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Resistance of Winter Wheat Cultivars to Eyespot and Characterisation of Causal Agents of the Disease

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Abstract

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The reaction of ten winter wheat cultivars grown in the Czech Republic to inoculation with *Oculimacula yallundae* and *Oculimacula acuformis* was evaluated in a small plot trial. In a parallel field trial the natural occurrence of stem-base disease complex in six of the tested cultivars was assessed. Lower severity of eyespot (and/or stem-base diseases) was observed in cultivars possessing the resistance gene *Pch1* (cvs Hermann, Annie, Princeps, Manager, and Rebell) in plots inoculated with *Oculimacula* spp. as well as in natural field conditions. A total of 468 wheat stem bases from the Czech Republic was screened by PCR to study the frequency of eyespot causal agents. The plants were colonised significantly more often by both species *Oculimacula yallundae* and *O. acuformis* together than separately.

Keywords: *Oculimacula yallundae*; *Oculimacula acuformis*; *Pch1* gene; molecular markers

Oculimacula yallundae (Wallwork & Spooner) Crous & W. Gams (anamorph *Helgardia herpotrichoides* /Fron/ Crous & W. Gams) and *Oculimacula acuformis* (Boerema, R. Pieters & Hamers) Crous & W. Gams (anamorph *Helgardia acuformis* /Nierberg/ Crous & W. Gams) are causal agents of eyespot, which is a very important disease of cereals. Eyespot can cause yield losses up to 40%. The pathogens can survive on plant debris more than 3 years and disease occurrence varies due to weather conditions (Matusinsky *et al.* 2009). *Oculimacula* spp. are part of the stem-base disease complex of wheat pathogens that includes *Rhizoctonia* spp., *Fusarium* spp., *Monographella nivalis*, and *Gaeumannomyces graminis*. In 2015–2016, eyespot was found in stem-base disease samples of wheat in all regions of the Czech Republic apart from North Moravia (according to Central Institute for Supervising and Testing in Agriculture).

O. yallundae and *O. acuformis* differ in morphology, pathogenicity, occurrence, and sensitivity to fungicides (Wei *et al.* 2011). Eyespot pathogens have a wide host range among cereals and grasses. Conidia produced on infested straw are the principal form of inoculum in the field (Leroux *et al.* 2012). Conidia are dispersed over short distances in rain splash droplets and can contaminate wheat from autumn to spring. Possible occurrence of secondary infections originating from conidia produced on plant lesions is still discussed. Ascospores play an important role as a source of genetic variability within pathogen populations, though they not probably constitute a major source of inoculum (Leroux *et al.* 2012).

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Typical symptoms are elliptical eye-shaped spots at the base of the plants that are usually visible near the end of stem elongation. However, fungicide treatment should be done at the beginning of stem elongation (BBCH 30–32) otherwise it is not sufficiently effective.

The incidence of both causal agents, their pathogenicity, life cycle, ability to adapt to selection pressures, and their sensitivity to fungicides have been studied in different countries, mostly in France and the UK. The changes in relative abundance of both species in connection with resistance to fungicides were studied by Leroux *et al.* (2012). In the early 1980s most strains belonged to the species *Oculimacula yallundae* in France. Then the relative abundance of *O. acuformis* gradually increased between 1985 and 1995. Since 1998, *O. yallundae* has become prevalent of the two species again in all French regions. The system of eyespot monitoring and management is well described in the UK. The epidemiological model for eyespot disease was developed and incorporated within The Agricultural Production Systems Simulator software (Al-Azri *et al.* 2015). *O. acuformis* isolates cause significant lesions on both wheat and rye, whereas *O. yallundae* isolates are more virulent on wheat (Lucas *et al.* 2000). Both pathogens can co-exist on one stem and can cause symptoms of eyespot that are visually indistinguishable on various plants of the family *Poaceae* (Fitt *et al.* 1987). No extensive study of *Oculimacula* populations has been done in the Czech Republic.

There are several known sources of resistance to eyespot. The most effective and also the most widely used resistance gene in commercial cultivars is *Pch1,* a single major gene mapped to the distal end of the long arm of chromosome 7D (Wei *et al.* 2011). It was transferred to wheat from *Aegilops ventricosa* (Maia 1967). The deleterious traits carried on the *Ae. ventricosa* introgression cause a yield penalty in the absence of the disease (Koen *et al.* 2002). The *Pch2* gene confers a moderate level of resistance. It was originally identified in the old French cv. Cappelle-Desprez, the first commercial cultivar resistant to eyespot. *Pch2* is located on the long arm of chromosome 7A (de la Peña *et al.* 1996). Although *Pch2* was found to confer resistance against both pathogen species (Klos *et al.* 2014), it was significantly less effective against penetration from *O. yallundae* than *O. acuformis* (Burt *et al.* 2010). Another resistance gene present in commercial cultivars is *Q.Pch.jic-5A*. It is located on chromosome 5AL of cv. Cappelle-Desprez. *Q.Pch.jic-5A* confers a moderate level of resistance at both seedling and adult plant stages (Burt *et al.* 2011). Additional quantitative resistances on chromosomes 1A, 2B, and 5D are known from cv. Cappelle-Desprez (Law *et al.* 1975). Few wheat cultivars from Germany (Lind *et al.* 1994) and from the Pacific Northwest (Peterson *et al.* 1974; Jones *et al.* 2000) were reported to show moderate resistance to eyespot apparently not inherited from cv. Cappelle-Desprez. Börner *et al.* (2006) detected resistance among Gatersleben genebank accessions of wheat collected from 1933 to 1992. These accessions may carry already known genes/alleles as well as new loci. Burt *et al.* (2014) obtained resistance suitable for introgression into elite wheat cultivars in a study of the collection of hexaploid wheat landraces assembled by the British botanist A. E. Watkins in the 1920s and 1930s from 32 countries across the world. A potential source of moderate resistance is a single dominant gene *Pch3*, located on the distal part of the long arm of chromosome 4V of *Dasypyrum villosum* (Yildirim *et al.* 1998). Multiple resistance genes are present on chromosomes 4V and 5V of *D. villosum*. Resistance to eyespot was identified also in other wheat relatives including *Triticum tauschii*, *T. monococcum*, *T. durum*, *T. dicoccoides*, *T. turanicum*, *Thinopyrum ponticum*, *Th. intermedium*, *Aegilops longissima*, and *Ae. kotschyi* (Cadle 1997; Figliuolo *et al.* 1998; Cox *et al.* 2002).

The aim of this study was to observe the reaction of selected winter wheat cultivars grown in the Czech Republic to inoculation with *Oculimacula* spp. We focused on wheat cultivars possessing the resistance gene *Pch1* and evaluated the influence of *Pch1* on infection by eyespot under field conditions. Samples of winter wheat stem bases and Czech *Oculimacula* spp. isolates were screened with molecular markers to find out the incidence of *O. yallundae* and *O. acuformis* in our population.

MATERIAL AND METHODS

Cultivar resistance. The reaction of nine winter wheat cultivars to inoculation with *Oculimacula yallundae* and *O. acuformis* was studied in a small plot trial in Prague-Ruzyně (Czech Republic) in 2014/2015 and 2015/2016. The set of tested cultivars included those registered in the Czech Republic, four of them (Annie, Hermann, Manager, Princeps) possessing the eyespot resistance gene *Pch1* according to the molecular marker *Xorw1* analysis (Dumalasová *et al.* 2015). In the second season, the new German cultivar Rebell was added because of its resistance

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to eyespot (*Pch1*). The inoculum for these trials was prepared from two isolates of *O. yallundae* and one isolate of *O. acuformis*. All isolates were obtained from South Moravia (Kroměříž, Czech Republic). The fungi were grown on sterilised barley grains. The inoculum was applied on experimental plots in November and April (40 g/m^2). The natural occurrence of stem-base diseases in six of these cultivars was assessed in a parallel field trial at the Úhřetice Breeding Station (Chrudim District, Czech Republic). Two experimental fields were used: the one had been sown with wheat for 1 year before, the other for 10 years. Moreover, the effect of seed treatment with Celest Extra Formula M (0.2 l/100 kg of seeds) was observed in both fields. The reaction of all cultivars was rated twice: for the first time at the growth stage of stem elongation (BBCH 32–33), when a 0 to 4 rating scale was used (0 – no symptoms, 1 – spot only on the outside of leaf sheath, 2 – spot on the second layer of leaf sheath, 3 – pathogen penetrates to the stem, spot covered by sclerotial cells, 4 – big spot with sclerotial cells); for the second time at the milk growth stage (BBCH 73–77) using a 0 to 5 rating scale (0 – no symptoms, 1 – one small spot, 2 – more spots covering maximally a half of the stem perimeter, 3 – spots covering more than a half of the stem perimeter, 4 – spot covering the whole stem perimeter, 5 – broken stem). In the field (Úhřetice), 25 randomly selected plants with 4 stems were assessed. In inoculated plots (Prague), 14 randomly selected plants with 4 stems were assessed. The data were analysed by the UNISTAT 6.5 package (UNISTAT Ltd., London, UK) and Statistica 12 (Statsoft, Tulsa, USA) – analysis of variance, Tukey's multiple comparisons test.

Sampling of stem bases and fungal isolates. A total of 468 samples of winter wheat stems were collected from the beginning of April to mid-July. Each sample was one stem base 50 mm long taken just above the soil surface. Stem segments were frozen at –30°C until DNA isolation. The samples for direct isolation were collected annually, mostly at the end of June. Fields were selected from a simple random sample of all winter wheat fields in the Czech Republic. Stem bases were cut from the infected wheat plants, surface-sterilised for 2 min in a 5% sodium hypochlorite solution and rinsed in sterile distilled water. The stem segments were then placed on commercial potato dextrose agar (PDA) containing ampicillin at a concentration of 50 µg/ml. Petri dishes were incubated at 18°C in darkness for 10 days. Developing colonies were transferred to new PDA plates for species identification by PCR.

Molecular determination of O. yallundae and O. acuformis. Infected stem bases and isolates were subjected to molecular diagnostics for the identification of *O. yallundae* and *O. acuformis*. Mycelia and plant samples were ground to a fine powder in a mortar using liquid nitrogen, homogenised and total genomic DNA was extracted using a Qiagen DNeasy® Plant Mini Kit. Species-specific primers were used as described previously for *O. yallundae* and *O. acuformis* (Nicholson *et al.* 1997). Each

Table 1. Reactions of winter wheat cultivars to inoculation with *Oculimacula* spp. (Prague-Ruzyně) in 2015 and 2016

2015								2016							
Cultivar	*Pch1*	(1)*	h.g.	cultivar	*Pch1*	(2)*	H.g.	cultivar	*Pch1*	(1)*	h.g.	cultivar	*Pch1*	(2)*	h.g.
Manager	+	0.00	a	Hermann	+	0.95	a	Hermann	+	0.00	a	Princeps	+	0.32	a
Princeps	+	0.00	a	Annie	+	1.64	ab	Annie	+	0.00	a	Manager	+	0.62	ab
Hermann	+	0.00	a	Princeps	+	1.86	b	Manager	+	0.00	a	Hermann	+	0.75	ab
Annie	+	0.05	a	Manager	+	2.16	bc	Princeps	+	0.04	a	Annie	+	0.82	ab
Bohemia	–	1.00	b	JB Asano	–	2.75	cd	Rebell	+	0.13	ab	Rebell	+	1.20	b
Turandot	–	1.13	b	Cubus	–	3.21	de	Bohemia	–	0.30	ab	Cubus	–	2.93	c
JB Asano	–	1.45	b	Potenzial	–	3.43	de	Cubus	–	0.68	b	JB Asano	–	3.16	c
Potenzial	–	1.59	b	Turandot	–	3.59	e	Turandot	–	1.48	c	Potenzial	–	3.23	c
Cubus	–	1.61	b	Bohemia	–	3.64	e	Potenzial	–	1.50	c	Bohemia	–	3.25	c
								JB Asano	–	1.80	c	Turandot	–	3.59	c

Pch1 – eyespot resistance gene (+ cultivar with *Pch1*, – cultivar without *Pch1*); *(1) = the first assessment, (2) = the second assessment; h.g. (homogeneous groups) – ANOVA, multiple comparisons by Tukey's method were used (P = 95%), the means in columns followed by the same letter are not significantly different from each other

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Table 2. Reactions of winter wheat cultivars to natural infection with stem-base diseases in field conditions (Úhřetice) in 2015 and 2016

2015								2016							
Cultivar	*Pch1*	(1)*	h.g.	cultivar	*Pch1*	(2)*	h.g.	cultivar	*Pch1*	(1)*	h.g.	cultivar	*Pch1*	(2)*	h.g.
Bohemia	–	0.47	a	Hermann	+	0.49	a	Hermann	+	0.45	a	Hermann	+	0.75	a
Hermann	+	0.49	a	Princeps	+	0.75	b	Annie	+	0.65	b	Rebell	+	0.84	a
Turandot	–	0.53	a	Annie	+	0.91	b	Bohemia	–	0.74	bc	Annie	+	0.94	ab
Annie	+	0.57	a	Turandot	–	0.98	bc	Rebell	+	0.75	bc	Cubus	–	1.14	bc
Princeps	+	0.59	a	Cubus	–	1.20	c	Turandot	–	0.85	c	Turandot	–	1.20	c
Cubus	–	1.80	b	Bohemia	–	1.46	d	Cubus	–	0.86	c	Bohemia	–	1.29	c

Pch1 – eyespot resistance gene (+ cultivar with *Pch1*; – cultivar without *Pch1*); *(1) = the first assessment, (2) = the second assessment; h.g. (homogeneous groups) – ANOVA, multiple comparisons by Tukey's method were used (P = 95%), the means in columns followed by the same letter are not significantly different from each other

reaction contained extracted stem base DNA, a negative control and a positive control of purified DNA from the mycelium of each fungal species. Aliquots (10 µl) of amplification products were electrophoresed through 1.7% agarose gels prepared using TAE buffer and stained with ethidium bromide. Differences in the frequency of positive or negative DNA detections in samples were evaluated using the Chi-squared test.

RESULTS

Cultivar resistance. Statistically significant differences in the reaction of the tested cultivars were found at both locations in 2015–2016 (Tables 1 and 2). Generally, cultivars that possess the eyespot resistance gene *Pch1* had statistically less severe disease symptoms than those without *Pch1*. Symptoms on resistant cultivars with *Pch1* were mostly nonspecific necrotic spots on stem bases that differed from typical elliptical eye-shaped spots. These typical symptoms on cultivars possessing *Pch1* were observed very rarely and only in the inoculated plots with *Oculimacula* spp. in Prague.

The highest level of resistance to eyespot was detected in the cultivar Hermann in Úhřetice in both years and in Prague in 2015. The highest mean level of disease in cv. Hermann was 0.95 in Prague on

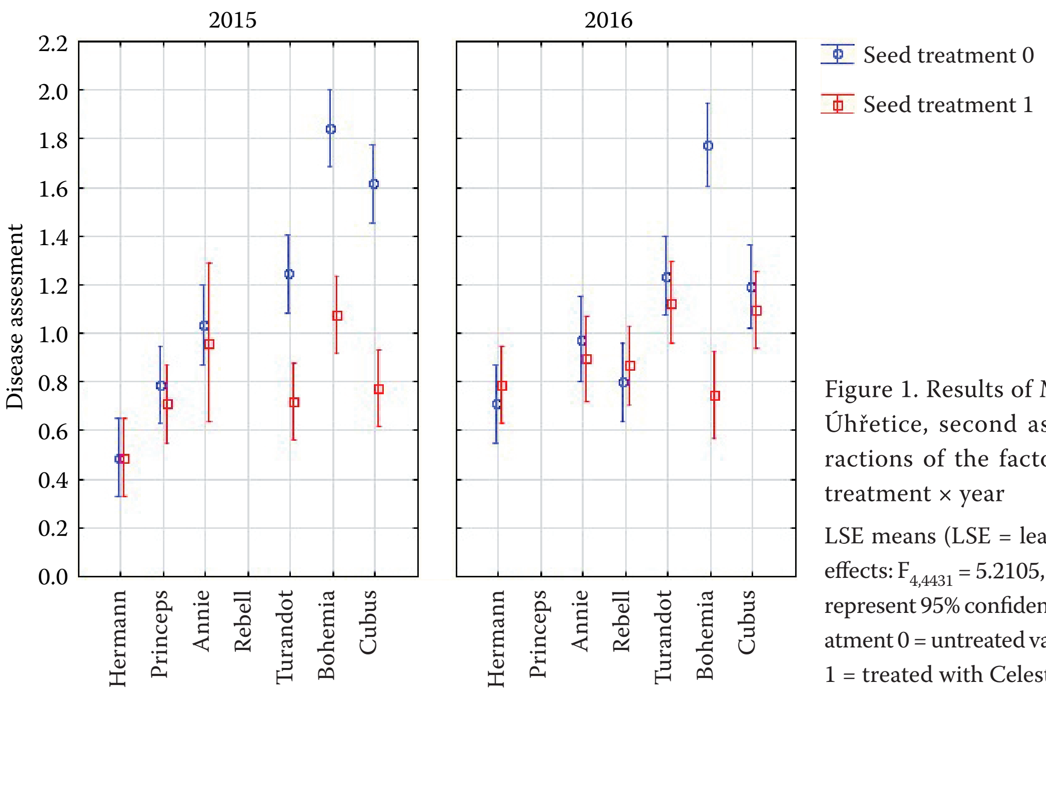

Figure 1. Results of MANOVA (locality Úhřetice, second assessment) – interactions of the factors cultivar × seed treatment × year

LSE means (LSE = least square errors), all effects: $F_{4,4431}$ = 5.2105, P = 0.00035, verticals represent 95% confidence intervals; seed treatment 0 = untreated variant; seed treatment 1 = treated with Celest Extra (see above)

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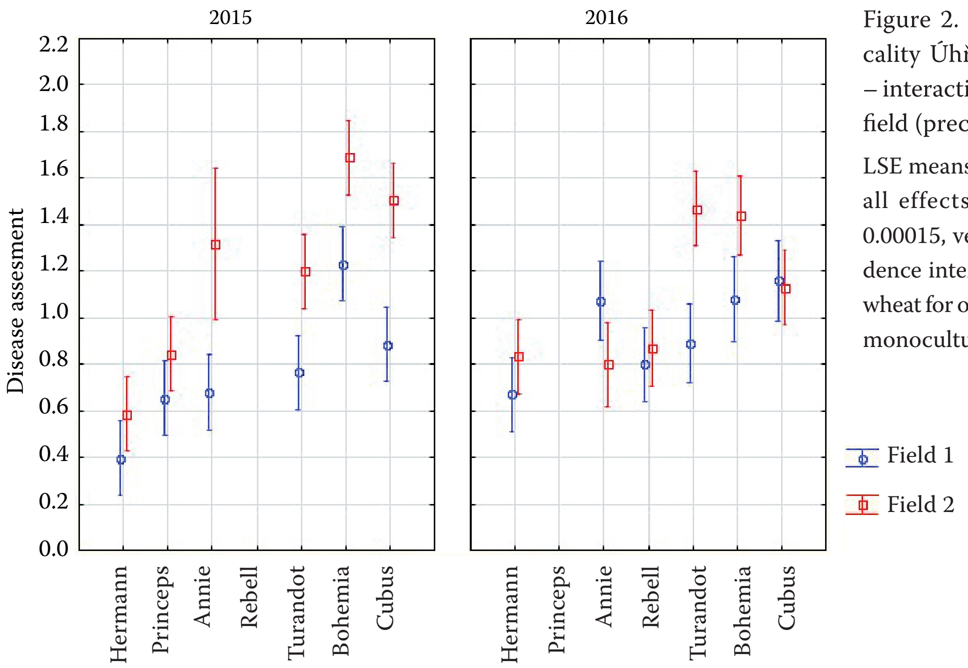

Figure 2. Results of MANOVA (locality Úhřetice, second assessment) – interactions of the factors cultivar × field (preceding crop) × year

LSE means (LSE = least square errors), all effects: F(4,4431) = 5.6813, P = 0.00015, verticals represent 95% confidence intervals; field 1 = field sown by wheat for one year before; field 2 = wheat monoculture for ten years

inoculated plots in 2015 (Table 1). The cultivars Princeps and Manager were less affected by eyespot than cv. Hermann in Prague in 2016. The second mean assessment of cv. Princeps was only 0.32 on inoculated plots. The most susceptible reaction to *Oculimacula* spp. was observed in cultivars Bohemia and Turandot on the artificially infected plots in Prague. Cv. Bohemia reached 3.64 in the second assessment in 2015 and 3.25 in 2016. The mean level of the disease in cv. Turandot was 3.59 on inoculated plots in both years. Bohemia was the most susceptible cultivar to stem-base diseases in the field in Úhřetice in both years but the values of disease symptoms was relatively low – 1.46 (2015) and 1.29 (2016).

There were also differences between the two assessments (Tables 1 and 2). Especially cv. Bohemia had a low level of the disease at the stem elongation stage and it was the most affected cultivar at the milk growth stage in both seasons. The statistical analyses by MANOVA (factors: cultivar, seed treatment, preceding crop, year) proved significant differences between cultivars and significant interactions between some factors in naturally infected fields in Úhřetice. The seed-treated variants were less affected by stem-based diseases than the untreated ones, except the cultivars Hermann and Rebell (Figure 1). In 2015, the effect of seed treatment was most important in cultivars without resistance gene *Pch1*. In 2016, this hypothesis was true only in the cultivar Bohemia. Significant differences between two experimental fields with different preceding crops were found (Figure 2). The field with wheat monoculture maintained for 10 years had more disease than the field that had been sown by wheat only for two years, with the exception of the cvs Annie and Cubus in 2016.

Table 3. Frequencies of *Oculimacula acuformis* (Oa) and *O. yallundae* (Oy) on the wheat stems

Oy \ Oa	No	Yes	Total
No	426 (91.03%)	12 (2.56%)	438 (93.59%)
Yes	12 (2.56%)	18 (3.85%)	30 (6.41%)
Total	438 (93.59%)	30 (6.41%)	**468 (100%)**

Chi-squared test: χ^2 = 153.44 (P = 0.000), r = 0.57 (P = 0.000); P-values lower than 0.05 refer to 95% confidence level

Molecular determination of O. yallundae and O. acuformis. There were 6.41% of *Oculimacula* spp. positive samples using PCR from the total of 468 collected wheat stems. *Oculimaculla yallundae* was detected in 12 samples alone, *Oculimacula acuformis* in another 12 samples alone (Table 3). Both species were detected together in the same stem in 18 samples. The results of the Chi-squared test demonstrate that both *Oculimacula* species occurred more often together in the same stem than separately (χ^2 = 153.44, P = 0.000) in our samples. Pearson's r = 0.57 (P = 0.000) expresses a close correlation.

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DISCUSSION

The current eyespot resistance genes do not provide any complete disease control, nevertheless, the difference between the amount of the disease of genotypes carrying *Pch1* and genotypes without *Pch1* was obvious in this study. The level of protection provided by *Pch1* is not 100% on inoculated plots, however, the lower level of eyespot incidence on cultivars containing the *Pch1* gene is statistically significant. Few contradictory results were reported regarding durability of resistance conferred by *Pch1* (Wei 2011). Increases in yield loss of cv. Madsen, which carries the *Pch1* gene, were observed over a 12-year period (Jones *et al.* 1995), whereas no isolate of the pathogen obtained from France was found virulent on *Pch1*-carrying genotypes (Saur & Cavelier 1995).

Our current survey of the *Oculimacula* spp. population has shown that both species are often found coexisting on the same wheat stem and the frequency of both species is similar in the Czech Republic. This fact can complicate the management of eyespot disease because of different resistance of both *Oculimacula* species to some fungicides. In the study of Ramanauskiene and Gaurilčikiene (2016) all 122 samples of cereal stem bases in Lithuania were colonised by both species *O. yallundae* and *O. acuformis* together except for one example. Previously Leroux and Gredt (1997) found that *O. yallundae* and *O. acuformis* co-occurred on up to 56% of the stems in a survey of wheat crops in France. Bierman *et al.* (2002) described a stable coexistence of about 50% of both species in unsprayed and carbendazim sprayed plots for five seasons in the UK.

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