

Session 3

Reflections and projections on a decade of aerobiological studies on British and Polish phoma stem canker disease of oilseed rape caused by *Leptosphaeria maculans*

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Introduction: Airborne fungal propagules can be readily measured when captured on rotating sticky tapes within Hirst-type volumetric spore traps. In the UK, at Rothamsted Research, the dynamics of such fungal ascospores and conidia have been monitored since the 2001 cropping season. In Poland, the SPEC project (www.spec.edu.pl) has operated, since 2004; Burkard and Lanzoni spore traps were situated near winter oilseed rape fields in 10 climatic zones of Poland for seasonal monitoring of airborne spore concentrations. Our collaboration has involved a sustained annual processing of tapes for spore counts followed by DNA extraction to enable the determination of pathogen species (*Leptosphaeria maculans* and *L. biglobosa*, associated with phoma stem canker, and *Pyrenopeziza brassicae*, the cause of light leaf spot) in air samples by both endpoint and quantitative Real-Time PCR, as well as LAMP, approaches. In addition, our collaboration has also evaluated extracted DNA samples for some pathogen avirulence allele combinations and for monitoring molecular changes in fungicide targets. We have since progressed beyond molecular aerobiology into exploring environmental and medical implications of airborne allergenic *Leptosphaeria* antigens and examined spatial aerobiology by determining the back-trajectory patterns of the movement of ascospores of species of *Leptosphaeria* in historical turbulent air currents across regional and national boundaries in Northern Europe. We are currently exploring cutting-edge biometric and biostatistical methods, backed with meteorological data, for interpreting the role of terrain and topography in the aerial spread of the inoculum of these destructive pathogens across geographical regions with the aim of achieving a better prediction, diagnosis and control of the diseases of oilseed rape (*Brassica napus*).

Materials and Methods: Samples of DNA extracted (CTAB method) from one half of tapes in volumetric spore traps from sites in Poland, and from Harpenden, UK, were used for these studies. The other half of the tapes was stained with Trypan Blue and examined daily with a light microscope for the abundance and frequencies of ascospores of *Leptosphaeria* species. Species-specific primers targeted against fragments of *internal transcribed spacer regions of ribosomal RNA (ITS)* or *β-tubulin* genes from *L. maculans* or *L. biglobosa* were used for PCR diagnostics and quantification with chemistries based on either the SYBR Green or dual-labelled fluorescent probes. The abundance and frequencies of *Pyrenopeziza brassicae* propagules was also determined. Primers designed for fragments of virulence alleles *avrLm1* and *avrLm6* as well as fragments of the membrane biosynthetic gene *erg11* (*ergosterol demethylase*) from *L. maculans* were further employed to characterise propagules captured on tapes from both countries. Furthermore, sequence alignment and bioinformatic comparisons of aeroallergenic proteins from Polish (ex Szczecin) and British (e.g. Worcestershire) *Leptosphaeria* ascospores were carried out against those from spores of proven allergenic fungal species such as *Aspergillus fumigatus*, *Cladosporium herbarum*, and *Penicillium citrinum*. This was done to relate the contribution of the annual massive discharge of *Leptosphaeria* ascospores into autumnal air to asthma and respiratory discomforts in general.

Results: Dual-labelled fluorescent probes proved to be more efficient than SYBR Green-based primers in the qPCR determination and identification of DNA from the fungal DNA associated with phoma stem canker disease of *B. napus*. For both countries, *avrLm1* and *avrLm6* alleles were easily detected in spore populations thereby

enabling characterisation of aerial *L. maculans* propagules at the race level even from the minute amounts of fungal DNA obtainable on segments of spore tapes. The ergosterol biosynthetic gene *erg11* was easily monitored over the evaluation period in samples from both countries. Comparative bioinformatics suggested strongly the allergenic propensities of surface proteins from the ascospores of *Leptosphaeria spp* thereby implicating these pathogens in autumnal respiratory challenges, given the relative aerial abundance.

Conclusions:

- Quantitative Real-Time PCR remains a reliable and increasingly affordable means of precise measure-

ment of DNA extracted from spores captured in air even though different methodological approaches exist.

- Pathogen DNA from spore tapes can be further characterised using molecular markers for phenotypes such as virulence, pathogenicity and fungicide resistance. The data generated are amenable to further robust interpretations with rapid advances in bioinformatics and modelling.

Concentration of ascospores of *Leptosphaeria maculans* and *L. biglobosa* in Poland in autumn 2015-2017

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Introduction: Poland is an economically important producer of oilseed rape. Nowadays, the intensive cultivation of crops creates some undesirable side effects such as the risk of the disease development, resulting from the increase in the amount of inoculum of certain pathogens. Among the numerous fungal diseases of oilseed rape, phoma stem canker is the most damaging one. It is caused by the *Leptosphaeria maculans*/*L. biglobosa* species complex. The disease may lead to considerable yield losses. The pathogens survive on debris from the previous season crop. Epidemic events are initiated in autumn by airborne ascospores released from the infected stubble. Wind-borne ascospores adhere to the leaf surface and germinate to produce germ tubes, which penetrate the leaf indirectly through stomata or wounds. In Europe the control of severe phoma stem canker epidemics relies on the application of fungicides. However, the effectiveness of fungicide application depends on the season and region. Applications may be mistimed or unnecessary and contribute to economic and environmental costs. More accurate targeting of pesticide sprays can help to maintain the profitability and sustainability of winter oilseed rape. The aim of our

work was to use aerobiological tools in the integrated pest management to control the disease.

Methods: Seven (2017) or eight (2015, 2016) Burkard 7-day volumetric spore samplers were installed in regions with the most intensive cultivation of oilseed rape and they were used for the monitoring of the concentration of *L. maculans* and *L. biglobosa* ascospores. To simulate and standardize field conditions, each spore trap was surrounded by the oilseed rape stubble from the previous season crop, infected by *Leptosphaeria spp*. Each year, the monitoring of ascospores release began on 1 September and ended in late November. The number of spores present per unit volume of air (m³) was calculated for each sampling day.

Results: The microscope observations of the number of *Leptosphaeria spp.* ascospores showed great differences between the geographical locations and years. A start of the season was defined as detection time of the first ascospores in the air, captured by the spore sampler. The earliest detection of the first ascospores was at the beginning of September, and at most sites and over most seasons, the ascospores were first observed in September. The number of days with

ascospores ranged from 26 in eastern Poland (Krasne, 2016) up to 76 (Radostowo, 2015). Large differences of the number of days with airborne ascospores were also found between seasons. The highest differences were found in Glebokie (from 41 days in 2016 to 72 days in 2015). The highest peak concentration of ascospores in 1 m³ of air ranged from 9 (Rarwino in 2016) to 45 (Charbielin in 2017) spores. Long lasting ascospore showers with numerous spores in air samples led to high cumulative daily mean of spore concentrations. The extreme differences in the number of ascospores collected in one cubic metre during autumn seasons

ranged from 39 spores/m³ observed in Krasne in 2016 to 339 spores/m³ in Charbielin during the same year.

Conclusions: Collected data indicated that time of the first ascospore release, the date of maximum spore concentration in the air and the quantity of spores differed among climatic regions and seasons. These parameters highly depended on the acreage of oilseed rape (the source of infected stubble), cultivars (resistant vs. susceptible) and meteorological conditions. To reduce the need for regular disease assessment, the decision support systems can be employed to forecast the disease progression and help timing of fungicide applications.

Modern tools for tracking of fungal spore transport in air masses, using *Leptosphaeria* spp. as an example

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Leptosphaeria maculans and *L. biglobosa* are fungal pathogens causing stem canker or so called blackleg disease in Brassicaceae. Due to the significant economic losses caused in Europe, North America and Australia an aerobiological monitoring of ascospores is being conducted. Information about the spore levels in the air and identification of the potential location of inoculum sources can greatly help to predict the exact time of the fungicide application and possible further span of the disease they cause. Here we present a combined source-receptor study on *Leptosphaeria* spp. spores by using the HYSPLIT model in combination with novel source maps by using a GIS framework. Observations were obtained with a 7-day volumetric spore for the period 2006-2010 from Szczecin, Poland. We identified two events of the long distance transport of fungal spores, which suggested that the major sources of *Leptosphaeria* spp. can be mainly found either in Northern Germany or Southern Denmark. Our study also suggested that some fractions of the ascospores may directly come from the application of the harvesting machines rather than natural processes of the spore release from fungal fruiting bodies. This has particular

relevance in relation to air quality during late summer and health as most people that are sensitive to fungal spores today live in the urban environment. However, the main aim of this study was to show the evidence for an eastward spread of the stem canker disease in Europe.

Moreover, a joint Polish-Ukrainian-British team of researchers performed a thorough study on the epidemiology of stem canker. The aim of this work was to pinpoint the most crucial weather parameters that are useful to build a forecast model allowing to evaluate the risk and to plan the appropriate plant protection measures. For the first time, the study compared real field situations at four locations across Europe, from humid British Island, through the region of intensive oilseed rape cultivation in West Pomerania of Poland (Central Europe, under the influence of the Baltic Sea) to continental and steppe regions of Ukraine (the new, fast expanding producer of oilseed rape in East Europe). The areas in Ukraine have never been subjected to such studies and have never been compared to the other sites of Europe or elsewhere. The study has started from the unification of monitoring methods and

self-training of all teams via the internet and personal trainings, to standardise the spore counts. In such way we have avoided false results concerning the amounts and variability of *Leptosphaeria* ascospores. In our study, 150 forecast models have been produced using Artificial Neural Networks (ANNs) and Multivariate Regression Trees (MRTs). Then, the best model was selected for each studied location, as well as for joint two-site combinations. Special emphasis was given to Dew Point Temperature. Although, both the temperature and relative humidity were also taken into consideration, we have hypothesised that the DPT parameter, which

is a combination of both, would play a key role in the disease development. The hypothesis was based upon the knowledge of the pathogen's biology. Based on previous studies, done by the other authors, it is known that the maturation of pseudothecia (teleomorphs of the fungus) takes place on oilseed rape stubble from the preceding growing season, and it is tightly connected with the moisture of plant stems. We have confirmed the impact of DPT; all MRT analyses with only one exception indicated its importance and half of them ranked DPT as a fundamental factor.

Molecular detection of *Ramularia collo-cygni* – the endophyte and pathogen of grasses

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Introduction: The symptoms of *Ramularia* Leaf Spot (RLS) were first recorded over 100 years ago in Italy, when the causal agent – the fungus *Ramularia collo-cygni* (Rcc) – was found on the leaves of barley. Mostly, the fungus was detected as an endophyte of barley, wheat as well as fodder and meadow grasses. The endophytic phase is usually asymptomatic until late in the growing season. The symptoms of the appearance of RLS are rectangular reddish-brown, square necrotic spots surrounded by a chlorotic zone. The spots are visible on both sides of the leaf blade. In the advanced stage, premature ageing of the leaves is observed. Rcc in its pathogenic stage leads to substantial loss of assimilation surface, resulting in the reduction of the seed yield and quality. The red colour of grass leaves is related to the production of anthraquinone toxins, referred to as rubelins. These toxins induce photodynamic necrosis of the leaf tissue. From the 1980s, the pathogen has greatly increased its distribution and impact. Reports indicate its occurrence in Europe, Canada, South America, South Africa and New Zealand. The development of molecular diagnostics has made it possible to detect Rcc and make it easier to distinguish RLS from other barley diseases, many of which have leaf blotch forms. A number of studies confirmed that the mycelium of Rcc moves from the infected seed to the developing plant tissue. Spores

may play an important role in a disease transmission between winter and spring cereals and in (increasing seed borne fungal levels?). It was found that the release of spores from winter and spring crops was related to high levels of the disease in both crops.

Materials and methods: The shape of Rcc spores is typical for several fungal species, making the visual estimation of Rcc in the environment, laborious, cumbersome and prone to significant errors. In the UK and Poland, these problems has been overcome by the use of aerobiological sampling methods (Burkard volumetric trap) combined with molecular tools. The DNA present in Rcc spores has been monitored with a quantitative PCR utilising TaqMan probes.

Results: The monitoring exercise showed that the concentration of Rcc spores in Poland is relatively low, as compared to the spores of *Cladosporium*, *Alternaria* or others, such as *Leptosphaeria*. The initial source of inoculum in the crop is most likely seed infection and the occurrence of spores in the later stage of the plant development may not contribute to the ultimate severity of RLS symptoms on plants. The highest concentration of Rcc spores was detected in the northern part of Poland in Radostowo (53°59,457'N, 18°43,781'E), near Zulawy Wislane. This is an area of high air humidity (with growing conditions?), similar to Scotland. In Scotland,

the major environmental driver of spore release was high levels of surface wetness in the crop. In Poland, the DNA present in *Rcc* spores was observed from April to June.

Conclusions: Both in Scotland and in Poland, it has been shown that *Rcc* DNA can be detected even in regions where the severity of RLS is negligible. Other studies have shown that *Rcc* can produce an *Astero-*

mella-type structure on the barley straw. The function of these structures has not been fully elucidated, but there is the preliminary evidence that it may be a place of sexual recombination of the pathogen and ascospore production. However, the molecular detection does not allow to distinguish asexual and sexual stages of this fungus.

Meteorological conditions at different troposphere levels during extremely high concentrations of *Alternaria* and *Cladosporium* spores

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Introduction: *Alternaria* and *Cladosporium* encompass at least several hundred of known species. Spores of these taxa commonly occur in the air almost all over the year and are considered to be the most important fungal inhalant allergens. The behaviour of spores in the air is governed by both local and large-scale weather conditions. **However, apart from long distance transport studies, the higher atmospheric levels are rarely analyzed in aerobiological studies.** The main objective of this study was to identify synoptic- (at different troposphere levels) and local-scale meteorological factors associated with extremely high concentrations of *Alternaria* and *Cladosporium* spores.

Methods: Airborne spores were collected by the volumetric trap of the Hirst design between 2000 and 2016 in Poznań, Poland. The trap was situated on the roof at a height of 33 m, approximately 1 km away from the city centre. *Alternaria* and *Cladosporium* spores were counted under the light microscope and then their hourly values were recalculated per 1 m³ volume of air. Extreme daily and hourly values were selected on the basis of probability criteria, i.e. the highest 5% and 1% of data and named as “high” and “extreme” respectively. We also assessed the timing of sudden changes in hourly concentrations by searching for an inflection point on the curve fitted to the data on

days with the largest intradiurnal spore concentration differences (5% of days). Different meteorological data were considered: (1) synoptic, large scale patterns of sea level pressure, 500 hPa geopotential height, air temperature recorded at 850 hPa level and precipitable water contained by troposphere column (2) local meteorological data obtained at Poznań weather station: hourly air temperature, relative humidity, wind speed and direction, cloudiness, precipitation, cloud types and ground temperature.

Results: The sudden increase in spore concentration during a day was very often associated with the occurrence of cumuliiform convective clouds. Even several *Cumulus humilis* clouds (the smallest cloud in *Cumulus* genus – a “fine weather cloud”) indicated increasing hourly spore concentration. If precipitation was recorded during the presence of convective clouds, the spore concentration remained mostly unaffected. During episodes with high spore concentrations the sea level pressure anomalies (compared to the summer mean) were positive over Finland and Baltic countries (up to 3.3 hPa) and negative over Germany and British Isles (-2.4 hPa). Positive anomalies of 500 hPa geopotential height occurred in Central and Eastern Europe (up to 84 m a.s.l.) but this was not enough to markedly change the average conditions in the middle troposphere. The

effect of local and synoptic conditions on the extreme level of spores showed the similar pattern in relation to both studied fungi taxa.

Conclusions: In this study we combined local, hourly meteorological data with large scale synoptic patterns to detect possible triggers of high/extreme airborne spore concentrations. Synoptic data revealed that the anticyclonic weather with mild positive changes of 500 hPa geopotential height favours high and extreme spore concentrations. High hourly concentrations are possible not only during a stable weather but also when

it is more dynamic. Our study supports the previous findings that the spore concentration increases before a thunderstorm events connected with *Cumulonimbus* convective clouds. In addition, for the first time, it documents that the appearance of *Cumulus humilis* clouds, which are signs of the free convection, can result in the sudden increase in spore concentrations.

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