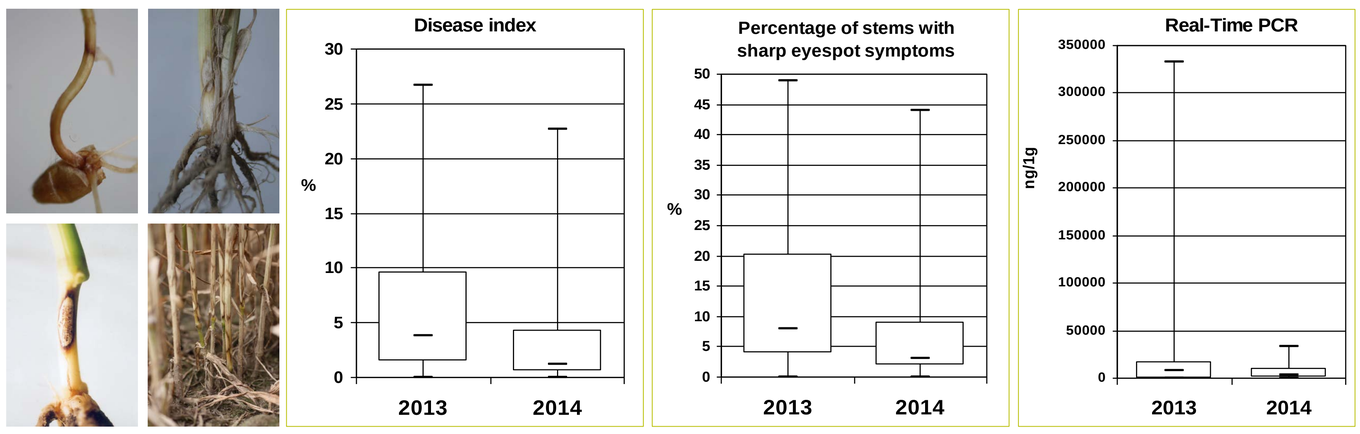
## Introduction

One of the diseases affecting wheat stem bases is sharp eyespot. This disease is caused by the soil-borne fungus *Rhizoctonia cerealis*. In cereals some role is also played by *R. solani*, which has a wide host range. Visual diagnosis of stem base diseases is very difficult, especially when the infection is caused by more than one pathogen. In such cases, one can easily make a mistake, especially in the early stages of the disease. In addition, symptoms may be obscured by the second pathogen.

The research was aimed to estimate sharp eyespot incidence in winter wheat crops in different agro-ecological condition of Poland, and to quantify sharp eyespot causal agents *R. cerealis* in the population using the Real-Time PCR (Q PCR) method.

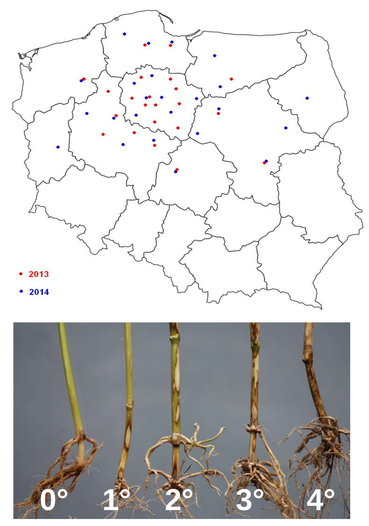
**The incidence of sharp eyespot (DI, %) in winter wheat and the amount of DNA of *R. cerealis* in wheat samples (Rc DNA ng /1 g of dry mass of stem base) - top, median, and bottom quartiles**



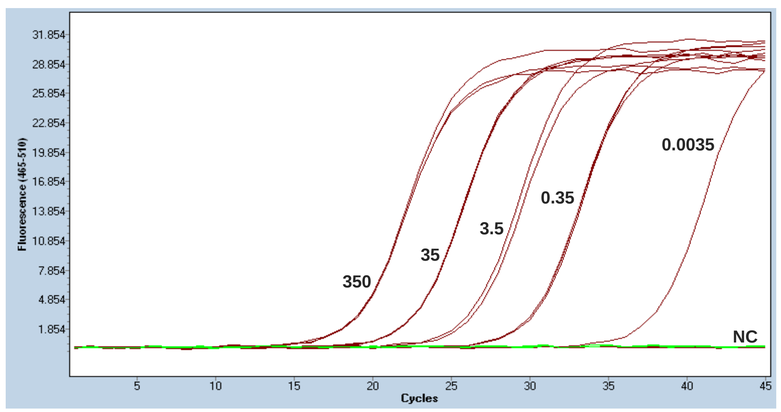

## Materials and Methods

Observations of the occurrence of sharp eyespot were performed during the 2013–2014 periods, on 45 winter wheat production fields. At the BBCH 75–77, along the diagonal of the field, random samples were taken. One sample, consisting of 100 stems, was taken from each field. The percentage of stems with symptoms of sharp eyespot was evaluated. The degree of the intensity of sharp eyespot was determined, applying the 0–4° scale. The degrees of infection were transformed into the Disease index (DI). The evaluation of the plant's health status was supplemented by using the quantitative Real-Time PCR (Q PCR) assay for specific, sensitive detection and quantification of *R. cerealis* in stem base samples. We used the specific primer RtubF4 (50-CCTA AATGAGTCTGGAGTAAGTC-30) and RtubR4 (50-GCTAGTGCGGTCAATGTATAG-30) amplified a fragment of 138 bp. Each sample collected from commercial fields was composed of 100 randomly selected stems. Stem segments approximately 0.5 cm in size were ground and homogenised in liquid nitrogen. Total DNA was extracted and purified from 50 mg homogenised sample in three replications using the modified method described by Doyle and Doyle (1990). The standard curve method was used for *R. cerealis* DNA quantification in stem bases of winter wheat. For development of standard curves *of R. cerealis*, DNA was isolated from pure *R. cerealis* culture, obtained from own collection. Individual standard curves were formed from different concentrations of DNA of the tested samples, having done 5 dilutions of the initial DNA sample. Q PCR was performed in LightCycler 480II using LightCycler 480 SYBR Green I Master Mix. Q PCR of one sample was performed in three replicates.

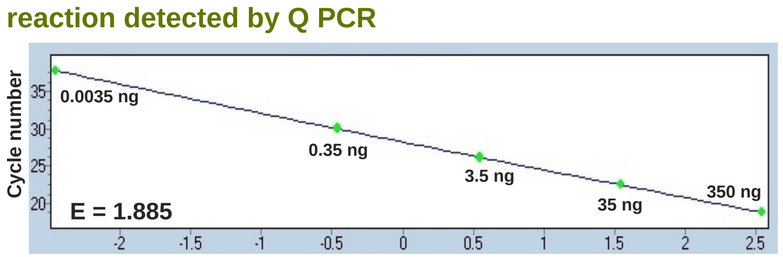
**Location of surveyed fields in Poland**



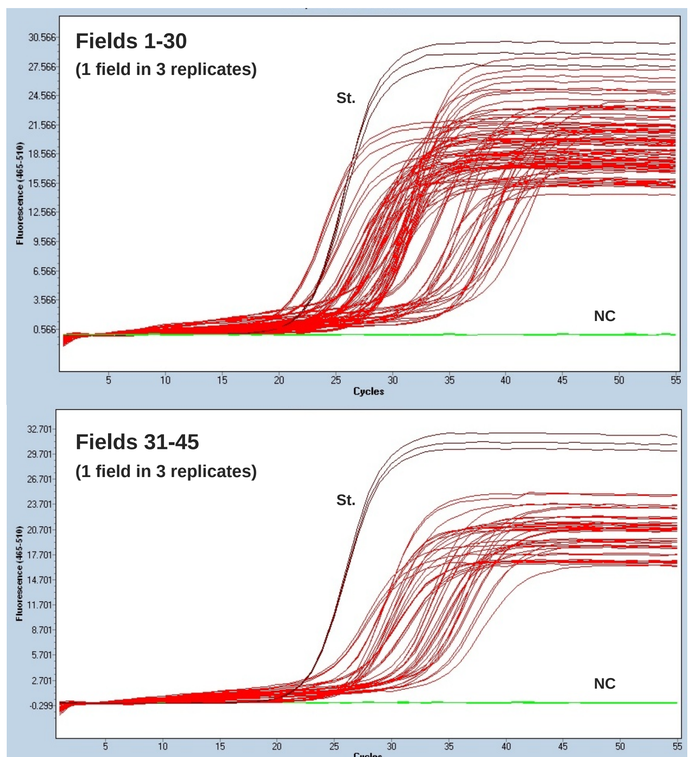

**Representative Real-Time PCR fluorescent curves for *R. cerealis*; DNA concentrations from 350 ng to 0.0035 ng, and negative control**




**Standard curve obtained by plotting the log amount of *R. cerealis* DNA (ng) vs. the threshold cycle of each reaction detected by Q PCR**



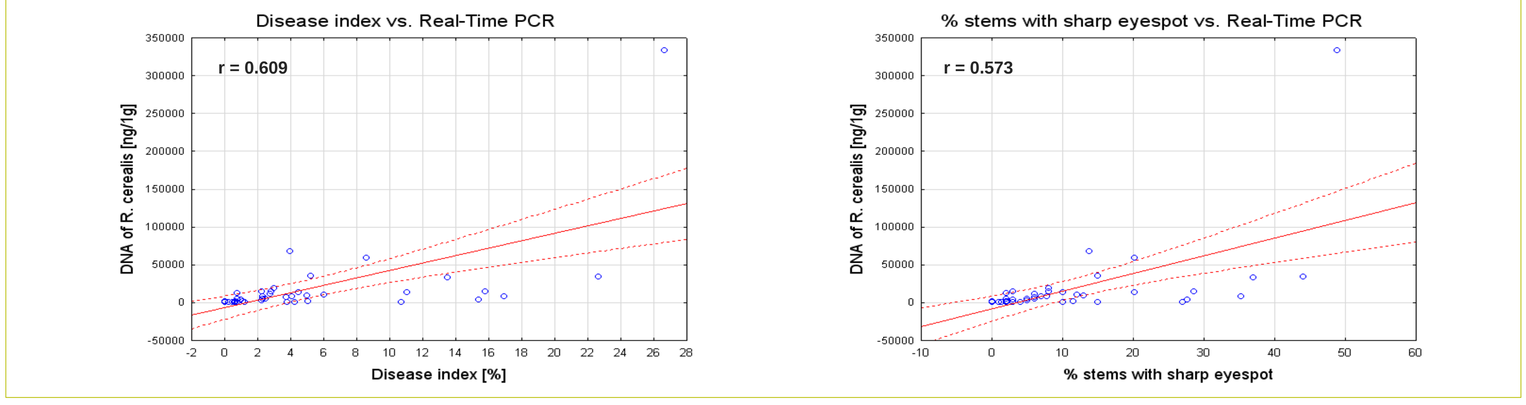

**Real-Time PCR amplification of *R. cerealis* DNA in analyzed stem base samples with SYBR Green I using RtubF4 and RtubR4 primers**



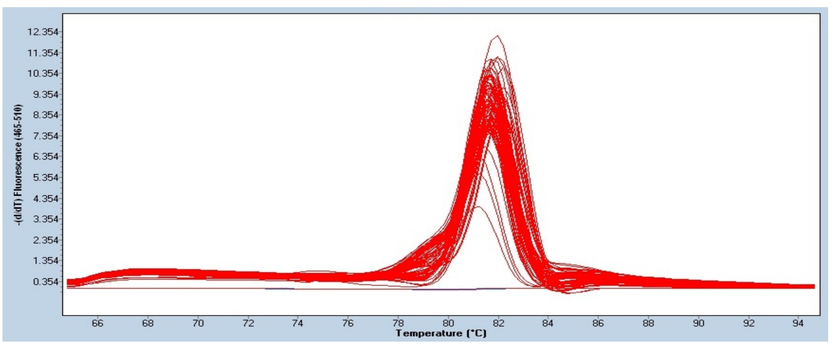

**The incidence of sharp eyespot (DI, %) in winter wheat and the amount of DNA of *R. cerealis* in wheat samples (Rc DNA ng /1 g of dry mass of stem base) collected in fields in 2013–2014**

| No. Field | 2013 | | | No. Field | 2014 | | |
|---|---|---|---|---|---|---|---|
| | Sharp eyespot | | Q PCR [ng/1g] | | Sharp eyespot | | Q PCR [ng/1g] |
| | DI | % | | | DI | % | |
| 1 | 3.0 | 8 | 19080 | 24 | 1.0 | 3 | 4200 |
| 2 | 17.0 | 35 | 8100 | 25 | 0.0 | 0 | 1908 |
| 3 | 2.3 | 5 | 4608 | 26 | 0.5 | 2 | 639.0 |
| 4 | 10.7 | 27 | 501 | 27 | 4.5 | 10 | 13230 |
| 5 | 4.1 | 8 | 8370 | 28 | 0.8 | 2 | 11940 |
| 6 | 8.6 | 20 | 58800 | 29 | 6.0 | 12 | 10140 |
| 7 | 0.0 | 0 | 46.2 | 30 | 2.3 | 5 | 2535 |
| 8 | 4.3 | 15 | 36.9 | 31 | 0.8 | 2 | 5220 |
| 9 | 2.8 | 8 | 14610 | 32 | 0.0 | 0 | 28.2 |
| 10 | 3.8 | 10 | 0.7 | 33 | 1.3 | 4 | 92.7 |
| 11 | 4.0 | 14 | 67200 | 34 | 0.3 | 1 | 145.2 |
| 12 | 0.0 | 0 | 4.9 | 35 | 2.8 | 6 | 11850 |
| 13 | 26.7 | 49 | 333000 | 36 | 3.8 | 6 | 6750 |
| 14 | 5.3 | 15 | 34800 | 37 | 5.0 | 13 | 9060 |
| 15 | 11.1 | 20 | 13890 | 38 | 0.5 | 2 | 0.02 |
| 16 | 0.7 | 2 | 213.3 | 39 | 2.3 | 3 | 14880 |
| 17 | 2.5 | 6 | 5310 | 40 | 0.7 | 2 | 1620 |
| 18 | 0.5 | 2 | 284.4 | 41 | 1.1 | 2 | 1878 |
| 19 | 15.8 | 29 | 14550 | 42 | 5.1 | 12 | 1212 |
| 20 | 0.0 | 0 | 33.9 | 43 | 0.6 | 1 | 393 |
| 21 | 13.5 | 37 | 33300 | 44 | 15.4 | 28 | 3990 |
| 22 | 0.8 | 3 | 203.4 | 45 | 22.7 | 44 | 33900 |
| 23 | 2.3 | 7 | 7680 | | | | |
| **Mean** | **6.1** | **13.9** | **27157.5** | **Mean** | **3.5** | **7.3** | **6164.1** |

**Correlation between visual diagnosis and with using Real-Time PCR**




**Melting curve profile of PCR products obtained by real-time PCR in tested samples (1-30). The Single peak at 82ºC with *R. cerealis* DNA as template indicates the specificity of the RtubF4 and RtubR4**




## Results

Sharp eyespot was identified in 88.9% of the crops surveyed. The incidence of sharp eyespot in winter wheat crops varied from 0.0% to 49% (DI=26.7%) depending on the year and location. Most infected stems were noted in 2013 in which it occurred, on the average, on 13.9% (DI=6.1%), and least in 2014 – on 7.3% (DI=3.5%). The highest incidence of sharp eyespot was identified in Minikowo (2013). Using the Real-Time PCR assays, we were able to quantify *R. cerealis* in naturally infested stem base samples. This technique to quantify *R. cerealis* is rapid and accurate and will be a useful tool for future studies of pathogenic *R. cerealis*. Using a specific primer pair we could specifically detect *R. cerealis* at quantities from 333000 ng to 0.02 ng DNA of *R. cerealis* in 1 g of dry mass of wheat stem bases. On average the highest amount of *R. cerealis* DNA was noted in 2013 (27157.5 ng/1g), and lowest in 2014 (6164.1 ng/1g). There was a positive correlation between the amount of *R. cerealis* DNA in wheat stem bases, the DI (r=0.609), and the percentage of stems with symptoms of sharp eyespot (r=0.573).