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Molecular and traditional tools in tracking spores, genes, and toxins that cause economic loss in crops

Abstract: Fungal pathogens such as Fusarium graminearum and Leptosphaeria maculans are significant worldwide agricultural crop pathogens in wheat (Triticum aestivum) and canola (Brassica napus), respectively. In addition, their virulence and host ranges have steadily increased over time, despite mobilization of significant efforts to control their spread and damaging effects on crop yield and end-use quality. Therefore, understanding the mechanisms in which they are transmitted at the plant, field, regional, and continental levels is crucial to provide both farmers and researchers with appropriate disease management strategies and cropping systems recommendations. F. graminearum (sexual state = Gibberella zeae) is dispersed by seed, crop residues, and the more recently studied mechanism of aerial spores. Traditional spore collection, identification and characterization tools are giving a way to more sophisticated apparatus', serving to decrease turnaround times on sample processing and increase the precision of pathogen tracking and genotypic characterization. This article broadly reviews a selection of novel applications of aerial spore collection and population mapping tools in F. graminearum (G. zeae) and L. maculans - both traditional and molecular - which demonstrate recent innovations in agricultural plant pathology.

It is with a heavy heart that one acknowledges the role of an agricultural plant pathologist to have been, purely and traditionally, observatory and reactionary; an emergency worker facing an endless cue of unpredictable fires. The entire discipline has been driven by epidemic agricultural catastrophes: infamous examples include potato late blight (caused by *Phytophthora infestans*) in Ireland and the depression-era wheat leaf rust epidemics in North America's Puccinia pathway [1], and more recently, the ravages of blackleg, or phoma leaf canker of canola caused by *Leptosphaeria maculans* in Australia [2] and western Canada [3], and the virulent Ug99 race of *Puccinia graminis*, causing stem rust in wheat on a potentially global scale [4] . This brief review article will use examples of identifying, tracking, and quantifying of selected fungal pathogens and their impacts on North American agriculture. We will show how and why novel applications of sometimes eccentric technologies are necessary for moving the discipline of plant pathology from that of a reactive science to one which informs about the structure and appearance of cropping systems into the 21st century [5].

As food, feed, and increasingly energy preferences ebb and flow, so do farmers' cropping practices. Increased market liberalization, access to global trade, and the associated political policies have often pressured and/or enticed first-world agricultural producers into crop production systems which, in the light of sound agronomic and cultural practices, are at best a compromise, and at worst, a disaster in waiting [6]. Political intervention in developing world agricultural and agri-food systems often pressures subsistence producers into the same scenario, albeit for different and more pragmatic reasons. These pressures, when combined with ever-present non-agricultural development, have expanded arable lands into what previously may have been deemed "unsuitable" or "inappropriate" for sustained cropping and grazing ventures [7]. Typically, these developments see similar trends to those already under cultivation, where new crops and crop types routinely displace natural and traditional agricultural fauna, leading to increased cropping intensities, simplified crop rotations, and homogeneity of crop cultivars. One must note that, above all else, these changes take place at rate orders of magnitude greater than that under which traditional host-pathogen interactions, or "pathosystems", have developed [8]. The manufactured evolution of agricultural ecosystems has along the way forgotten to inform it's resident and transient pathogens that the end goal would no longer be persistence and succession, but rather production of anthropocentric end-use products in grains, oilseeds, pulses, and horticultural crops [5].

Prediction rather than reaction to epidemic development is as preferable in agriculture as any other sub-discipline of epidemiology. It shares the same fundamental difficulties in developing predictive host-pathogen and system-level models: Outbreaks can occur unexpectedly, their spread varies greatly with weather, climate, and proximity to other hosts, and sudden changes in the virulence or range of pathogen population [9]. Agricultural epidemio-

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logy, however, is uniquely susceptible to these vacillations due to the aforementioned structural instability of contemporary cropping systems [10]. Suitable hosts are often present across large, functionally homogenous areas of similar crop types and cultivars, in relatively similar growth stages, or, from the perspective of a pathogen, temporal levels of susceptibility [11]. The plant pathologist thus plays an integral role in informing the larger agricultural community of how present cropping systems, crop types and cultivars can be managed under constantly evolving pathogen pressures, then extrapolating how present agricultural practices at the field, regional, and global levels will influence pathogen population dynamics in the future [12]. This duality necessitates that the pathologist should be equipped with the tools to monitor and model pathosystems at all relevant levels - in the field, within a region, and across a continent - in order to develop productive and sustainable management strategies, now and for the future.

There is a rather standard process of understanding which influences research into aerially-dispersed agricultural plant pathogens. It is initiated with the identification of the pathogen in situ, and then correlating the presence of a damaging pathology with a causal agent, which, in the case of fungal pathogens, is usually a spore. One could say that the work of Pasteur (1861) lay the groundwork for this process by showing that air-borne sources of inoculum caused pathologies of formerly unknown origin. Succeeding seminal works by Gregory, Stepanov, Hirst, Edmonds, and Cox [13] all involved in characterizing that phenomena in basic terms relevant to agricultural production: when and under what conditions pathogens occur, upon which hosts, or crops, and lastly, why? What were the origins of these particles? What mechanisms facilitated their liberation, transit, and adhesion upon impact to hosts enabling their growth and, occasionally, reproduction? Although seemingly rudimentary, these questions are complex to resolve and are revisited with every change in cropping practice, climate norm, and pathogen virulence. Isard et al. [14] remarked that despite their ubiquity and significance, the study of biological organisms' movement via atmospheric pathways is largely not understood, and at present, this knowledge gap may be extended down to the ground level for many economically important crops.

It is here, at the ground or field level that most of the processes causing loss or contamination of crop production takes place. For research purposes, this is an infinitely more convenient environment in which to conduct observations and experiments on spore-disseminated fungal pathogens: It is upon primary and secondary, or alternate, hosts that the pathogen is at least temporarily in spatial stasis. Spore collection in the field ranges from simple, passive, and qualitative methods to more advanced active, time controlled, and electronically monitored quantitative apparatus. As we shall see, all have utility in contemporary plant pathology research.

Tracking microscopic spores within a crop can often be a difficult maneuver. Inferring their pathogenic effects upon a crop is even more challenging; if a regional agricultural landscape can be characterized as patchy - a mixture of fields, crops, soil types, often interspersed by non-agricultural wooded or grassy areas, waterways, and human settlements [6] - an analogous varied landscape, albeit with smaller features, can also be seen within a crop [15]. Thus, what variation one observes among different areas of a region, in terms of pathogen infection and crop damage, can also be quantified within an individual field.

An example of intra-field disease gradients can be found in Fernando et al. [15] while studying the sexual state of Fusarium graminearum (teleomorph = *Giberella zeae*), the causal agent of head blight in wheat and pink ear rot of corn. To infer the effects of different types of artificial inoculum upon the incidence and severity of *F. graminearum* infection in wheat, two types of active spore samplers were employed: a Burkard 7-day sampler was used in one site in a downwind direction immediately adjacent to the inoculated area, while in another ones, series of Burkard high-throughput jet spore traps with Fusarium-selective media were placed in a linear fashion downwind of the prevailing wind direction. Spore trap measurements were correlated with a two-dimensional matrix of wheat head samples which were assessed for both disease infection (number of heads infected per sampling area) and incidence (percentage of infected seeds per head). In addition to the novel matrix sampling, the researchers used a phenotypically unique race of the pathogen (showing yellow coloured mycelia) to avoid the possibility of exogenous inoculum sources confounding to their disease observations. Resultant data on spore production and disease infection were pooled to produce isopath contour maps, showing that the infection gradient of *F. graminearum* is reflective of spore deposition, and that prevalent downwind infections were higher than upwind, showing characteristic qualities of a monocyclic disease.

Spatially sensitive experiments as described above have initiated a cascade of follow-up research. Reflecting the growing consensus that cropping practices play a key role in the development and spread of a fungal pathogen, work has been done to model the effects of both crop rotation and tillage practices upon potential *F. graminearum* infection in subsequent wheat crops [16]. It was shown that cropping practices which left the larger amount of crop residue on the soil post-harvest (shorter or more intense crop rotations and decreased tillage) contributed to higher levels of Fusarium head blight in subsequent crops, and in another trial, that increased residue density facilitated further intra-field distribution of point-source inoculation with G. zeae clones [17]. Both of these artificially inoculated experiments exhibited higher levels of infection, with greater intra-field distribution than it would be expected using natural inoculum sources [18].

Now that a direct relationship between *F. graminearum / G. zeae* spore presence and subsequent host infection under both inoculated and natural circumstances, a higher elevation perspective was required: Where did non-resident aerially dispersed spores originate if not from a field in which the pathogen was present? When do they arrive, and under what conditions? And should the pathogen be resident in a field, what is the effective distance that spores can travel? The next natural research question is to begin identifying a pathogen in periods of transit, either from alternate to primary (in this case, agricultural crop) hosts, or in the case of polycyclic agricultural pathogens, from primary host to secondary via concurrent infection cycles within a growing season.

Schmale and Bergstrom [19] established aerial spore activity beyond an individual field by showing that viable spores of the corn ear rot pathogen *G. zeae* were deposited within corn canopies on a nightly basis during the susceptible silking (flowering) period. They accomplished this objective using the simplest of devices; selective media-filled petri dishes suspended upon pedestals. In a follow-up study using AFLP (Amplified Fragment Length Polymorphism) molecular techniques to characterize the genetic structure of the regional (New York state) *G. zeae* population, it was concluded that upon sampling both atmospheric and terrestrial populations and comparing them to known genetic lineages of *G. zeae*, they appeared to be one large continental interbreeding population [20]. Such genetic exchange by necessity infers previously-hypothesized long-distance aerial spore dispersal [21] for genetic transfer and recombination. This agreed with the conclusions of simultaneous work done on *Puccinia graminis*, the causal agent for stem rust of cereal crops [22]. In Oregon, U.S.A., researchers used a similar downwind spore trap arrangement

as Fernando et al. [15] in coordination with an air pollution model, and hypothesized that up to 12.5% of aerially dispersed spores of *P. graminis* were advected (distributed horizontally in air currents) 200 km away from their source.

Intercepting spores in transit or upon arrival is not the only evidence pointing out a long-distance pathogen dispersal by wind or other vectors. Wheat head blight caused by F. graminearum produces three strain-specific tricothecene mycotoxins which incite a number of metabolic pathologies when ingested by humans or livestock. These strain-specific toxins, or "chemotypes" (nivelanol (NIV), 3A deoxynivelanol (3ADON), and 15A deoxynivelanol (15ADON) [23] are presently undergoing a shift in population structure in the Canadian prairies from 15ADON to the more toxic 3ADON chemotype. After mapping province-wide populations in Manitoba, Canada using SRAP techniques of PCR products, researchers mused that the introduction of the 3ADON chemotype may have been caused by a "wheat seed shipment and long-distance spore transportation among different locations" [23].

If characterizing a pathogen's aerial spore dispersal and subsequent potential for infection and reproduction is challenging with two feet on solid ground, doing so in the air is dually so. The plenary boundary layer (PBL) consists of the biologically rich above-ground elevations between 50 and 1000m [11,24,25], in which several recent research efforts have used both aerial RPV (Remote Piloted Vehicles) and UAV (Unmanned Aerial Vehicles) equipped with spore collection media apparatus' to intercept and map aerial spore plumes [26-28]. Schmale [29] recently combined spore plume interception using multiple coordinated UAV's with tricothecene chemotyping described previously [23] to find that nivelanol (NIV)-producing strains of *F. graminearum* were suspended between 40 and 320 metres above ground, when no NIV--infected wheat plants had been observed in the region. This validates assumptions that long-distance aerial spore dissemination of F. graminearum (or for that matter, pathogens with other phenotypically similar aerial spores) can occur [30], and that this transport mechanism facilitates changes in pathogen population structure (as evidenced by genotype and chemotype).

Researchers now have at their disposal a multitude of aerial spore sampling mechanisms. Similarly rapid advancements in DNA-based identification technologies have created an entirely new toolbox for pathogen characterization beyond traditional microscopic methods [31]. Host-pathogen interactions of *Leptosphaeria maculans* (blackleg) with canola are mediated by gene-for-gene relationships [32], where modifications or deletions of *L. maculans* avirulence (Avr) genes will render the host crop with corresponding resistance (R) genes susceptible to infection. Using quantitative PCR (qPCR) techniques, Van de Wouw et al. [33] validated that variations in allelic frequency can be accurately quantified in L. maculans aerial spore populations, and that qPCR is a valuable mechanism for monitoring resident or transient pathogen population shifts. Similarly, Kaczmarek et al. [34] proved the potential of qPCR for differentiating between *L. maculans* and the phenotypically close *L. biglobosa* with speed and accuracy previously unattainable using microscopy and enzymatic assays.

When appropriate aerial sampling methodology and thorough, real-time genomic analyses are combined with comprehensive environmental data at the crop, field, and regional levels, the possibility of real-time pathogen monitoring and management recommendations becomes very realistic. Several recent reviews have comprehensively addressed the topic of integrating novel and near real-time monitoring tools with real-time crop protection technologies and decision-making tools [9,13,31,35,36]. Though noble in scope, they consistently fail to recognize that the fundamental structures of contemporary agricultural systems - larger, more homogenous and specialized cropping enterprises, reduced crop genetic variability, and input-dependent intensive cropping systems - have been the driving force behind the outcomes which prompt epidemiological research in the first place. Until the deeper understanding of plant pathogens in a global agricultural community is applied proactively to avoid, for instance, *F. graminearum's* extreme selection pressure and nearly unlimited host availability of the central USA, the discipline of plant pathology, and aerial spore research in particular, will fall far short of its true potential as an informant of long-term sustainably-structured agricultural systems.

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