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Advances in Plant Pathology

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Contributors

Muzafar Ahmad Sheikh, Teresa Coutinho, Lucy Novungayo Moleleki, Divine Yufetar Shyntum, Siphathele Sibanda, Jianfeng Gu, Luis Ignacio Cazón, Juan Andrés Paredes, Alejandro Mario Rago, Moussa El Jarroudi, Louis Kouadio, Jürgen Junk, Bernard Tychon, Mustapha El Jarroudi, Clive Bock, Philippe Delfosse, Manish Kumar, Pradeep Kumar, Mizuho Nita

Notice

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Meet the editor

Dr Josphert N. Kimatu is the director of Research, Innovation, and Technology and a senior lecturer at the School of Pure and Applied Sciences, Department of Biology, South Eastern Kenya University, Kenya.
He received his PhD degree in Plant Molecular Epigenetics from the Northeast Normal University (NENU) in Changchun City, China; his MPhil degree in Forestry Pathology from the Moi University, Kenya; his BSc degree in Botany and Zoology from the Moi University, Kenya; and his PGDE degree from the Maseno University, Kenya.
He completed his post-harvest management in maize, legumes, and rice certificate course in four modules from the Stellenbosch University in South Africa, the Sydney University in Australia, and the Kwame Nkrumah University of Science and Technology in Ghana in 2012. He studied agricultural education in Sweden and biodiversity at the Technical University of Munich in Germany. He is a Commission for University Education trained external reviewer of Kenyan universities.
He has undertaken research in over nine crops and fodder plants and is widely published in peer-reviewed journals, university-level book chapters, and three reviews in plant cytosine DNA methylation, plant polyploidy, and hybrid necrosis. Moreover, he has presented findings in seminars and workshops in Kenya, Uganda, the USA, and South Africa.

Contents

Preface

Plant pathology is the study of microorganisms that interfere with the normal metabolism of plants. It includes etiology, pathogenesis, epidemiology, and integrated pest management. Research in plant pathology has advanced from morphological and physiological analysis to more molecular studies involving interactions of gene products.

A plant is described as diseased when there are calculable deviations in physiology, structure, functions, and a deviation of its perceived economic value. Pathogens interfere with plants in many ways including obtaining nutrients, interfering with metabolic pathways, and through the production of metabolites, toxins, enzymes, etc. The diseased plant might slow down its water uptake, food translocation, mineral absorption, flowering, photosynthesis, fruiting, gaseous exchange, seed setting, food storage, respiration, and even its defense system.

This book combines the old approaches of plant-pathogen interactions with studies in molecular and physiological changes in plants, which have been triggered by climate change, increase in pesticide resistance, pathogen mutations, invasive species, plant biotic and abiotic adaptations, and plant breeding strategies. Recent advances in molecular biology and bioinformatics are enabling plant pathology to be studied using new technologies, such as RNAi, epigenetics, and nanotechnology. This book highlights modeling of plant diseases, quorum sensing, newly identified plant pathogens, counter defenses of plant viral pathogens, pesticide resistance and diagnostic approaches of plant diseases.

Scientists have recently discovered interesting plant interactions between plants and microorganisms in symbiotic relationships. These relationships involving endophytes and exophytes have enabled plants to survive and adapt in diverse environments.

Dr Josphert Ngui Kimatu, BSc., Mphil, PDGE, PhD
South Eastern Kenya University
Kitui County, Kenya

Section 1

Advances in Fungal Plant Pathology

Chapter 1

Modeling the Main Fungal Diseases of Winter Wheat: Constraints and Possible Solutions

Moussa El Jarroudi, Louis Kouadio, Bernard Tychon, Mustapha El Jarroudi, Jürgen Junk, Clive Bock and Philippe Delfosse

Additional information is available at the end of the chapter

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Abstract

The first step in the formulation of disease management strategy for any cropping system is to identify the most important risk factors. This is facilitated by basic epidemiological studies of pathogen life cycles, and an understanding of the way in which weather and cropping factors affect the quantity of initial inoculum and the rate at which the epidemic develops. Weather conditions are important factors in the development of fungal diseases in winter wheat, and constitute the main inputs of the decision support systems used to forecast disease and thus determine the timing for efficacious fungicide application. Crop protection often relies on preventive fungicide applications. Considering the slim cost–revenue ratio for winter wheat and the negative environmental impacts of fungicide overuse, necessity for applying only sprays that are critical for disease control becomes paramount for a sustainable and environmentally friendly crop production. Thus, fungicides should only be applied at critical stages for disease development, and only after the pathogen has been correctly identified. This chapter provides an overview of different weather-based disease models developed for assessing the real-time risk of epidemic development of the major fungal diseases (Septoria leaf blotch, leaf rusts and Fusarium head blight) of winter wheat in Luxembourg.

Keywords: mechanistic model, stochastic model, integrated pest management

1. Introduction

Plant disease epidemics involve changes in disease intensity in a host population over time and space. Acquiring comprehensive information on this process is necessary to understanding

the factors that cause epidemics. However, even a complete set of data on disease intensity does not automatically lead to insights into the epidemic process. Furthermore, the information regarding risk of disease needs to be communicated stakeholders who can subsequently take management decisions to protect the crop when risk of an epidemic is deemed high. Various mathematical models are used to summarize the essential features of the data or measurements of interest regarding disease development. Models for biological or physical processes can be developed using several methods. Empirical models are developed to describe an observed process, phenomenon, or relationship between variables using established statistical principles, and do not use previously developed theory or concepts to establish the relationship between the response variable and predictor variables. In contrast, mechanistic models are developed based on a theory, hypothesis, or concept of how a phenomenon or process occurs. Data are later considered after the mechanistic model is developed and might be used to improve the theory on which the model is based.

2. Challenges in predicting plant disease epidemic development

In many of the models that are discussed in this chapter, diseased individuals are grouped in three categories. After infection of the host takes place, the infected individual first goes through a phase where the disease develops and "grows" in the individual, but the infected individual does not produce propagules or infectious units. The infected individual is in a latent state. After the latent period, the infected individual becomes an infectious individual, meaning that it now produces infectious units that have the potential to cause subsequent infections. "Disease forecasting," "disease prediction," and the development of "disease warning systems" are activities familiar to plant disease epidemiologists [1–6]. Having identified the factors that lead to epidemics, it is of great importance to use this information to provide a basis for the management of plant disease. The level of disease risk to which a crop is exposed may be influenced by many factors, some of these are beyond the control of growers, but some factors are integral components of crop production systems and can be managed to minimize that risk.

2.1. Seasonality and the disease cycle

Many cropping systems are cyclical or seasonal. With annual plants, the crop is planted and harvested at specific times each year. Planting a specific (or a few) genotype(s) results in an abrupt increase in population of susceptible individuals. While harvesting immediately decreases both the population of susceptible individuals and the population of latent, infectious individuals. In the period between harvest and planting, the pathogen has to survive either as propagules or on living or dead plant material left in the field, in the soil, or in other locations. Crops are exposed to a risk of infection from pathogens, the outcome of which is economic loss when the epidemic increases above a certain threshold, which results from reduction in both the quantity and quality of crop yield. In this chapter, we are interested in quantifying the risk of infection to which a crop is exposed as a basis for deciding whether intervention aimed at

disease suppression is justified. Aspects of this process differ from pathogen to pathogen, from crop to crop, and from location to location [4]. Goulds and Polly [7] and Binns et al. [8] draw a distinction between crop protection based on either curative or preventative action. Without necessarily wishing to adhere rigidly to this dichotomy, it is nevertheless clear that in some cases, sample data are the most important components of the information on which decision making is based. In others, data relating to the host and the environment often play a more important role, and the evidence on which a decision is made about the need for appropriate control action is therefore likely to be more wide ranging. The first step in the formulation of a disease management strategy for any cropping system is to identify the most important risk factors among those on the long list of possible candidates. This is facilitated by basic epidemiological studies of pathogen life cycles, and an understanding of the way in which weather and cropping factors affect the quantity of initial inoculum and the rate of the pathogen life cycle. To be able to identify risk factors, we need information both on the candidate risk factors and on the definitive status of the crops in which they are studied.

2.2. Basis of decision making

Jones [9] discussed a decision-making guideline based on impact on yield for fungicidal control of eyespot disease of winter wheat (*Triticum aestivum* L.). Treatment was considered to be worthwhile if ≥20% of tillers were diseased at growth stage (GS) 30–31. Accordingly, the recommendation was for a sample of tillers to be collected at the appropriate growth stage and a decision of whether to treat was made based on the percentage of tillers with symptoms of eyespot disease, in relation to the specified threshold. Decision making was based on a two-stage cluster sampling procedure, collecting a total of 50 tillers for the assessment [7]. The economic threshold is the level of risk exposure at which crop protection measures should be applied, in order to prevent the economic injury level from being reached. An economic threshold may be used to identify circumstances in which it becomes economically advantageous to apply crop protection measures. The economic threshold is a discrete choice threshold: the only options are to apply crop protection measures or to withhold them. However, the choice between these two options must be made before it is known for sure whether a crop will sustain economic loss resulting from reductions in the quantity and quality of yield. Thus, the economic threshold may be used as a basis for deciding whether or not crop protection measures are required, at a time when it is still possible to keep damage below the economic injury level. Weather-based systems, or weather-based systems combined with other disease or agronomic variables have been developed in various areas in Europe to determine whether fungicide sprays should be applied to prevent the risk of epidemics that might otherwise lead to yield loss. For example, Audsley et al. [10] developed a model in the UK based on weather, host resistance and inoculum pressure to project effects on green leaf area, which was coupled with effects on yield loss as a decision support system for Septoria leaf blotch, powdery mildew, and yellow and brown rusts.

In this chapter we specifically provide an overview of different weather-based disease models developed and used for assessing in real time the risk of epidemic development for the major fungal diseases (i.e., Septoria leaf blotch, powdery mildew, leaf rusts and Fusarium head

blight) of winter wheat in Luxembourg. A description of the models is provided along with the constraints associated with their use for in-season disease monitoring. The challenges faced using weather-based models in a changing climate are also discussed.

3. Main fungal diseases of wheat in Luxembourg and associated decision support systems

Wheat represents one of the most widely cultivated cereals with a production area of 215 million ha worldwide [11]. Unfortunately, wheat diseases remain a major constraint to wheat production [12]. Crop protection often relies on calendar-date applied, preventive fungicide applications, and small grain cereals are typically treated with two or three foliar fungicide applications in Luxembourg and Belgium [13, 14]. The marginal cost/revenue ratio for winter wheat and the potential negative impacts that overuse of pesticides can have on the environment are compelling arguments to minimize inputs, including fungicides. Effective estimation of the risk of disease epidemic development can minimize the number of fungicide spray applied, leading to a more sustainable and environmentally friendly system of wheat production. Using tools to develop integrated pest management can lead to fungicides being applied only at particular stages that are at risk of infection, and only when the pathogen has been correctly identified (accurate identification and/or estimation of severity of disease can be critical to effective management). Diseases of wheat that have become economically important in Luxembourg include Septoria leaf blotch (SLB) caused by *Zymoseptoria tritici Roberge in Desmaz.*, wheat leaf rust (WLR) caused by *Puccinia triticina Eriks.*, wheat stripe rust (WSR) caused by *Puccinia striiformis Westend. f. sp. Tritici Eriks.*, and Fusarium Head Blight (FHB) caused mainly by *Fusarium graminearum*. The control of the diseases caused by these pathogens is a high priority to minimize yield and grain quality losses.

3.1. Septoria leaf blotch

The majority of the SLB disease prediction systems proposed for the management of *Z. tritici* assume that the main risk of infection of the upper leaves (the most critical for grain fill [15]) comes from the inoculum that developed on the leaves during the winter and spring before the extension of the stem [16]. These prediction systems are based solely on rainfall occurring during stem extension, without considering the development of individual leaves [17–19]. The importance of rain and splash dispersal for development of severe SLB has been demonstrated in several studies (e.g., [16, 20–22]). Shaw and Royle [19] suggested that the amount of Septoria inoculum at GS31 (first node detectable) [23] was only a partial guide to forecast the inoculum available during the expansion of the last two leaves. The progression of the disease on the upper leaves depends on the sensitivity of the cultivar, and the period of infection (infections occurring during and/or just after the emergence of these leaves could lead to severe impacts if the weather conditions are favorable) [24]. The mechanisms by which the pathogen population increases on the upper leaves are determined by the interaction of plant growth, the meteorological conditions allowing the dispersal of the inoculum and thus opportunity for new infections, and the availability of that inoculum in sufficient proximity to the upper leaves [19]. El Jarroudi et al. [20] suggested that the greatest risk to a wheat crop occurs

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from infections arising between the emergences of leaf 2 (L2) and the flag leaf and roughly two latent periods before these leaves would naturally begin senescence. If the upper leaves are infected early in the cropping season, they are likely to suffer much more severe disease for two reasons: a) there is sufficient time for the pathogen to have more than one multiplication cycle on the leaves, with a longer time during which dissemination and infection may occur, resulting in premature loss of leaf area; b) these leaves are closer to the sources of the inoculum and extreme splashing events will no longer be necessary to disperse sufficient number of spore onto a susceptible tissue that is higher in the crop canopy. Furthermore, the structure of the wheat plants and the position of the source of the inoculum on specific leaves relative to each other are constantly changing and thus the risk of disease progression is dynamically complex and specific to each crop, cultivar and season [22]. In addition, the life of the upper leaves is considerably shortened by secondary infections resulting from the inoculum produced by primary lesions in the same leaf layer [19]. The detection of spores of *Z. tritici* during the season demonstrates the need for a predictive model [25, 26]. Both asexually produced pycnidiospores and sexually produced ascospores of *Z. tritici* are known to cause disease in wheat [22, 27], with ascospores being aerially dispersed over relatively long distances, and the pycnidiospores being primarily splash dispersed. Furthermore, the ascospores have an impact not only as primary inoculum in autumn and winter [27], but also as secondary inoculum at the end of spring and in summer. This airborne inoculum could help to colonize the upper leaves without the need for splash-dispersed pycnidiospores or could exacerbate the damage caused by splash-dispersed *Z. tritici* (**Photo 1**) due to the presence of the additional ascospore inoculum [28].

Due to the potential for yield loss from SLB, growers tend to spray fungicides several times during the winter wheat season to protect their crops. The development of resistance in

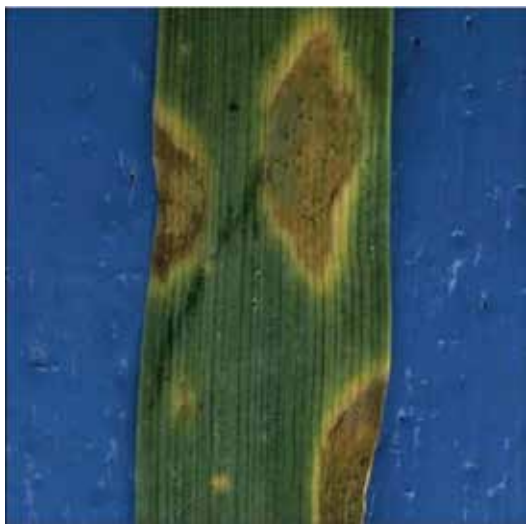

Photo 1. Symptoms of Septoria leaf blotch caused by *Zymoseptoria tritici* on leaf L3 of the cultivar Achat. The black dots in the tan lesions are the pycnidia that produce the splash dispersed pycnidiospores (photo taken on May 30, 2007 at Everlange, Luxembourg; photo credit: El Jarroudi M.).

Z. tritici to the main fungicides used for its control [20] has been demonstrated in many countries. Moreover, actual disease severity does not always justify a fungicide spray. In years with a low disease risk, a lower fungicide dose could be used [29]. There are several weather-based Decision Support Systems (DSSs) available to help a grower decide whether a fungicide application is required [30–32]. These models rely mainly on rainfall measurement, or in some case more comprehensively on weather data, without considering the development of the different leaf layers during stem elongation [18, 33–37].

However, many models neglect the periods of interruption of acceptable temperature or humidity for infection which are important factors in disease development, and can be an indispensable element in developing more accurate models. According to Shaw [38], interruptions in periods at 75% relative humidity for 48 h slightly reduced the efficiency of the infection process, but interruptions at 50% relative humidity resulted in major effects, but still allowed infection to occur. To simulate infection, some models take daily conditions [39, 40], while others, for example the PROCULTURE model are based on hourly weather conditions [14, 20].

3.1.1. The PROCULTURE model

The PROCULTURE model is an interactive web-based, field-specific, DSS based on the mechanistic modeling of the development of the last five leaf layers of the wheat plant coupled with the progress of SLB on these layers [14, 20, 41, 42]. A descriptive flowchart of the model is presented in **Figure 1**. The main inputs include weather data (hourly air temperature, rainfall and relative humidity) and field-specific data including the location, sowing date and cultivar susceptibility. Field observations are also important since a fine-tuning of the model may be required based on the actual growth stage (around the first node stage, GS32) and the severity of SLB on the particular leaf layer as specified by the model. The model considers infection to have occurred when, during a 2 h rainfall event, precipitation for the first hour is at least 0.1 mm (to allow for the swelling of pycnidia), followed by a second hour with at least 0.5 mm precipitation (**Figure 1**), leading to the release and splash dispersal of the conidia [14, 20]. In addition, after rainfall, relative humidity should be higher than 60% during the following 16 h [20, 43] and the temperature should remain above 4°C for 24 h [20] for germination and infection.

The evaluation of the PROCULTURE model at several sites in Belgium [14, 44] and Luxembourg [20] demonstrated that the model can explain disease progression in the canopy (**Figure 2**) and can be used to advise farmers when to apply fungicides during stem elongation, as the three upper leaves emerge. The need for and timing of a single fungicide spray using the PROCULTURE model is based on the observed disease severity earlier in the cropping season (i.e., severity on the lower leaves L5-L4 at GS 31–37, L1 being the flag leaf), the susceptibility of the cultivar, past and forecasted weather conditions, and the predicted development of leaves based on the output of the PROCULTURE model. Furthermore, historical data (weather and disease incidence and severities) were used as a basis for similarity analysis to further evaluate the risk of severe disease development. Given the threshold level of observed disease severity (namely on the lower leaves) and weather conditions (actual and forecasted), an advice for fungicide treatment was taken and fungicides applied only if required to protect the upper leaves. For example, a 5% of emergence of L3 coinciding with SLB symptoms on L5 and a rainfall event, results in a greater risk that L3 will be affected by

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SLB during full emergence. Consequently, a fungicide treatment against the risk of SLB is recommended if a latency period of the disease is completed at 75% emergence and favorable weather conditions forecasted. Overall, the assessment of the infection periods achieved an accuracy of 85%. The results showed that the PROCULTURE model satisfactorily recommended none or a single fungicide treatment at each study site, regardless of geographical location or possible variability among the fungal diseases involved [45].

3.1.2. Spatialization of PROCULTURE alerts using radar rainfall

The PROCULTURE model is being used in early warning systems in Belgium and Luxembourg to define, in real time, the risk of SLB developing on the upper leaves of winter wheat during stem elongation. However, setting up an operational network for recommending the optimal time for fungicide application requires a representative network of weather stations throughout the region where the DSS will be used. In our studies (e.g., [20, 46]) overestimation or underestimation of the risk of SLB progression could often be traced back to differences in rain events captured by the tipping-bucket rain-gauges at the weather station compared with the rainfall to which a particular field was actually exposed. Rainfall data could be interpolated between weather stations, but precipitation between fields are characterized by high spatial and temporal variability [47, 48], making the interpolation unreliable [49, 50].

Radar may provide a solution for improving the interpolation of precipitation data [51, 52]. Over the past few years, radar-derived estimates have been increasingly used in disease forecasting applications as an alternative to gauge-derived measurements [51, 53].

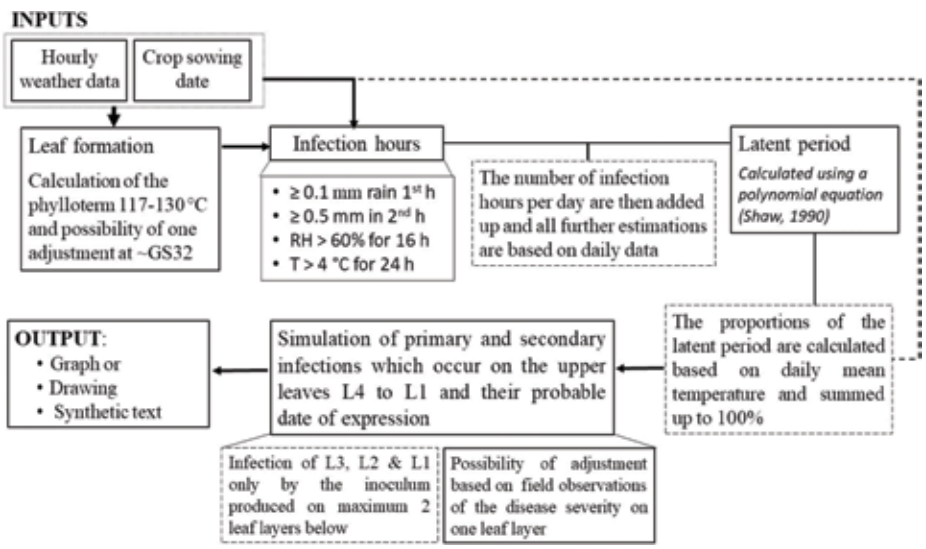

Figure 1. Descriptive flowchart of the PROCULTURE model for predicting the risk of Septoria leaf blotch (SLB) infection events. T: Air temperature; RH: Relative humidity.

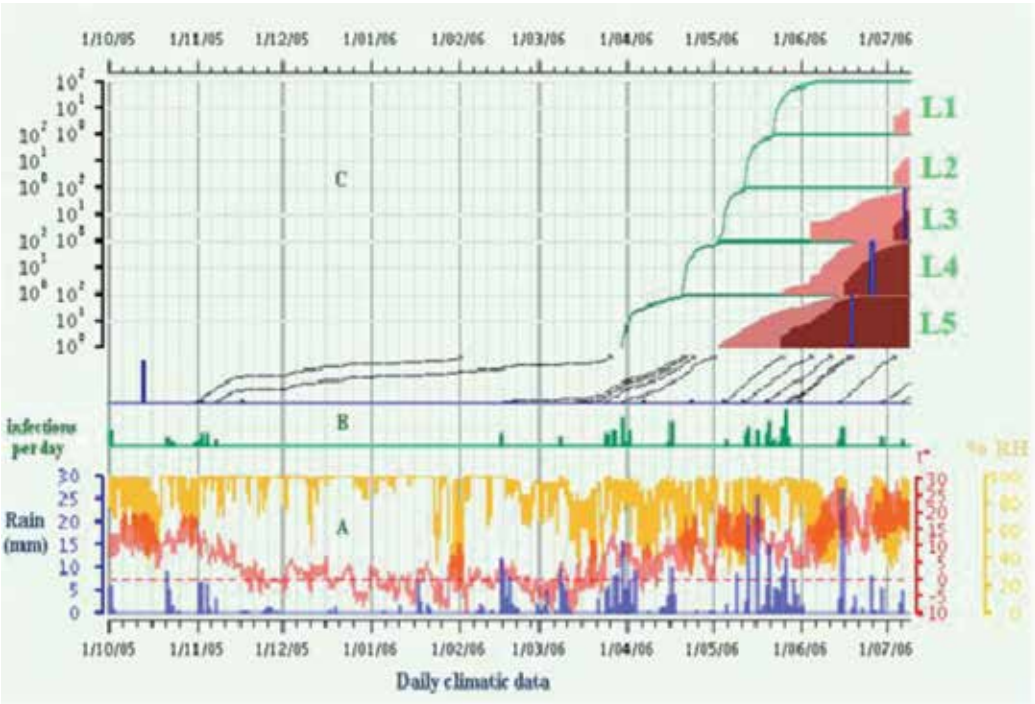

Figure 2. Output of the Septoria risk simulation model from 2006 in winter wheat fields at Reuler Luxembourg. A: Observed daily values of air mean temperature (°C) and rainfall (mm). B: Number of hours per day with a > 80% probability of infection. C: Lines: Leaf area development (0–100%) of leaves L5–L1 (flag leaf is L1). Gray: Accumulation of hours of primary infection expressed on leaves L5–L1 (maximum of 100 h) (Reuler is one of the representative sites of winter wheat cropping regions in Luxembourg selected for field experiments in the framework of the SENTINELLE project. It is located in the northern part of Luxembourg).

Mahtour et al. [42] validated the simulation of infection periods for *Z. tritici* calculated by PROCULTURE using radar-based rainfall measurements. The duration of periods with a high probability of infection by *Z. tritici* was calculated by PROCULTURE and using radar rainfall data for these trials was similar to that based on gauge measurements (**Table 1**). A better spatial representation of precipitation will inevitably improve present DSSs. Consequently, the DSSs could more accurately be the basis for recommending appropriate fungicide applications. If the results of the radar-based rainfall measurements combined with PROCULTURE are confirmed for a larger precipitation dataset and a larger number of stations, the sole use of radar data in the disease-warning system will be considered in the future. The results from this work should encourage research on additional radar-based rainfall applications for diseases of other crops.

3.2. Wheat leaf rust

WLR is of major historical significance and is of economic importance worldwide. It is the most widespread of the three species of rusts causing significant yield losses over large geographical areas [54–59]. Several studies in major cereal-producing areas have revealed

Field sites	Observation period	Year	Events[a]	Duration of infection period[b]		POD_{so}[c]		FAR_{so}[d]		CSI_{so}[e]	
				Gauge	Radar	Gauge	Radar	Gauge	Radar	Gauge	Radar
HUMAIN[f]	21/05 to 05/07	2003	18	60	62	0.93	0.83	0	0	0.93	0.83
	03/05 to 28/06	2004	16	46	40	0.73	0.87	0	0	0.73	0.87
	20/05 to 15/07	2005	8	24	27	0.86	1.00	0	0.12	0.85	0.87
			42	130	129	**0.84**[h]	**0.90**	**0**	**0.04**	**0.84**	**0.86**
USELDANGE[g]	13/05 to 29/06	2003	15	56	44	0.87	0.80	0	0	0.87	0.80
	16/05 to 09/07	2004	18	48	48	0.72	0.78	0	0	0.72	0.78
	14/05 to 12/07	2005	15	33	32	0.71	0.86	0.09	0.07	0.67	0.81
			48	137	124	**0.77**	**0.81**	**0.03**	**0.02**	**0.75**	**0.80**
BURMERANGE[g]	17/05 to 03/07	2003	10	30	22	0.70	0.70	0	0	0.70	0.70
	05/05 to 13/07	2004	15	43	55	0.73	0.93	0	0	0.73	0.93
	12/05 to 04/07	2005	12	24	28	0.91	0.83	0	0	0.91	0.83
			37	97	105	**0.78**	**0.82**	**0**	**0**	**0.78**	**0.82**
REULER[g]	—	2003	—	—	—	—	—	—	—	—	—
	27/05 to 05/07	2004	10	45	32	0.70	0.70	0	0	0.70	0.70
	16/05 to 11/07	2005	11	24	23	0.82	1.00	0	0	0.82	1.00
			21	69	55	**0.76**	**0.85**	**0**	**0**	**0.76**	**0.85**
All			148	433	413	0.79	0.84	0.01	0.02	0.77	0.83

[a]Number of infection events deduced from visually observed symptoms in the field sites on the upper three leaves.

[b]Total number of hours with a high probability of infection simulated by PROCULTURE.

[c]Probability of Detection of infection by *Z. tritici* is the number of cases where infections are both simulated and observed against the number of infections observed. Perfect forecast = 1.

[d]False Alarms Ratio of infection by *Z. tritici* is the number of observed infections not simulated against the number of infections observed in the field. Perfect forecast = 0.

[e]Critical Success Index of *Z. tritici* infection takes into account both false alarms and missed events. The POD_{so}, FAR_{so} and CSI_{so} show the infection occurrence comparison between infection periods (on the last 3 leaves) determined by visual observations and simulated by the PROCULTURE model using measurements from four rain-gauges or radar-based estimates. Perfect value = 1.

[f]Site in Belgium.

[g]Site in Luxembourg.

[h]Average for each field site over three cropping seasons indicated in bold.

- No data.

Table 1. Comparison of the performance when using rain-gauge or radar-based rainfall measurements in the PROCULTURE model for estimating the risk of infection events in winter wheat by *Zymoseptoria tritici* at four sites during three cropping seasons in Luxembourg and Belgium [42].

that epidemics of WLR occur under (i) favorable conditions for overwintering spores as a source of primary inoculum, (ii) rapid and abundant production of wind-dispersed urediniospores, and (iii) a complex interaction between environmental conditions and host resistance [54, 60]. The dispersal of foliar pathogens and WLR in particular around a spore source has been described in many studies, sometimes confirming dispersal over large distances [61] but most often at the spatial scale of an infected plant or group of plants [62, 63], or even a single leaf [64]. Although these studies give valuable insights to allow understanding of epidemic spread of diseases like WLR and to parameterize simulation models, they most often do not take into account the local structure of the host crop and its potential effect on disease distribution [64].

Two different approaches have been used to forecast development of epidemics of WLR. Some forecasting systems consider the effect of weather on the disease by means of empirical rules, flow charts [65], disease indices [66, 67], or regression equations [68, 69]. Other models forecast severity of WLR on the basis of the dynamic of the epidemic, using a fixed relative growth rate of the disease [70–72].

Moisture and temperature are reported to be the most important meteorological parameters influencing the development of epidemics of WLR [73]. Nevertheless, the genetic resistance of wheat cultivars is critically important factor in determining the impact of the disease [74]. Urediniospores are deposited by wind or rain on the adaxial and abaxial surfaces of wheat leaves. Rain on, or turbulence around the leaf surface allows the dispersal of urediniospores. In addition, wet deposition (spore scavenged from the air by rain) is considered an important mechanism of crop contamination by some rusts [75]. Although most rainfall events promote spore dispersal in the field, heavy rain may also induce the leaching of spores deposited on leaves and may totally deplete the lesions in the process [76]. When the urediniospores of WLR are in contact with susceptible wheat leaves, the success of infection requires a minimal duration of surface wetness, which varies as a function of temperature [50, 77]. De Vallavieille-Pope et al. [77] showed that optimum temperatures for uredospore germination ranged from 12 to 15°C and that the germination process ceased above 35°C. As noted, the presence of free water on the leaf surface is essential for urediniospore germination. In an earlier study, Eversmeyer [78] proposed an optimum temperature of 16°C for completion of the infection process by uredinisospores of *P. triticina*, with infection needing a dew period of at least 3–4 h. In the same study, it was shown that the latent period for WLR ranged from 8 to 20 days for air temperatures between 10 and 20°C. The process of infection has an approximately linear relationship with the sum of base 0 degree-days. It has also been demonstrated that germination of urediniospores of *P. triticina* could be delayed or inhibited by increasing light intensity [78, 79]. For this reason, infections occur preferentially at night (**Photo 2**).

Considering these data, an empirical approach for simulating infection by WLR and progress of the disease on the upper three leaf layers has been proposed and validated in Luxembourg [2]. The model used only weather data logged between 8 pm to 5 am based on the assumption that spore germination is inhibited by light. Each infection event was deemed to require a period of at least 12 consecutive hours counted on at least two nights with air temperatures ranging between 8 and 16°C and a relative humidity greater than 60% (**Figure 3**). Moreover, the hourly rainfall totals during these 12-hour periods must be less than 1 mm to avoid the leaching of spores present on leaves. Furthermore, the primary infection in a field requires a

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Photo 2. A leaf showing symptoms of infection by *Puccinia triticina,* causing pathogen of wheat leaf rust (photo taken on June 2009 at Burmerange, South Luxembourg; photo credit: El Jarroudi M.).

light rain (0.1–1.0 mm) in the first hour of an infection event supposing that this rainfall allows the first deposition of the inoculum in the field. This light rain event is not a necessity once the primary infection has occurred. The model has led to a DSS that allows optimizing timing of applications of the fungicide for controlling WLR in fields in Luxembourg.

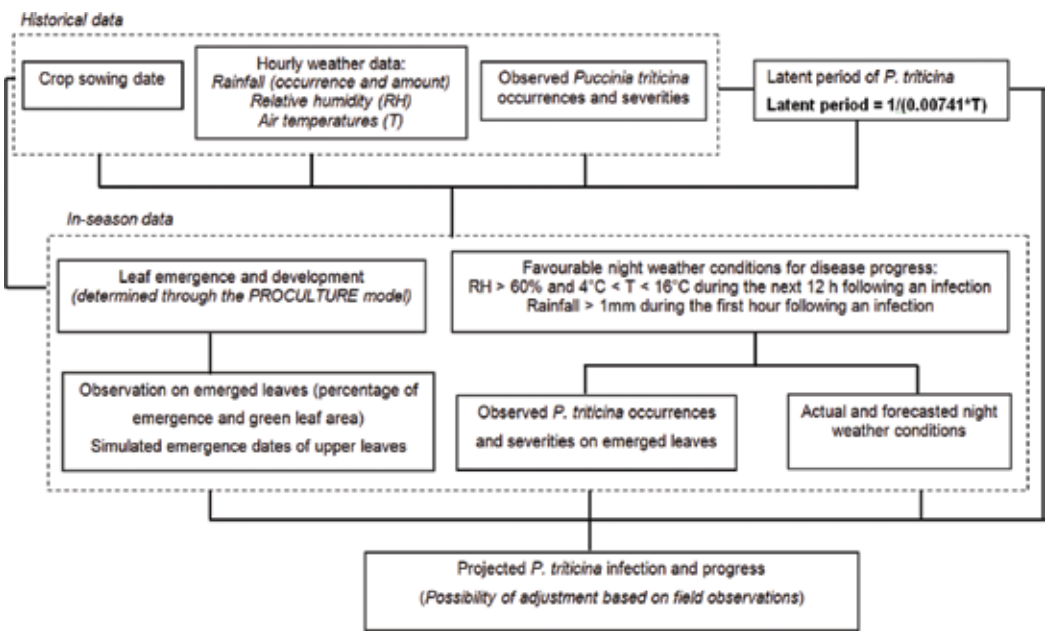

Figure 3. Descriptive flowchart of the model used for predicting wheat leaf rust (WLR) infection events caused by *Puccinia triticina* [80].

The presence of primary inoculum in the air is not considered as a limited factor in this model. We assumed that spores of *P. triticina* are already present in fields during the period of study. A fine-tuning of the DSS will include an effective assessment (i.e., spore dispersion estimates) for the spores in the same field, since spores from outside the field are only required to initiate the first infection (exogenous inoculum). Indeed, the assessment of the model coupled with detection of spores showed that the infection periods on susceptible cultivars (**Figure 4**) were well predicted [81].

Thus, the detection of airborne inoculum by sensors and its coupling to a reliable model of dispersion could help improve forecasting the occurrence of WLR. In Belgium, a recent study on the spatio-temporal distribution of the airborne inoculum of *P. triticina* indicated that infection on the three youngest leaf layers could originate from endogenous and/or exogenous inoculum. The first symptoms observed on crops can be the result of either infection by urediniospores carried upwind by air masses from distant infected fields or the consequence of sporulating lesions occurring in the fall and remaining active after the winter [82]. Airborne inoculum was generally detected in fields during the growing season between March and May (during the spring green-up). Various densities of airborne inoculum were observed depending of the site and the year, and the severity of WLR on the upper leaf layers during the grain filling was strongly influenced by the density of spores collected during the development of these leaf layers [83].

Molecular diagnostics combined with sampling of airborne inoculum could be exploited to more accurately predict the risk of epidemics in wheat agro-ecosystems. Strategies for controlling WLR in fields include the use of resistant cultivars. But a prolonged period of monitoring WLR involving susceptible cultivars and favorable night conditions conducive to spore production, dispersal of, and infection by *P. triticina* with subsequent development of WLR should demonstrate the capability of the DSS in these situations. Junk et al. [84] studied the potential infection periods of WLR in a changing climate at two selected sites in Luxembourg (Burmerange and Christnach) using a weather threshold-based model for infection and development and progress of WLR that involved hourly night-time data for air temperature, relative humidity and rainfall. Their findings revealed that highest proportions of favorable days for infection with *P. triticina* and development of WLR in the future would occur during spring and summer at both sites, with the proportions more marked at Burmerange.

3.3. Wheat stripe rust

WSR is an example of a disease of world-wide importance and ability for long distance dispersal. Crop pathogens with worldwide prevalence and potential for long distance migration and thus invasions into new areas may pose a serious threat to food security regionally or globally [85]. WSR of wheat is among the most important crop diseases causing a continuous threat to crop production [86, 87]. Worldwide. the virulence and race diversity of populations of *P. striiformis* is apparent. Races from regionally prevalent lineages cause epidemic outbreaks resulting in widespread economic losses in wheat production [85, 88]. Virulence to most of the characterized resistance genes has been observed in Europe, reflecting the large-scale deployment of these genes in Europe in the past [89–93]. More recently, the footprint of epidemics of WSR appears to be moving into non-traditional, warmer and dryer areas suggesting a wider range of adaption [85]. Based on an ostensibly representative selection of isolates of WSR collected from the United States (and genetically similar isolates from Denmark, Mexico and

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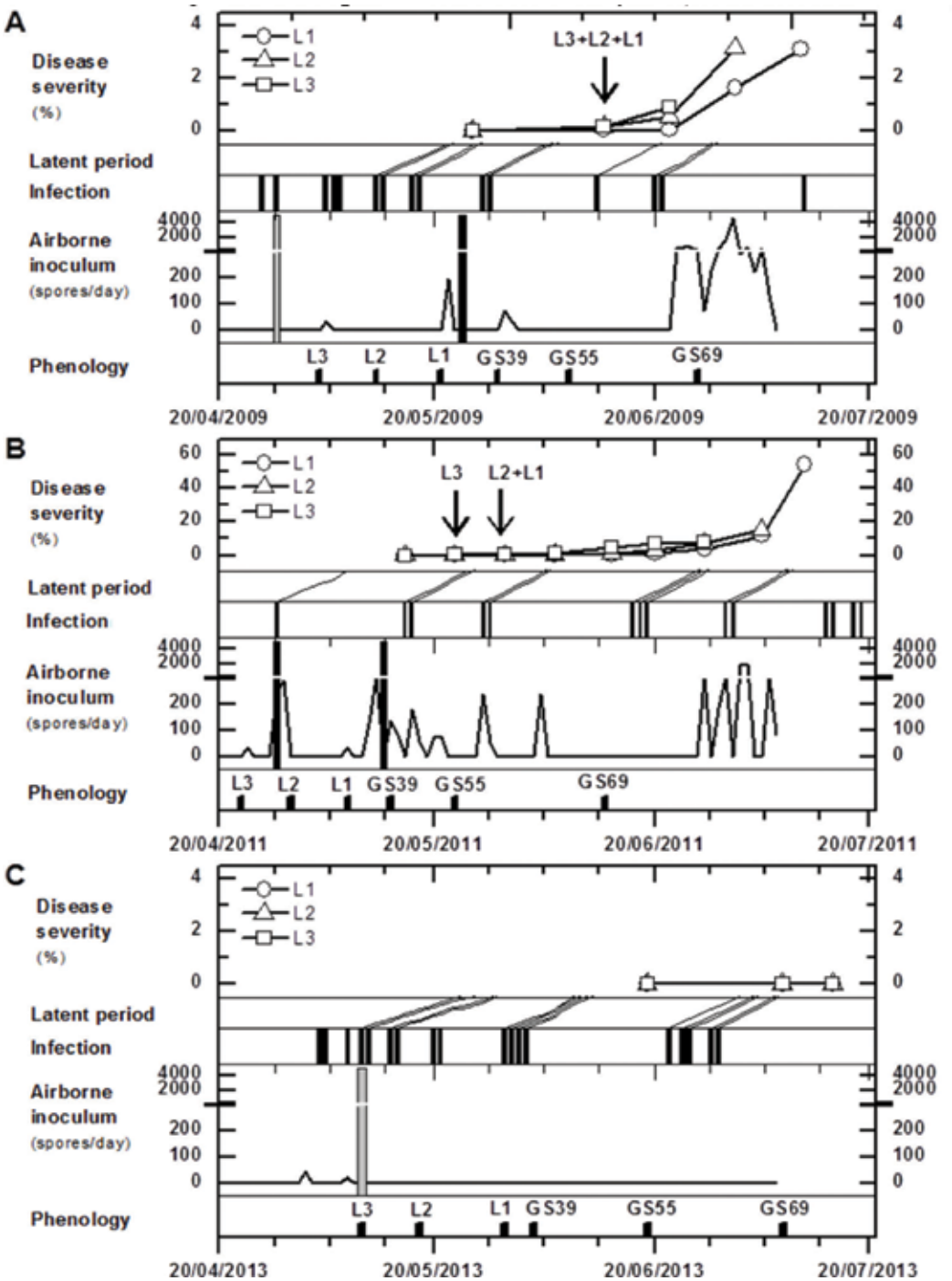

Figure 4. Severity of wheat leaf rust (WLR) on the three upper leaves in wheat plants. Severity of WLR, infection by, and latent periods of *P. triticina* were determined based on favorable night weather conditions at Perwez, Belgium in 2009 (a), 2011 (B) and 2013 (C). The arrows show the time of the first disease observation in the field. Phenology of the plants including the appearance of the three upper leaves is represented at the bottom of each figure. The airborne inoculum trapped in the field allows determination of when the "inoculum condition" was reached (black bars). The gray bars symbolize the moment when the "rain conditions" of the original model were reached. (source: [81]).

Eritrea) before and after 2000 [94], it was demonstrated that isolates collected after 2000 were more aggressive and had adapted to produce more urediniospores in a shorter time period, and at higher temperatures. The pathogen has been highly mobile and the geography of its genetics has changed and expanded, especially since 2000. Multiple new incursions of the pathogen have been reported in Australia and South Africa [95, 96] and international movement of spores of *P. striiformis* from Europe (in 1979) and North America (in 2002) has been implicated on the clothing of travelers [97]. Indeed, in 2011 a new race of *P. striiformis*, named "Warrior," was detected in various European countries including France, Germany and the UK [93]. Since urediniospores of P. striiformis can spread over large distances [98], the race Warrior is probably already present in Luxembourg. Confirming the existence of Warrior in commercial Luxembourgish wheat fields was not part of this study.

In most seasons, environmental conditions during spring and early summer are conducive to the production of large quantities of spores of *P. striiformis*, which are dispersed from distances of a few centimeters to thousands of kilometers (**Photo 3**), where they might reach a susceptible host plant [76, 98]. The sporulation capacity and infection efficiency of *P. striiformis* are affected mainly by air temperature, leaf-wetness duration and light intensity [77]. Urediniospores of *P. striiformis* require a relative humidity near saturation for at least three hours to germinate [99] and are sensitive to an interruption of the wet period during germination [77]. The presence of free water on the leaf surface is also essential for spore germination [77, 99, 100]. Thus, rain is often considered conductive to disease spread because rain events are generally followed by extended periods of leaf wetness [76, 99].

The model developed is based on a stepwise approach (**Figure 5**) consisting of (1) the determination of the potential range of weather conditions conducive to WSR in Luxembourg using a stochastic approach, and (2) the determination of optimum classes of combined weather variables

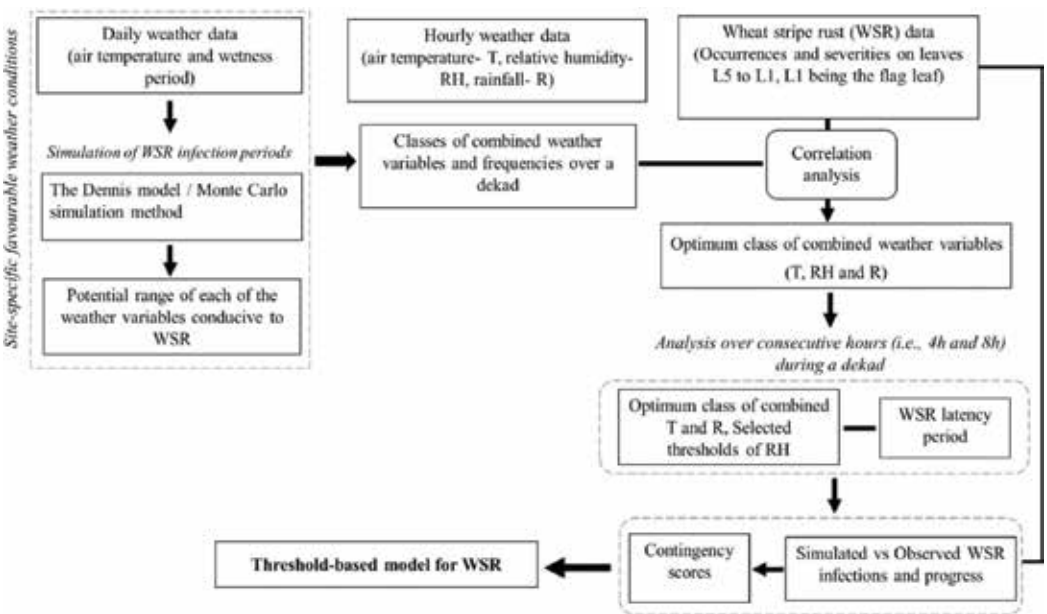

Figure 5. Descriptive flowchart of the modeling approach for predicting infection events of wheat stripe rust caused by *Puccinia striiformis* [13]. Air temperature (T), relative humidity (RH) and rainfall (R).

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Photo 3. Fungicide treated and non-treated plots of winter wheat and a leaf (inset) showing symptoms of wheat stripe rust caused by *Puccinia striiformis* (photo taken on 2015 in Burmerange, South Luxembourg. Photo credit: Beyer M.).

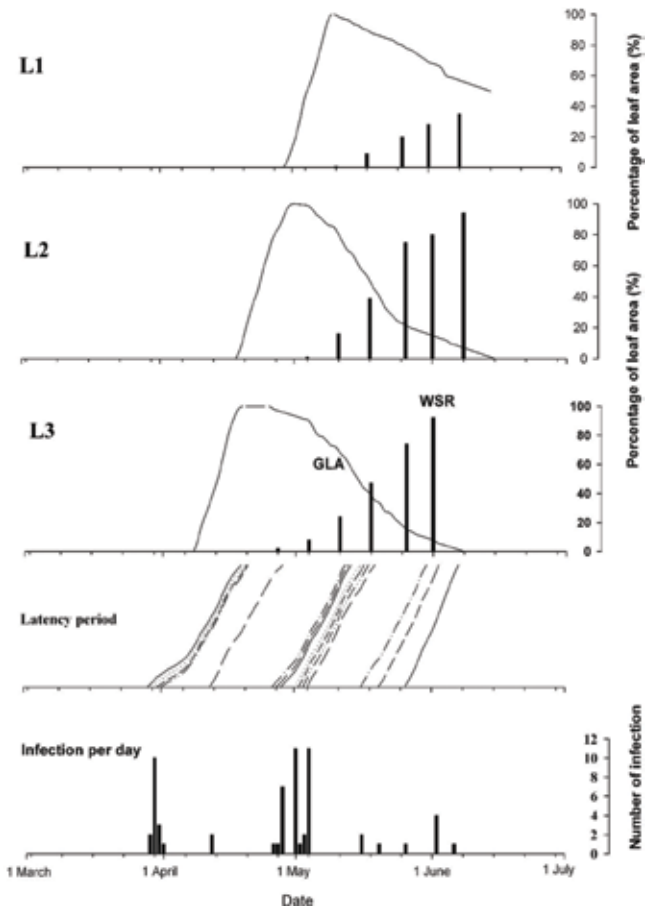

Figure 6. Example of simulated infection events by *Puccinia striiformis* (cause of wheat stripe rust (WSR)), observed green leaf area (GLA) and severity of WSR on the three upper leaves (L3 to L1, L1 being flag leaf) at Burmerange, Luxembourg during the 2015 cropping season. The severity of WSR is expressed as percentage leaf area diseased [13].

(air temperature (T), relative humidity (RH) and rainfall (R)) conducive to the disease and building of a weather threshold based model for predicting WSR infection events [13].

The threshold-based model for development of WSR was evaluated using independent data from experiments in Luxembourg in 2002–2015 [13]. Infection days and latency periods for *P. striiformis* (**Figure 6**) were calculated based on periods when the combined favorable weather variables (4°C < T < 16°C, RH > 92% and R ≤ 0.1 mm) were met. The overall performance of the threshold-based weather model developed in this study is quite similar to that developed for WLR across the same geographical region. Although the findings are area-specific and may differ in other geographic regions, the underlying hypothesis and approach can be extended to different locations and/or explored for other economically important fungal diseases of other crops.

3.4. Fusarium head blight

Besides the yield loss that it can cause, FHB can negatively affect the entire human food and animal feed chain through the contamination of wheat grains with mycotoxins. Contamination with fumonisins can result in grains unusable for consumption or for further processing into bakery products, breakfast cereals, pasta, snacks, beer or animal feed, etc., [101–106].

Photo 4. Fusarium growth on wheat (Photo credit: Giraud F.).

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Weather is a critical factor influencing FHB. Frequent rainfall, high humidity and warm temperatures, coinciding with flowering and early kernel filling, favor infection by *Fusarium* spp. and development of the disease [107]. Numerous research and survey reports have shown that the main environmental factors influencing the development of FHB (**Photo 4**) are temperature and humidity/wetness [108, 109] It has been speculated that the difference observed in severity of FHB between 2007 and 2008 (**Figure 7**) (21.0 ± 17.8% versus 13.5 ± 16.2%) may, at least in part, be explained by the warmer temperature observed in 2007 (11.9°C) compared to 2008 (9.4°C) [103, 110]. Climatic factors can also influence the impact of fungicide application and its effect on Fusarium strain population [111].

Many studies have highlighted the relationship between the severity of FHB in specific fields where certain cereals particularly maize, were the previous crop [103, 112]. Maize residues are a host for several *Fusarium* species and thus provide a source of inoculum for infection of any susceptible crops planted in that land [113, 114]. Suitable cultural practices (e.g., crop rotation) aiming to reduce inoculum borne plant residues could be effective in controlling FHB in winter wheat fields.

A simulation model for predicting the periods of infection by *Fusarium* spp. was developed and evaluated at various sites in Luxembourg during 2007–2009 [115]. Like the models developed for other fungal diseases, the main inputs are T, R and RH. Information on the cultivar and the previous crop are also considered while using the model outputs for recommending fungicide sprays (i.e., the model is only used when sensitive cultivars are planted after maize or sorghum).

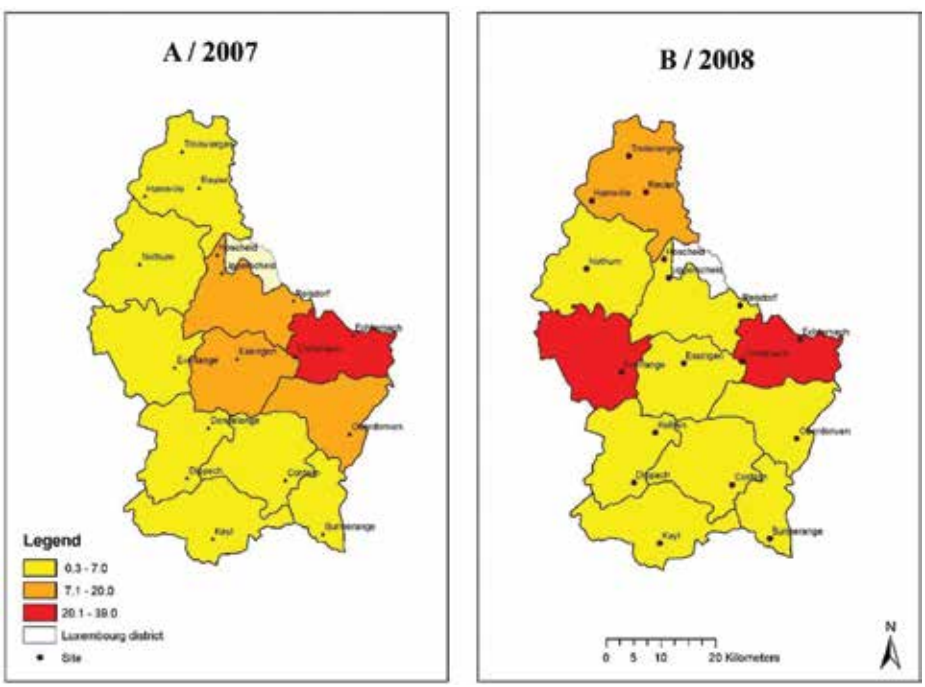

Figure 7. Incidence of fusarium head blight (% infected wheat spikes), caused by *Fusarium* spp. in various districts of Luxembourg (n = 17) in 2007 (a) and 2008 (B) as assessed between GS 77 and GS 87 ([45]).

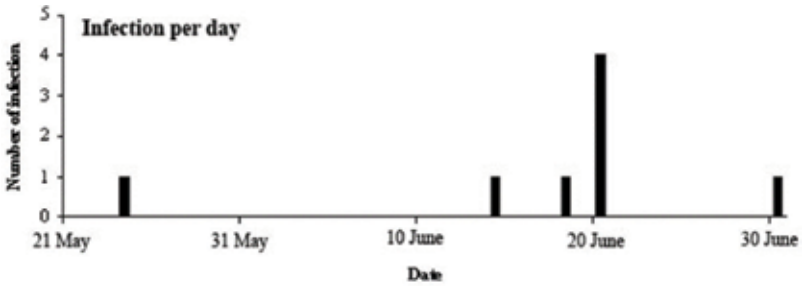

Figure 8. Example of simulated infection events by *Fusarium spp.* at Reuler, Luxembourg during the 2007 cropping season.

An example of the number of infection events by *Fusarium* spp. is depicted in **Figure 8**. Because of the changes in the composition of Fusarium population across sites and other site-specific characteristics related to the climate and topography, a mixed performance of the model. Thus, knowledge of the spatial patterns of epidemics of FHB, along with information on the Fusarium species involved are crucial to developing improved control and management measures relevant to each region, as in Luxembourg [116]. Furthermore, management strategies based on fungicide application should also take into account the effect chemical treatments may have on toxin induction by Fusarium species [103, 111]. Management tools in the future might include a weather-based DSS to help predict and eventually manage FHB.

4. Concluding remarks

Meteorological variables are most often used as the input data for disease forecasting models of fungal diseases of winter wheat in Luxembourg and elsewhere. For disease risk assessments at the regional scale, the meteorological data in these forecasting models must originate from local weather stations which are part of a meteorological networks consisting of automatic weather stations (AWSs). However, the choice of location for an AWS within a field or the distance between AWSs locations are both factors that hamper accurate forecasting of fungal diseases at regional scales. Moreover, techniques used to interpolate weather data from a set of neighboring sites suffer from some potential sources of error, e.g., difficulty in capturing small scale variation, failure to account for local topographical features, etc.

With the changes in the patterns of world climate expected during the coming decades [117], the pattern of corresponding distributions of fungal diseases will be affected accordingly. Thus, new challenges are emerging that need to be addressed. Climate change affects pathogen biology not only directly but also indirectly through effects on host development and phenology. Modeling to predict new disease threats is expected to be beneficial since many years of data are needed to prepare appropriate solutions to developing issues. However, although the impacts of climate change on crop disease are being studied, uncertainties inherent in crop disease models remain largely unexplored and unreported [118]. Moreover, acclimation to future climatic conditions by both the pathogen and the host can significantly alter the outcome of the plant–pathogen interaction [119].

Wheat diseases present a constant and evolving threat to food security. Decision-support tools based on in-season disease monitoring and disease progress models in relation to weather variables present various advantages for managing the development of epidemics of those diseases, while limiting potentially harmful side effects of excessive fungicide applications while ensuring economic benefit. Embedded in operational warning systems for plant disease monitoring, DSSs could provide a valuable service to the farmer community for pest and disease management through integrated and environmentally friendly methods.

Author details

Moussa El Jarroudi[1]*, Louis Kouadio[2], Bernard Tychon[1], Mustapha El Jarroudi[3], Jürgen Junk[4], Clive Bock[5] and Philippe Delfosse[4]

*Address all correspondence to: meljarroudi@ulg.ac.be

1 Department of Environmental Sciences and Management, Université de Liège, Arlon, Belgium

2 International Centre for Applied Climate Sciences, University of Southern Queensland, Toowoomba, QLD, Australia

3 Laboratory of Mathematics and Applications, Department of Mathematics, Université Abdelmalek Essaâdi, Tangier, Morocco

4 Department Environment and Agro-Biotechnologies, Luxembourg Institute of Science and Technology, Belvaux, Luxembourg

5 USDA-ARS-SEFTNRL, Byron, GA, United States

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Chapter 2

The Biology of *Thecaphora frezii* Smut and Its Effects on Argentine Peanut Production

Luis Ignacio Cazón, Juan Andrés Paredes and Alejandro Mario Rago

Additional information is available at the end of the chapter

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Abstract

Thecaphora frezii was first reported in 1962 in wild peanut from Aquidauana, Mato Grosso do Sul, Brazil. In Argentina, it was first detected in 1995 in commercial crops from the central-northern area of Córdoba province. The fungus can survive in the soil as teliospores. When peanut gynophore penetrates the soil, their exudates disrupt telial dormancy; *T. frezii* penetrates and colonizes the tissues and replaces the cells with teliospores. Since its first report, peanut smut prevalence has gradually increased in peanut areas to reach a 100% in 2012. Currently, it is the most important peanut disease in Argentina, not only for its destructive power on crop but also for its quick spread throughout the growing region of Córdoba and the lack of effective tools for its management. It is important for additional research to find effective agronomical practice that reaches high control efficiencies. The collaboration of all those involved in Argentinian peanut production systems is necessary for the management of peanut smut to be successful.

Keywords: peanut smut, teliospores, peg, basidiospores, phytopathology

1. Introduction

Peanut is an herbaceous plant from South America. Its origin is located specifically in southeastern Bolivia and northwestern Argentina, where its parental species are found in wild habits [1]. In 1753 the cultivated species of peanut was classified as *Arachis hypogaea* L. in two subspecies, *hypogaea* and *fastigiata* [2]. *Arachis hypogaea* belongs to the family Leguminosae, subfamily Papilionoidea, and gender Arachis [3]. Peanut is an annual plant, and its growth habits are described as bunch, decumbent, or runner. The bunch types can

reach 40 cm in height. They have an upright growth habit with flowering on the main stem and lateral branches. Runner types can reach 120 cm in diameter, and they are considered to have a prostrate growth habit and do not flower on the main stem. Decumbent varieties have an intermediate growth habit between a runner and bunch [4]. Peanut has vegetative and reproductive stages. The vegetative stage involves germination and formation of stems and leaves. Reproductive stage goes from flowering (R1) to the obtaining of overripe fruit (R9) [5, 6]. The flowering process covers almost 80% of the peanut life cycle and overlaps with the fruiting period. After the flower fertilization, the cells located under the receptacle divide rapidly, giving rise to the gynophore or "peg." Gynophore grows toward the ground by stimulation of the light, carrying at its end the ovary protected by a layer of lignified cells [7]. Once introduced into the soil, the elongation stops, and the fruit begins to develop. This requires darkness, mechanical stimulation of the medium, humidity, and the presence of calcium [1]. In Argentina, peanut represents one of most important local economies. More than 92% of Argentine peanut production and processing is in the center of the country, mainly in the province of Córdoba. In this region, more than 12,000 jobs are directly or indirectly related to peanut production [8, 9]. In 2016/2017, peanut was cultivated over 328,600 ha, producing a total of 1.17 MT of peanut and an average yield of 3.69 T/ha. Peanut industry is characterized as an "exporter industry" in Argentina. More than 80% of Argentine production is exported to the European Union (mainly the Netherlands, Germany, England, Spain, Italy, Greece, and France) and other countries such as the USA, Canada, China, and India. All these facts show that peanut industry is not only important to Argentina but also the world peanut market [10]. In Argentina, during the 1980s, peanut production changed to adapt to the demand of the international market for edible peanut. New cultivars were used, passing from bunch-type cultivars to runner types. However, the prevailing climatic conditions were conducive for the development of soilborne fungal diseases [11, 12]. Therefore, peanut production was moved to more southern areas of Cordoba in the early 1990s to avoid the consequences of the production issues in the northern region [13]. During this migration process, emerged peanut smut caused by *Thecaphora frezii*. It was first detected in commercial peanut in the northern producing areas in Córdoba province and then established on the central region where the main grain processing industries are located [14]. Currently, Argentina is the only country that has reported peanut smut in commercial crops. Both Bolivia and Brazil, however, have only reported cases of smut in wild peanuts [15–17]. *T. frezii* was first reported in 1962 in wild peanut samples from Aquidauana, Mato Grosso do Sul, Brazil [15, 18] (**Figure 1**). In that time, fungus was classified based on disease symptoms and morphology of teliospores. 51 years later this classification was confirmed using molecular tools [19, 20]. In Argentina, *T. frezii* was first detected in commercial crops of peanuts from the central-northern area of Córdoba province: Pampayasta (32°15′07″S 63°39′20″W), Villa Ascasubi (32°10′00″S 63°53′00″W), and Ticino (32°41′25″S 63°23′14″W) [14]. By this time, the presence of affected pods was more frequent year by year in different plots across the peanut area, to finally being found in all production fields in the 2011/2012 growing season [21, 22]. 2 years later, the prevalence was 100% in Argentinian production area, including Salta, Jujuy, La Pampa, and San Luis [23]. During the last 10 years, this disease has caused significant decreases in yield production in Argentina, resulting in 51% losses in some locations [13, 21, 22, 24].

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Figure 1. Seed and wild peanut pod totally damaged by smut (mass of teliospores replacing the grain tissue).

2. Peanut smut symptoms and disease assessment

The smut symptoms are very characteristics on peanut and easy to identify. Affected pods shows hypertrophy and spongy consistence when the infection is highly severe. The wall of pods tends to thin, and the grains inside could be totally or partially transformed in a reddish-brown smutted mass (**Figure 2**). According to the symptoms, it is possible to assess the disease in mature pods (R8) from a given field. In this stage, the disease expression is very clear [17]. There are two parameters to consider when quantifying the disease intensity in affected fields: in terms of incidence and severity. The first is the proportion of infected pods out of a total sample, and the second is the proportion of damaged pod tissue. Disease severity can be estimated using a diagrammatic scale representing five different severity levels [25]. Ordinal levels of 0, healthy pods; 1, normal pod with a small sorus in single kernel; 2,

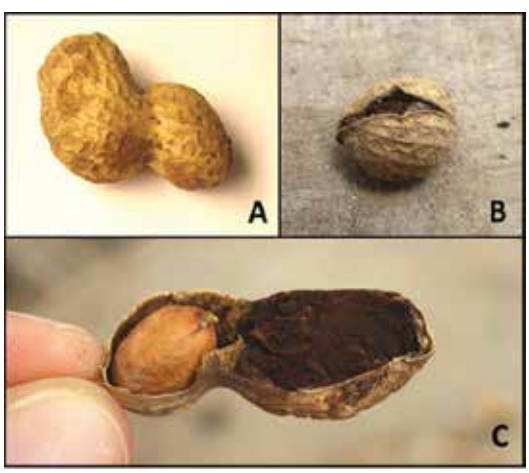

Figure 2. Peanut pods affected by smut. A: Hypertrophied pod due to smut. B: Totally smutted pod. C: Partially smutted pod.

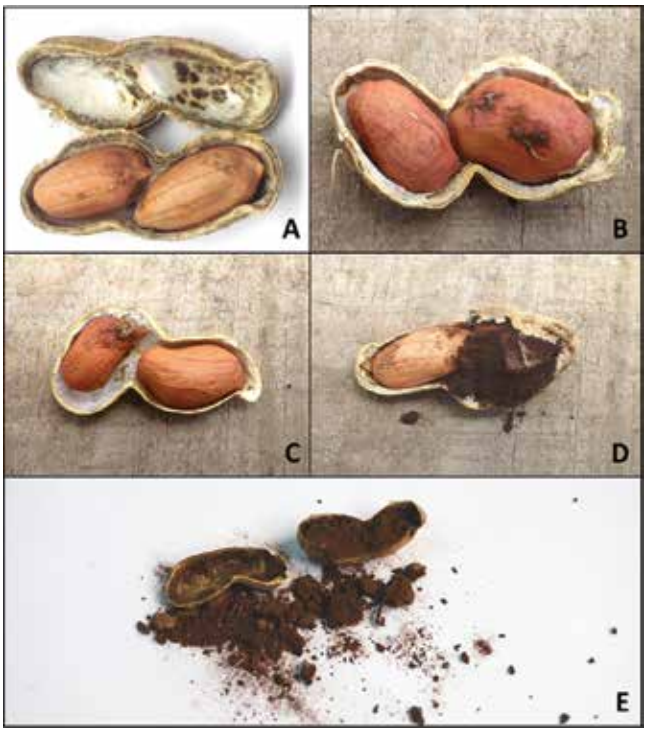

Figure 3. Peanut smut severity scale. A: level 0. B: level 1. C: level 2. D: level 3. E: level 4.

deformed or normal pod with half of the kernels affected; 3, deformed pod and a completely smutted kernel; and 4, deformed pod, two completely smutted kernels (**Figure 3**). Combining both parameters, incidence and severity, it is possible to calculate "intensity" [26]. For disease assessment, [27] surveyed different fields in Córdoba production area in two consecutives growing seasons (2015/2016 and 2016/2017) and determined the amount of samples according the regional average incidence. They concluded that regions with low incidence values need to be assessed using the highest amount of samples than regions with high incidence values. It is important to emphasize that the evaluation of the disease in the field is a tool to know the final sanitary status. To avoid high levels of disease, it is necessary to adopt other management strategies prior to harvest.

3. The pathogen life cycle

T. frezii can survive in the soil as teliospores; those are resistant structures that enable the fungus to be infective for many years in the soil. Ref. [28] studied the infection capacity of teliospores in the field, and they observed that it can be infective for more than 4 years. Smut spores are brown, 20–40 μm in size, and have an echinulate surface [29]. They are thick-walled structures that enable the fungus to survive in different environmental conditions (**Figure 4**).

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When peanut pegs penetrate the soil, their exudates promote spore germination and initiate local infections [30, 31]. The process of teliospore germination includes the formation of a probasidium, followed by a basidium which forms basidiospores. When basidiospores germinate, compatible haploid germ tubes fuse and produce a dikaryotic infective mycelium that penetrate the peanut gynophore in the soil, colonize the tissues, and replace the cells with reddish-brown teliospores [32, 33]. When the affected mature pods open, start the dispersion process (**Figure 5**). There are three important dispersion methods: wind, machinery and seeds. During the harvest activities, a cloud of dust is generated. Teliospores are transported by wind to adjacent fields. According to the Ref. [34], teliospores can travel at least 400 m depending on the wind rate. Peanut processing plants are one of the most important sources of teliospores. In the shelling process, a totally smutted pods release millions of spores that are transported by wind. Long-distance dispersion is attributed to infested machinery and infected seeds. Infested machinery can carry teliospores from one infected field to another located in other provinces or bordering countries [17]. Using the seeds, the pathogen can disperse even to other continents through exportation activities [35]. Teliospores can infest externally asymptomatic seeds or in small lesions that are not detected in the process of selection of seeds [31, 36].

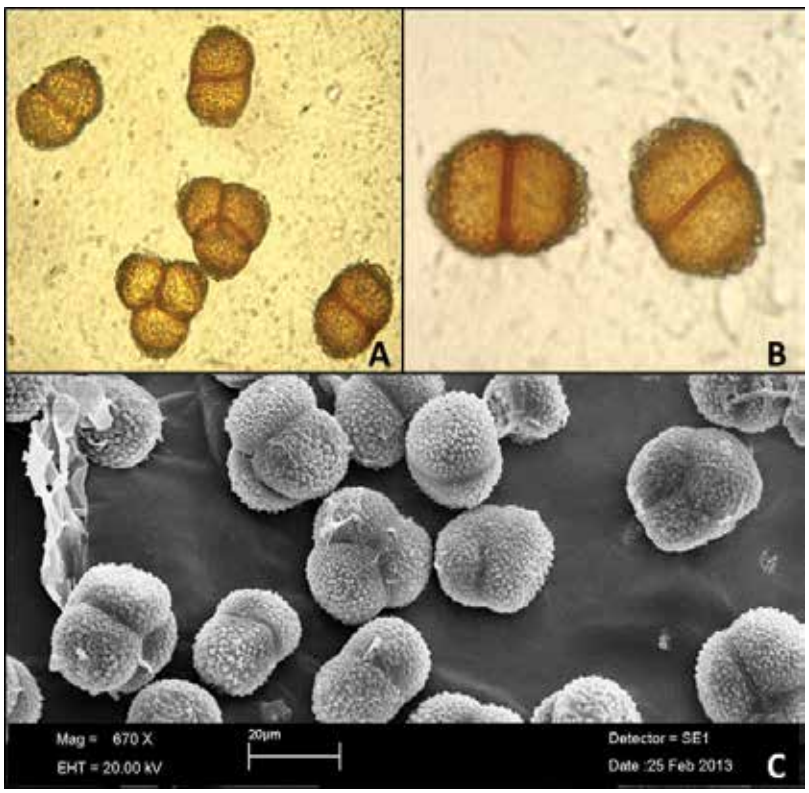

Figure 4. Teliospores of *Thecaphora frezii*. A, B: Teliospores observed under light microscope 40X. C: Scanning electron micrographs of multicellular teliospores.

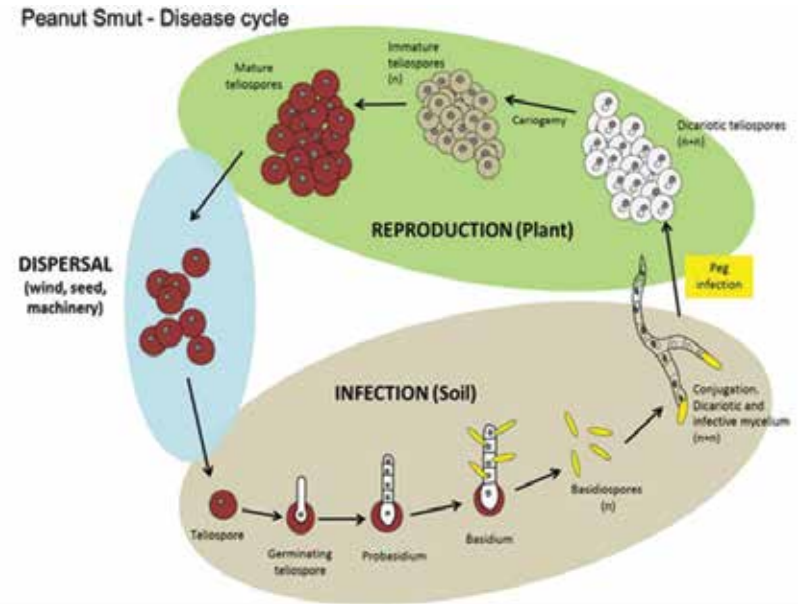

Figure 5. Peanut smut disease cycle [17].

4. Distribution of the disease and yield losses

Peanut smut is distributed across the entire production area in Argentina [22]. It was first reported in the north of Córdoba [14] and from this region began to expand. The first survey was performed in 2008 [31]. The data show that the prevalence was 10% in 1997 and increased to 24% in the next 10 years. In 2012, the prevalence was 100% in Córdoba, and 2 years later, the prevalence was 100% in Argentina peanut area, including Salta, Jujuy, La Pampa, and San Luis [22, 23]. To determine the yield losses in Córdoba province, [37] assessed peanut smut in 40 fields from peanut area in 2015/2016 growing season. The data show yield losses of 27.419 tons (USD 14.151.800), representing 3.15% of the total production. In some fields, yield losses of 35% with incidence values to 52% could be observed. The most affected region was in the north of Córdoba peanut area, with average incidence of 17% and yield losses of 21.894 tons. They observed that disease intensity decreases southward. This gradient is because the new production areas are there, away from processing plants, and a much smaller history of peanut crop than north (**Figure 6**). There are some studies about the yield loss estimation. Peanut smut incidence above 14% can be considered as a damage threshold, and it is estimated that 1% increase in incidence can correspond to a 1.2% decrease in yield. The loss estimation in field with low inoculum density is erratic, whereas the correlation was high between the losses and the n° teliospores/gr. of soil in field with high inoculum density [38]. In Ref. [37], peanut smut was evaluated in different fields, and a linear relationship (R^2 0.92–0.97) between estimated yield losses and disease intensity was observed.

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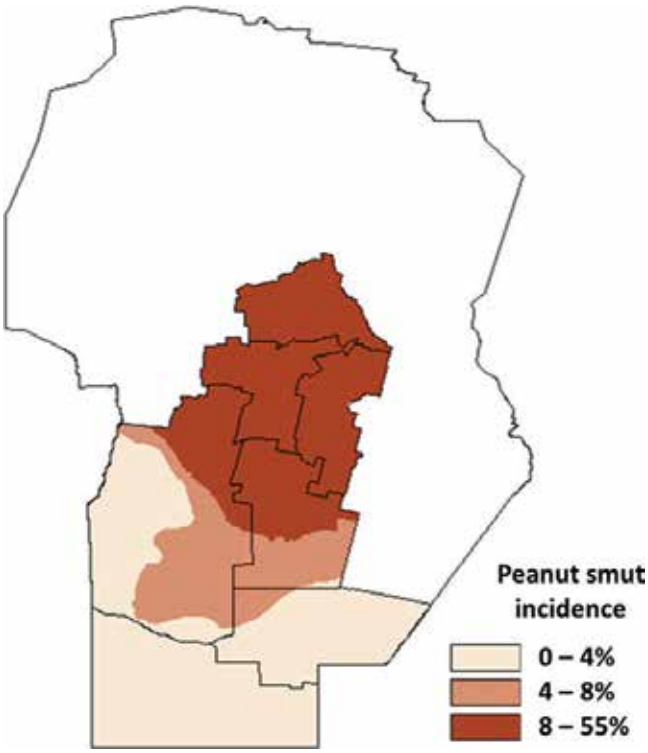

Figure 6. Gradient of disease incidence decreasing southward. Different colors represent peanut smut incidence found in different zones [37].

5. Teliospore detection

Detection and quantification of spores, both in soil and seeds, represent an important tool to epidemiological management of disease. Peanut smut is considered a monocyclic disease since there is no secondary inoculum produced in the same growing season and polyetic since annual inoculum accumulation affects subsequent seasons [17]. Knowing the amount of teliospores in the soil, it is possible to predict the incidence of the disease in the harvest [24]. On the other hand, by determining the amount of inoculum transported by seeds, it is possible to identify the contribution of teliospores to the field, which increases the probability of occurrence of the disease in future peanut plantations. In 2008, the detection of teliospores from soil samples using a microscope was performed [31]. The same technique was also employed to quantify spores on peanut seeds [39]. Using molecular methods, in 2014, a PCR protocol to detect teliospores was described. This begins with washing off a kernel sample with distilled water, separating supernatant water from kernels and extracting fungal DNA from the obtained pellet. PCR amplification is then performed using specific primers designed for *T. frezii*. This method is highly sensitive and can detect the presence of ten teliospores (10^{-4} pg. DNA) from a sample of 400 kernels. Its specificity is achieved by using primers that do not hybridize with the DNA of other seed-borne pathogens, such as *Sclerotinia minor, S.*

sclerotiorum, *Sclerotium rolfsii*, or *Fusarium solani* [40, 41]. These primers can be adapted for teliospore quantification using real-time PCR (RT-PCR), with a detection sensitivity of two teliospores in a sample of 400 seeds [42]. The development and use of detection techniques are important, because Argentina is the only country in South America that has reported this disease, hence the importance of implementing effective peanut smut management strategies that can minimize yield losses and contaminations of exportation products [8, 35, 43].

6. Peanut smut management

In the last years, various researchers have been studying different strategies for peanut smut control. Among them are the development of resistant cultivars, cultural practice, chemical and biological control.

6.1. Host resistance

High levels of infestation in the soils of the northern Córdoba peanut area and the lack of commercial fungicide that provides high disease control make the genetic resistance the main tool for an integrated management approach [17, 44]. Currently, 100% of cultivars widely planted are susceptible, which have favored *T. frezii* to quickly spread throughout the growing region of Córdoba [45, 46]. There is differential response of some cultivars against peanut smut. Pepe ASEM-INTA cultivar had 34% disease incidence and Colorado Irradiado-INTA 71% under favorable conditions [47]. Granoleico, the most widely distributed cultivar on Córdoba peanut area, shows incidence of 50% in high infested soil [27, 37, 47–49]. In 2011, it was reported that wild species *Arachis correntina* and *Arachis valida* show resistance to *T. frezii* [32]. Recently, INTA released a new cultivar, Ascasubi Hispano, with high tolerance to peanut smut (less than 2% of affected pods in high infested soil), but is not high-oleic cultivar. Among the tools used to facilitate the transfer of resistance are the molecular methods. In 2015, molecular markers associated with the tolerance to peanut smut were found [50]. Marker-assisted selection represents an important tool for breeding programs, as it would save time and money in the development of smut-tolerant cultivars [51]. There are cultivars highly tolerant to peanut smut in the final stages of evaluation [52]. This material would also be useful to prevent the spread of the pathogen to new areas of production [17]. Another aspect to consider is the ability of *T. frezii* to adapt to new cultivars. Cazón (*unpublished*) developed molecular markers to study the temporal variation of *T. frezii* isolates from different years and locations. If the rate of genetic variation of the pathogen is high, the resistance of the new cultivars against smut could be broken. Because of this, it is necessary that breeding programs continue to develop peanut smut-resistant cultivars.

6.2. Cultural practice

These strategies were focused on the reduction of initial inoculum. For peanut smut management, crop rotation schemes of more than 3 years without peanuts showed low *T. frezii* teliospore density in soil [53]. In addition, peanut crops preceded by corn exhibited lower

incidence of peanut smut than those preceded by soybean [35]. Different authors used deep tillage to burying teliospores 20 cm of depth reducing disease incidence, since peanut pods develop at a planting depth between 5 and 7 cm [54, 55]. Other practices were focused on modifying the soil chemical and physical properties. The objective is making the soil suppressive to *T. frezii*. The use of gypsum ($CaSO_4$) and dolomite to modify the pH of soil can contribute to a partial reduction of peanut smut intensity [21]. Phosphate-containing products were assessed to reduce the smut damage. This provided a control efficiency of 16% reduction in peanut smut intensity [56, 57].

6.3. Biological control

This area is not highly developed to peanut smut management. There are only some experiments done that used bioformulations based on *Trichoderma harzianum.* Control efficiency reaches 24% in incidence and 25% in severity [58, 59]. Researchers of IPAVE-CIAP-INTA have assessed the bioformulations based on *Bacillus subtilis* in different doses combining with soil amendments. Therefore, cultural practices and biological control still need to be studied in greater depth for sustainable and economic management of the disease.

6.4. Crop protection fungicides

The first experiences were performed in vitro using seed treatment fungicides. Teliospores were germinated using leaves and fruit extracts with the addition of PDA medium (39 gr/l). Later, a single colony was picked in media with different fungicides and evaluated the mycelial growth (**Figure 7**). All tested fungicides were effective, but the result could not be extrapolated to the field [33]. This is because the infection process occurred when fungicides from seed treatments have lost their protective effects [17]. The highest control efficiencies were achieved using crop protection fungicides in soil-directed applications. This is because the infective processes occur in the soil, where teliospores infect the gynophore [25, 31, 44]. Refs. [37, 60-62] show that these strategies were more effective at controlling peanut smut than leaf applications. They performed night spraying because in this moment, peanut leaves fold up, and the soil surface is easily reached [17, 63, 64]. Using strobilurin/triazole mixtures like

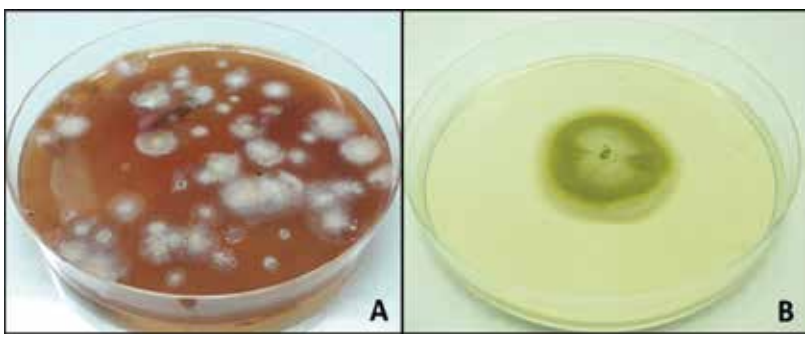

Figure 7. Colonies of *T. frezii*. A: Teliospores germinated in PDA medium (39 gr/l) composed by fruit extracts. B: *T. frezii* micelial growth of 7 days old plated on PDA medium (39 g / l).

Figure 8. Experimental granulate fungicide applied on peanut.

picoxystrobin + cyproconazole, control efficiencies reach 47% at a rate of 900 cc/ha or 1000 cc/ha in two applications in R2 (beginning peg) [65] and 10 days [17, 47]. An experimental granular fungicide with a slow release for longer protection during crop pegging was assessed [26]. Control efficiency reached 42% in incidence if the granulate is applied at flowering (**Figure 8**). Another study performed in INTA affirms that using high dose and night spraying for the first late leaf spot (*Cercosporidium personatum*) application ensures a 35% smut incidence reduction [37]. Control efficiencies close to 50% for peanut smut is an acceptable value, so accompanied by efficient molecules, correct dose and good times, and application technologies, chemical control could contribute to integrated disease management [17].

7. Conclusions

Argentina is the only country that has reported the disease in cultivated peanuts. Currently, it is the most economically important peanut disease in the country. This is due to the characteristics of the pathosystem. These represent an important prejudice since the peanut industry has clearly agroexporting characteristics. The alert generated by smut in the producing countries is mainly due to the destructive power of the disease in the crop but also to the lack of efficient strategies to control the disease and the speed with which the pathogen spreads throughout the Argentinean peanut area in a short time. There were many factors that contributed to the spread of the pathogen. Among them are the low diversification of cultivars used in recent years, the lack of crop rotations and the use of nonspecific fungicides. One of the most important facts is related to the increase of production scale in the 1990s. Many small-sized growers that used to harvest their own seeds were removed from the production system since they could not afford the costs of change of scale. Big growers produced and processed peanuts on a larger scale which included both healthy and diseased fields. This encouraged seed contamination and spread of the pathogen spores. Based on the current

understanding about the pathosystem and effectiveness of various techniques described, it is possible to recommend disease management tactics to minimize yield losses:

i. The use of pathogen-free seed: This is important to not increase the amount of teliospores in the soil.

ii. Plant peanuts in fields that have low inoculum density of *T. frezii* teliospores: In Córdoba province the healthiest area is in the south, to correspond to new production areas.

iii. The fields chosen to cultivate peanut are not to be close to peanut processing factories: Peanut processing plants are the most important sources of teliospores. Those teliospores can be transported by wind to adjacent fields.

iv. Spray fungicide mixtures including strobilurins and triazoles: azoxystrobin and cyproconazole are the most effective fungicides. Soil-directed spraying at the beginning of crop pegging and 7 days after.

It is important that additional research will be focused in determining the most effective agronomical practice with the most suitable application technologies that reach high control efficiencies. It is necessary that the recommended agricultural practices will be implemented in an entire peanut area, like a regional management. The development of molecular tools is important to facilitate the transfer of resistance to new cultivars and speed up the processes of obtaining varieties with good agronomic characteristics. The collaboration of all those involved in Argentinian peanut production systems is necessary for the management of peanut smut to be successful.

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Conflict of interest

All authors declare no conflict of interest about this publication.

Author details

Luis Ignacio Cazón[1]*, Juan Andrés Paredes[1] and Alejandro Mario Rago[1,2]

*Address all correspondence to: cazon.ignacio@inta.gob.ar

1 Instituto de Patología Vegetal, CIAP – INTA, Córdoba, Argentina

2 Facultad de Agronomía y Veterinaria, UNRC, Córdoba, Argentina

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Section 2

Advances in Bacterial Plant Pathology

Chapter 3

Systematic Identification of the *Xylophilus* Group in the Genus *Bursaphelenchus*

Jianfeng Gu

Additional information is available at the end of the chapter

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Abstract

The pine wood nematode (PWN) *Bursaphelenchus xylophilus* (Steiner & Buhrer, 1934) Nickle, 1970 is the agent responsible for pine wilt disease (PWD). This nematode has been killing native pine trees (*Pinus densiflora*, *P. thunbergii*, *P. luchuensis*) in Japan since the early twentieth century. It is the number one forest pest in Japan and has been spread to China, Korea, Portugal, and Spain. The nematode is native to North America (Canada, USA, Mexico) and is thought to have been carried to Japan at the beginning of the twentieth century on timber exports. Up to now, the genus *Bursaphelenchus* Fuchs, 1937 comprises nearly 120 species (14 groups). Around 14 species very similar to *B. xylophilus* are put together and named the *xylophilus* group. This chapter presents the grouping history, subspecies or genetic types in species of the *xylophilus* group, and an identification key for 14 species of the *xylophilus* group, ITS-RFLP identification, and other molecular identification methods are also discussed.

Keywords: morphology, molecular, ITS-RFLP, DNA barcoding

1. Introduction

Pine wilt disease (PWD), which is caused by pine wood nematode (PWN), *Bursaphelenchus xylophilus* (Steiner & Buhrer [6]) Nickle [1], has been devastating Japanese pine forests since the beginning of the twentieth century. For many years, the mass mortality of pine trees was supposed by attacks of beetles. Until 1971, *Bursaphelenchus* sp. was demonstrated as the causal agent of PWD by inoculation tests on *Pinus* spp. [2], and subsequently the nematode was described as *Bursaphelenchus lignicolus* [3]. After that, the PWN was first reported in the United States in 1979 [4]. Extensive surveys revealed the widespread distribution of the

nematode throughout the country [5], but no epidemic was found, and the disease occurred only on a few exotic pine species. The PWN was later proven to be the same species as the one described in Florida in 1934 [6], the name was then changed from *B. lignicolus* to *B. xylophilus* [7], and it has been indigenous to North America [8].

Later, the disease has spread into China in 1982, Korea in 1988, Mexico in 1993, Portugal in 1999, and Spain in 2011 [9], and it is now still a potential threat to pine forests worldwide.

In nature, *B. xylophilus* is spread from tree to tree through the activity of adult stages of wood-inhabiting longhorn beetles of the genus *Monochamus* (Coleoptera: Cerambycidae) for short distance. This transmits the nematode either to the shoots of living trees during maturation feeding either by sex or by oviposition of females. But human activity is responsible for the long-distance spread. It is widely accepted that national and international trade of pine logs and related packaging wood is the causal of PWN spreading, so national and international regulations (e.g., ISPM 15: FAO, 2003, revised 2009) were accompanied by intensive sampling and laboratory investigations for the presence of PWD in imported wood worldwide in order to significantly reduce the risk of the pest's spread. So, it is important to identify *B. xylophilus* to manage its further spreading and conduct early eradication plan.

Before 2000, there were only other two closely related species: *B. fraudulentus* Rühm [10] and *B. mucronatus* Mamiya and Enda [11] (*B. kolymensis* Korenchenko [12] was later considered as being synonymous with *B. mucronatus*). For a long time, in diagnostic protocol of *B. xylophilus*, it was morphologically compared with only *B. mucronatus* and *B. fraudulentus*, many PCR-based methods also used only these three species samples.

Since 2000, with further study of packaging wood and phoretic insects, more *Bursaphelenchus* species were discovered. Now, there are 110–120 known species in this genus [9] and 14 species in the *xylophilus* group. *B. xylophilus* (Steiner & Buhrer [6]) Nickle [1]; *B. fraudulentus* Rühm [10] (J. B. Goodey, 1960); *B. mucronatus* Mamiya and Enda [11]; *B. conicaudatus* Kanzaki et al. [13]; *B. baujardi* Walia, Negi et al. [14]; *B. luxuriosae* Kanzaki and Futai [15]; *B. doui* Braasch et al. [16]; *B. singaporensis* Zhang et al. [17]; *B. macromucronatus* Gu et al. [18]; *B. populi* Tomalak and Filipiak [19]; *B. paraluxuriosae* Gu et al. [20]; *B. firmae* Kanzaki et al. [21]; *B. koreanus* Gu et al. [22]; and *B. gillanii* Schönfeld et al. [23].

2. Grouping history

Giblin and Kaya [24] first separated five groups within *Bursaphelenchus* mainly according to spicule morphology; the *xylophilus* group contains three species, namely, *B. xylophilus*, *B. mucronatus*, and *B. fraudulentus*, all have large, paired, arcuate spicules with a sharply pointed rostrum, and a disk-like expansion, cucullus, and females of this group have a vulval flap (**Table 1**). Braasch [25] studied the morphological relationship between European *Bursaphelenchus* species in order to provide key characters for their taxonomic identification. She considered the number of incisures in the lateral field as a basic grouping feature, together with other features like spicule shape, number and position of caudal papillae, presence and size of a vulval flap, and the shape of female tail. Among the 28

Species	Main characters	Typical spicule shape	Typical female tail
B. xylophilus (R form)	Female tail cylindrical, terminus broadly rounded, without mucro (if there's a mucro, usually less than 2 μm)		
B. xylophilus (M form)	Like R form, but all females have a mucro, less than 3 μm on average (1.5–4.2 μm)		
B. fraudulentus	Spicule cucullus not clearly expanded, female tail cylindrical, c′ = 2.7–3.4, mucro usually present, about 1.5~2.6 μm		
B. mucronatus kolymensis	Female tail cylindrical or subcylindrical, mucro usually offset from the tail, mean length more than 4 μm		
B. mucronatus mucronatus	Female tail subcylindrical or conical, mucro not offset from the tail, about 4~7 μm		
B. conicaudatus	Female tail conical, ventrally bent, mucro about 2~3 μm, at the ventral position		
B. baujardi	Similar to *B. conicaudatus*, female tail conical, a small mucro present, length not clear		
B. luxuriosae	Female tail conical and clearly ventrally bent, terminus without mucro, roughed or irregular		
B. paraluxuriosae	Similar to *B. luxuriosae*, but female tail only slightly bent, without mucro, spicule without cucullus		

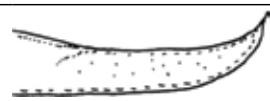
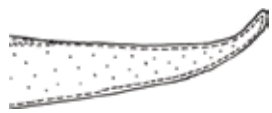
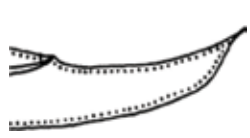
Species	Main characters	Typical spicule shape	Typical female tail
B. doui	Spicule length in chord 34~44 μm, the middle part nearly straight, female tail variable, usually show a mucro at the ventral position, about 2~4 μm		
B. singaporensis	Female tail without mucro, spicule length along the curved median line 41–48 μm, condylus continuous with the dorsal spicule line		
B. macromucronatus	Female tail conical, straight mucro usually continuous with tail, about 4.5 μm(2.5~6.5 μm)		
B. populi	Vulval flap ventrally bent with its distal half sunken in a conspicuous, sharp depression immediately posterior to the vulva		
B. firmae	Female mucro thick, terminus bluntly pointed		
B. koreanus	Spicule length along the curved median line 35–44 μm, condylus set off from dorsal spicule line, female tail conical and ventrally bent with slightly pointed, irregular, or roughened terminus		
B. gillanii	Female tail conical, mucro 5–7 μm, wide at the base		

Table 1. Main morphological characters of the ***xylophilus*** group.

conifer-inhabiting European species, she proposed eight groups. The *xylophilus* group (*B. xylophilus*, *B. mucronatus*, and *B. fraudulentus*) can easily be separated from all other species by the presence of four incisures, the typical shape of spicules, the special position of the caudal papillae, and the large vulval flap of females.

Ryss [26] considered that those characters like lateral lines, number and position of caudal papillae, and vulval flap are available for only some of the nominal species; thereby, their utility is limited. So, he studied 75 valid species of the genus *Bursaphelenchus* known that time. Only based on spicule structure, he sorted this genus into six groups: *hunti*, *aberrans*, *eidmanni*, *borealis*, *xylophilus*, and *piniperdae* groups. For the *xylophilus* group, its spicule is characterized by capitulum flattened anteriorly, small condylus, dorsal contour of the lamina distinctly angular in last third, and cucullus usually present (except in *B. crenati*). He listed ten species: *B. xylophilus*, *B. abruptus*, *B. baujardi*, *B. conicaudatus*, *B. crenati*, *B. eroshenkii*, *B. fraudulentus*, *B. kolymensis*, *B. luxuriosae*, and *B. mucronatus*. Later study showed that *B. abruptus*, *B. crenati*, and *B. eroshenkii* were definitely different from the *xylophilus* group [27]. *B. crenati* has a different position of the caudal papillae (the double pair in front of the bursa is missing), the presence of a vulval flap is questionable, and the spicules do not show a cucullus. Additionally, it is transmitted by a bark beetle, a scenario not typical for the *xylophilus* group. *B. eroshenkii* has five incisures in the lateral field, only five caudal papillae (seven in the *xylophilus* group) and no vulval flap [28]. The spicule shape of *B. abruptus* is not typical.

Braasch [29] stated that the *xylophilus* group of the genus *Bursaphelenchus* can be clearly distinguished from other species of the genus by the presence of four lateral lines, the presence of a vulval flap in females, a characteristic shape of the male spicules, and the arrangement of the seven caudal papillae. An identification key of the nine species of the *xylophilus* group was presented, and *B. kolymensis* was considered to be the European type of *B. mucronatus*.

Later, with the development of the molecular methods, especially sequencing technique, more *Bursaphelenchus* sequences are available in the GenBank. Based on morphological characters and phylogenetic analysis [27], the genus is divided into eight groups with four incisures in the lateral field (*xylophilus*, *okinawaensis*, *africanus*, *fungivorus*, *cocophilus*, *kevini*, *tokyoensis* and *sexdentati* groups), four groups with three incisures (*eggersi*, *eremus*, *hofmanni*, and *leoni* groups), and two groups with two incisures (*abietinus* and *sinensis* groups). Most of the groups are well separated by both morphological and molecular studies.

3. Subspecies or genetic types in species of the *xylophilus* group

Bursaphelenchus mucronatus Mamiya and Enda [11] was first found from pine trees in Japan. Braasch [30] reported for the first time *B. mucronatus* in timber imports from Siberia and in forest trees in Germany. These populations (later on named "European genotype" or "European type") had shown morphological and morphometric deviations from Japanese *B. mucronatus* isolate. Separate species status for Japanese and European *B. mucronatus* was postulated on the basis of sequence differences of an amplified fragment of the heat shock 70A gene [31]. However, successful mating experiments of a European *B. mucronatus* with a Japanese isolate argued against this idea [32].

Braasch et al. [33] proposed the two *Bursaphelenchus mucronatus* types to be subspecies. The European type is named *B. mucronatus kolymensis*, and the East Asian type is named *B. mucronatus mucronatus*. The earlier described *Bursaphelenchus kolymensis* corresponds to *B. mucronatus kolymensis* in morphological characters. The two subspecies show morphological

differences in the shape of female tail, length of mucro, position of excretory pore, and also small differences in spicule shape. They can be distinguished by their ITS-RFLP patterns based on restriction fragments obtained with enzymes *Rsa* I and *Hae* III. Based on sequence analysis of ribosomal ITS1/ITS2, LSU D2/D3, and mitochondrial COI regions, a clear subdivision of the two isolate groups (subspecies) has been confirmed.

Since the report of a mucronate ("M") form of *B. xylophilus* detected from balsam fir (*Abies balsamea*) in Minnesota and Wisconsin, USA [34], uncertainty in morphological distinction of *B. xylophilus* from related species became evident. For a long time, it is morphological and molecular characters are not clear. Gu et al. [35] made a morphological and molecular study based on five isolates of "M" form of *Bursaphelenchus xylophilus*, together with the round-tailed ("R") form of *B. xylophilus* and *B. mucronatus* (both subspecies), and founded that the spicules of these species (types or forms) are similar. The "M" form of *B. xylophilus* is distinguished from the "R" form of *B. xylophilus* by a distinct mucro at the female tail end. It differs from the *B. mucronatus kolymensis* by slightly shorter female tail mucro and position of excretory pore. It is distinguished from *B. mucronatus mucronatus* by female tail shape and shorter female tail mucro. The conventional five restriction endonucleases (*Rsa* I, *Hae* III, *Msp* I, *Hinf* I, and *Alu* I) used for obtaining ITS-RFLP patterns of *Bursaphelenchus* species cannot distinguish the "M" and "R" form of *B. xylophilus*, but the two forms can be differentiated by the use of two additional restriction endonucleases (*Hpy*188 I and *Hha* I). The molecular phylogenetic analysis based on the sequences of D2D3 LSU rDNA, ITS1/2 region, and mtCOI revealed that the "M" form of *B. xylophilus* is genetically closest to the "R" form of *B. xylophilus*, and that their sequence divergence is small.

4. Morphological characters of the *xylophilus* group

According to Braasch et al. [27], the *xylophilus* group is characterized by four lateral lines; seven caudal papillae; conspicuous P4, P3, and P4 papillae adjacent to each other (double pair) just anterior to bursa; spicules long, slender, and semicircular with angular lamina in posterior third; capitulum fattened with small condylus and distinct rostrum; cucullus present (for *B. fraudulentus* and *B. paraluxuriosae*, spicule cucullus is not clearly visible); and large vulval flap.

But lateral lines and caudal papillae are not easy to be seen sometimes, so typical male spicule shape and female vulval flap should be the main grouping characters [35]. In all known *Bursaphelenchus* species, only *B. masseyi*, *B. trypophloei*, and *B. abruptus* may be confused with *B. xylophilus* group. All their females have a vulval flap, but their spicules are not typical. *B. trypophloei* and *B. masseyi* differ in having relatively short rostrum, and the angular contour of the dorsal lamina is usually indistinct. *B. abruptus* differs in different ventral curvatures of the spicules.

5. Morphological identification of *B. xylophilus* with a key

Usually, R form of *B. xylophilus* is distinguished from other species by cylindrical female tail with bluntly rounded terminus, without mucro, or in some cases, some females will show a mucro, which is less than 2 μm.

But the mucro character of the R form of *B. xylophilus* is not always stable; it depends on different hosts and environmental situations. Braasch [36] reported that when an R form *B. xylophilus* isolate (US15) was re-extracted from trees 3 months after inoculation experiment, 35% of females were round-tailed, 8% had conical tails, and 17% had a distinct mucro (up to 4–5 μm), whereas 40% had a very small mucro of 1 μm length. Zheng et al. [37] reported that an R form *B. xylophilus* was detected from a pine tree in Ningbo, China; all females had a distinct mucro, ranging from 0.5 to 2.9 μm (mean 1.7 μm), but the mucro disappeared after culturing on *B. fuckeliana*. Gu et al. [35] also reported an R form *B. xylophilus* isolate (4049); about half of the females detected from packaging wood had a round tail, and the other half showed a

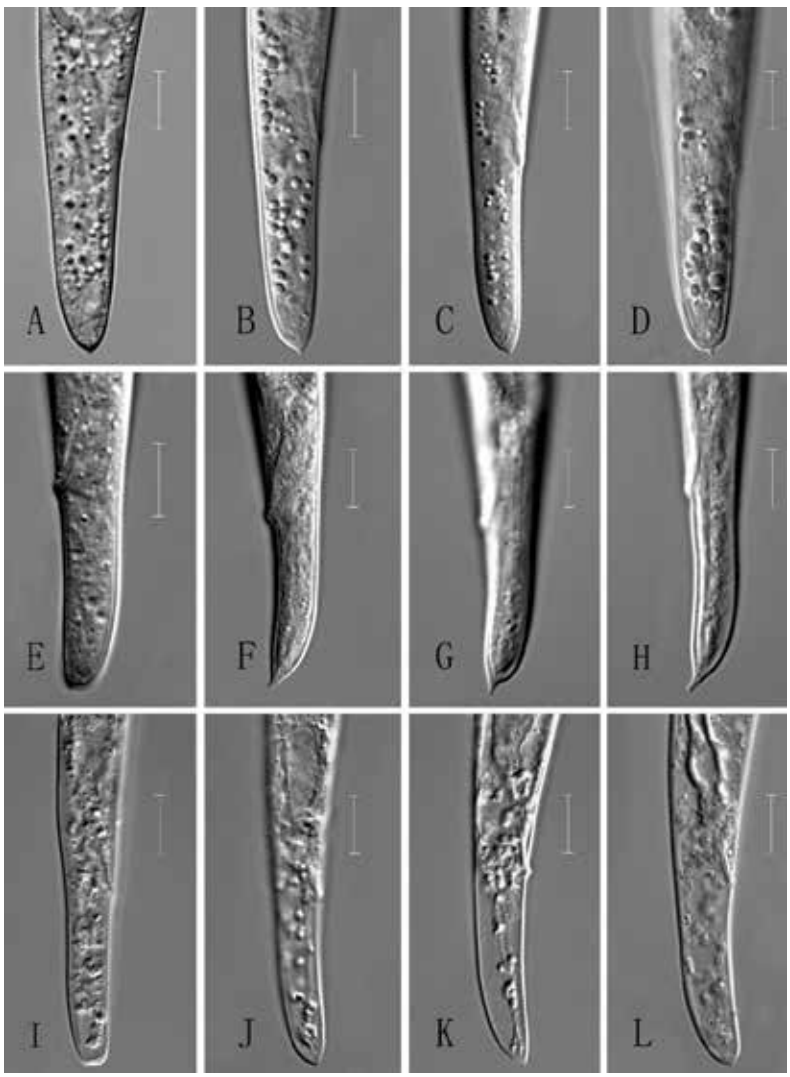


Figure 1. Light photomicrographs of female tails of "R" form of *Bursaphelenchus xylophilus* (isolate 4049) in different situations: A–D, detected from the packaging wood; E–H, after culturing on *B. fuckeliana*; and I–L, after culturing on *Pestalotiopsis* sp. (scale bars = 10 μm).

very small mucro about 0.5–1 μm long. But after culturing on *B. fuckeliana* for 1 month, more than half of females showed a mucro of about specimens, a mucro of less than 0.5 μm long, or no mucro. However, after being cultured on *Pestalotiopsis* sp., apart from some round-tailed females, most females had a bluntly pointed tail terminus (**Figure 1**).

Typical R form of *B. xylophilus* can be distinguished from other species of the *xylophilus* group by the female tail shape. *B. populi* sometimes also shows a cylindrical female tail without mucro, but they can be separated by the vulval flap ventrally bent with its distal half sunken in a conspicuous, sharp depression. Identification of the M form of *B. xylophilus* is more difficult. Females in mucronate populations generally show a mucro on the female tail end, on average 2.2–3.0 μm long (1.5–4.2 μm). Its mucro shape does not change even after culturing for many years. The M form of *B. xylophilus* is morphologically most similar to the *B. mucronatus kolymensis*. It is distinguished from it by slightly shorter mucro on female tail (mean 2.2–3.0 μm *vs.* 3.0–5.0 μm) and the position of excretory pore. Up to now, M form of *B. xylophilus* has only been reported in North America, and its report in China and Taiwan is still questionable. Due to a certain variation in characters between populations and different hosts and environmental situations, it is essential to perform molecular test in case of doubt.

The following dichotomous key of species of the *xylophilus* group is based on the female tail shape (conical or cylindrical, with or without mucro, and mucro length), vulval flap shape (straight or bent), and spicule size and shape (with or without cucullus).

1.	(a) Posterior to the vulva	*B. populi*
	(b) Vulval flap bent and to the vulva not clear	2
2.	(a) Spicule cucullus not clearly expanded	3
	(b) Spicule cucullus expanded	4
3.	(a) Female tail cylindrical, c′ = 2.7–3.4, mucro present	*B. fraudulentus*
	(b) Female tail conical, c′ = 4–5, without mucro	*B. paraluxuriosae*
4.	(a) Average c′ > 4, female tail conical	5
	(b) Average c′ < 4, female tail cylindrical, subcylindrical, or conical	9
5.	(a) Female tail without mucro	6
	(b) Female tail with mucro	8
6.	(a) Spicule length along the curved median line 27–30 μm	*B. luxuriosae*
	(b) Spicule length along the curved median line more than 35 μm	7
7.	(a) Spicule length along the curved median line 35–44 μm, condylus set off from dorsal spicule line	*B. koreanus*
	(b) Spicule length along the curved median line 41–48 μm, condylus continuous with the dorsal spicule line	*B. singaporeinsis*
8.	(a) Stylet with small knob, excretory pore ranging from median bulb to hemizonid, c′ = 3.6–5	*B. conicaudatus*
	(b) Stylet without small knob, excretory pore at the position of median bulb, c′ = 3–4	*B. baujardi*

9.	(a) Spicule length in chord 34~44 μm, the middle part nearly straight	*B. doui*
	(b) Spicule length in chord <34 μm, the middle part slightly ventrally curved	10
10.	(a) Female tail cylindrical, terminus broadly rounded, without mucro (some females may possess a short process at the tail terminus, usually less than 2 μm	*B. xylophilus* (R form)
	(b) Female tail cylindrical, subcylindrical, or conical, terminus with mucro, more than 2 μm	11
11.	(a) Mucro usually continuous with tail	12
	(b) Mucro usually offset from tail	15
12.	(a) Spicule condylus dorsally not offset, body slim (a > 40)	*B. mucronatus mucronatus*
	(b) Spicule condylus dorsally offset, body stout (a < 40)	13
13.	(a) Female mucro terminus pointed	14
	(b) Female mucro terminus bluntly pointed	*B. firmae*
14.	(a) Female tail straight	*B. macromucronatus*
	(b) Female tail slightly bent, dorsally stronger bent than ventrally	*B. gillanii*
15.	(a) Mucro mean length more than 4 μm	*B. mucronatus kolymensis*
	(b) Mucro mean length less than 3 μm	*B. xylophilus* (M form)

6. Identification of the *xylophilus* group species with ITS-RFLP method

Application of ITS-RFLP analysis to *Bursaphelenchus* species identification was first described in 1998 [38, 39]. In this technique, a region of ribosomal DNA (rDNA), containing the internal transcribed spacer regions ITS1 and ITS2, is amplified by PCR method with forward primer F194 5′-CGTAACAAGGTAGCTGTAG-3′ (Ferris et al.) and reverse primer 5368 5′-TTTCACTCGCCGTTACTAAGG-3′ (Vrain) [40, 41], and, subsequently, the PCR products were digested with five restriction endonucleases *Alu* I, *Hae* III, *Hinf* I, *Msp* I, and *Rsa* I to get the restriction fragment length polymorphisms. Using the same set of five restriction enzymes, species-specific ITS-RFLP reference patterns were compiled for 11 *Bursaphelenchus* species in 1999 [42] and extended to 26 species in 2005 [43]. The technique has proven to be a valuable tool in identification of nematodes isolated from imported wood in quarantine control or forest surveys [44–47]. Wolfgang et al. (2009) produced ITS-RFLP reference profiles of 44 *Bursaphelenchus* species [48], including two intraspecific types in each of *B. mucronatus* and *B. leoni*. Though in the case of *B. corneolus*, *B. lini* (later identified as *Deviburs aphelenchus lini*), *B. singaporensis*, *B. sexdentati*, and *B. doui* [49], additional bands in the patterns of certain isolates or individual nematodes were observed which may be explained by ITS sequence microheterogeneity, i.e., the presence of ITS sequence variants within the number of rDNA tandem repeats, but they did not seriously impair identification of species based on the overall reference patterns. ITS-RFLP analysis has proven valuable not only for differentiation of the pathogenic pine wood nematode, *B. xylophilus*, from related species but also useful in

other *Bursaphelenchus* identifications. In many recent descriptions of new *Bursaphelenchus* species, ITS-RFLP profiles have been used as additional species identification criteria.

The abovementioned traditional ITS-RFLP method cannot separate M and R form of *B. xylophilus*, but according to Gu et al. [33], the two forms can be differentiated by the use of two additional restriction endonucleases (*Hpy188* I and *Hha* I).

7. Other molecular identification methods

Besides RFLP method, many species-specific PCR and real-time PCR methods were developed for *B. xylophilus* identification [50–58]. By real-time PCR [59] or loop-mediated isothermal amplification (LAMP) methods [60], *B. xylophilus* can be detected directly from wood. But we should notice that those methods were developed years ago, now more species in the *xylophilus* group are known, and the results may be questionable. And when molecular tests are used for quarantine purposes to detect *B. xylophilus* in wood products, it is essential to recognize that both live and dead nematodes can be detected by these tests.

More recently, Ye et al. [60] developed a real-time PCR assay for PWN identification [61]. Based on DNA sequence analysis on the ribosomal DNA small subunit, large subunit D2/D3, internal transcribed spacer (ITS), and mitochondrial DNA cytochrome oxidase subunit one on the aphelenchid species, they developed a rapid and accurate PWN identification method targeting the ITS-1. A total of 97 nematode populations were used to evaluate the specificity and sensitivity of this assay, including 45 populations of *B. xylophilus*; 36 populations of 21 other species of *Bursaphelenchus* which belong to the *abietinus*, *cocophilus*, *eggersi*, *fungivorus*, *hofmanni*, *kevini*, *leoni*, *sexdentati*, and *xylophilus* groups and one unassigned group from a total of 13 groups in the genus *Bursaphelenchus*; 15 populations of *Aphelenchoides besseyi*, *A. fragariae*, *Aphelenchoides* species, and *Aphelenchus avenae*; and one population of mixed nematode species from a soil sample. This assay proved to be specific to *B. xylophilus* only and was sensitive to a single nematode specimen regardless of the life stages present. This approach provides rapid species identification necessary to comply with the zero-tolerance export regulations.

Nucleic acid sequencing methods have undergone tremendous advances over the past decade. Now, many 18S, ITS, and 28S gene sequences have been determined for *Bursaphelenchus* species, and they are deposited in the GenBank database (http://www.ncbi.nlm.nih.gov/). In general, the comparison of those genes with reference data using sequence and phylogenetic analysis allows classification of nematode samples and establishing identification. Determinations of clades to which samples belong and the level of the interspecific variation are two approaches used together for molecular identification.

DNA sequencing method has been used widely in the last decade. But this method is not standard: different target genes and different primers are used, and sequences are analyzed with different methods in different labs.

DNA barcoding is a generic diagnostic method that uses a short standardized genetic marker in an organism's DNA to aid species identification. An organism is identified by finding the closest matching reference record in a database containing large amounts of barcode sequence

data. The first genetic marker to be described as a "barcode" was the mitochondrial cytochrome c oxidase I (COI) gene which is used for species identification in the animal kingdom [62]. According to Quarantine Barcoding Of Life (QBOL) project financed by the Seventh Framework Program of the European Union (www.q-bank.eu), first, a 1600 bp fragment of the small subunit (SSU) 18S rDNA gene can be PCR amplified and sequenced using primers 988F, 1912R, 1813F, and 2646R [63]. The obtained sequence data is used for identification to the genus and sometimes to species level. However, in some cases the SSU does not contain sufficient variation for identification to the species level, and additional sequences of the LSU (28S) rDNA or COI gene may be required to confirm the identification.

He and Gu [64] evaluated the applicability of 28S, 18S, and ITS loci as candidate DNA barcode markers for the *xylophilus* group of the genus *Bursaphelenchus*; they demonstrated that the average intraspecific divergences of 28S (not distinguishing two subspecies of *B. mucronatus*), 28S (distinguishing two subspecies of *B. mucronatus*), 18S, and ITS were 0.0071, 0.0030, 0.0007, and 0.0043, respectively, and, for interspecific divergences, were 0.0476, 0.0454, 0.0052, and 0.1556, respectively. The genetic distances between intraspecific and interspecific divergences of 28S and 18S loci showed some overlapping, but ITS loci had some degree of barcoding gap. The NJ trees from 28S and ITS loci with reliable bootstrap value could effectively separate 14 species of the *B. xylophilus* group into an independent branch. Furthermore, 28S locus could identify two subspecies of *B. mucronatus* well. The NJ tree of 18S locus demonstrated that *B. gillanii*, *B. firmae*, and *B. mucronatus* were mixed and difficult to be separated each other. In conclusion, 28S and ITS loci were suggested as candidate barcode genes for the *B. xylophilus* group due to their larger barcoding gap and higher species resolution.

When sequencing is more easy, quick, and cheap, and more sequences are available in the database, DNA barcoding will be the best way for species identification for genus *Bursaphelenchus*, even for other genera in the future.

8. Conclusion

After devastating a vast area of pine forests in Asian countries, the pine wilt disease was spread into European forests in 1999 and was causing a worldwide concern. To date, about 120 species of the genus *Bursaphelenchus* have been described, and 14 groups is suggested. About 14 species very similar to *B. xylophilus* are put together and named the *xylophilus* group. The *xylophilus* group is characterized by four lateral lines; seven caudal papillae; conspicuous P4, P3, and P4 papillae adjacent to each other (double pair) just anterior to bursa; spicules long, slender, and semicircular with angular lamina in posterior third; capitulum fattened with small condylus and distinct rostrum; cucullus present or not clearly visible; and large vulval flap. Subspecies (*B. mucronatus kolymensis* and *B. mucronatus mucronatus*) and two genetic types ("M" form and "R" form of *B. xylophilus*) exist in the group, and the mucro character of *B. xylophilus* is not always stable, which depends on different hosts and environmental situations, making identification complicated. Usually, R form of *B. xylophilus* is distinguished from other species by cylindrical female tail with bluntly rounded terminus, without mucro, or in some cases, some females will show a mucro, which is less than 2 μm. Due to a certain variation in characters between populations and different hosts and environmental situations,

it is essential to perform molecular test in case of doubt. ITS-RFLP identification and other molecular identification methods are also discussed; DNA barcoding by using the 28S and ITS loci will be a reliable and convenient method in the future.

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Author details

Jianfeng Gu

Address all correspondence to: jeffgu00@qq.com

Technical Centre, Ningbo Entry-Exit Inspection and Quarantine Bureau, Ningbo, Zhejiang, P. R. China

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Chapter 4

Quorum Sensing in Gram-Negative Plant Pathogenic Bacteria

Siphathele Sibanda, Lucy Novungayo Moleleki,
Divine Yufetar Shyntum and Teresa Ann Coutinho

Additional information is available at the end of the chapter

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Abstract

Plant pathogenic bacteria regulate expression of specific genes through quorum sensing (QS). Some bacteria encode a single or more than one QS system while others encode a single LuxI and two or more LuxR homologs. Not all plant pathogenic bacteria encode the LuxI and in these situations the LuxR modulates cell behavior in a cell density manner by utilizing signal molecules that are produced by their plant hosts. The advantage of having more than one system is still not well understood. However, it has been speculated that it is essential for regulation of QS traits in different environmental conditions. Quorum sensing systems in plant pathogenic bacteria include those that use acyl homoserine lactones, 3-hydroxy palmitic acid methyl ester or methyl 3-hydroxypalmitate, virulence factor modulation genes and diffusible signal factors. This chapter discusses the various QS systems in Gram-negative plant pathogenic bacteria, notably those listed as the top 10 plant pathogenic bacteria that cause significant reduction in yields and inflict economic losses in agriculture. In addition, it explores the various biological processes influenced by QS and the extent of QS regulons in these bacteria.

Keywords: plant pathogenic bacteria, quorum sensing, signal molecules, QS regulon, inter kingdom signaling

1. Introduction

Bacteria are able to adapt to constantly changing environmental conditions by altering expression of genes that are crucial for fitness, adaptation and survival [1]. Some environmental changes encountered by bacteria include temperature, pH, osmolarity and nutrients availability [2]. Such environmental changes encountered by bacteria are best dealt with by

a group effort instead of by individual cells [3]. Most bacteria thus respond to fluctuations in both biotic and abiotic environments by altering gene expression in a process termed quorum sensing (QS). Quorum sensing refers to a process where bacteria accumulate, detect and respond to small diffusible communication signals called autoinducers [3]. The amount of signal molecules is directly proportional to the population cell density of the signal-producing bacteria [2]. Quorum sensing results in communication between cells in a population and leads to simultaneous coordinated behavior within a population [3].

The sequencing of genomes of plant pathogenic bacteria coupled with research on pathogenicity factors in different bacteria has revealed the involvement of QS in the regulation of virulence genes. The role of various QS systems is related to several phenotypes, and has been described (for examples see [4–24]) using methods such as site-directed mutagenesis or transposon mutagenesis. However, these methods fall short of clearly showing the biological pathways or genes that influence the observed QS phenotypes. One method used to circumvent this limitation is to determine the entire regulon controlled by QS using several techniques such as microarrays and RNA-Seq. These studies have unraveled the genes under the control of QS in several bacteria. This chapter focuses mainly on QS systems found in Gram-negative plant pathogenic bacteria, notably those listed as the top 10 most significant plant pathogenic bacteria [25]. In addition, it will explore the various biological processes in bacteria that are influenced by QS and highlight the difference in the size of the QS regulon for the different systems going from only a few genes to 26% of the transcriptome as exemplified by the *in planta* QS regulon of *Pectobacterium atrosepticum* (*Pa*) [26].

2. Overview of QS in the top 10 plant pathogenic bacteria

Plant pathogenic bacteria cause a reduction in yields and inflict economic losses in agriculture [25]. The key to a successful plant infection is regulation of pathogenicity traits. Plant pathogenic bacteria regulate expression of specific genes through QS. The major QS signals that have been characterized in plant pathogenic bacteria include acyl homoserine lactones (AHLs) and diffusible signal factor (DSF). Some plant pathogenic bacteria have a single QS system while others have more than one QS system. The advantage of having more than one QS system is still not well understood. However, it has been speculated that this could be beneficial for the regulation of QS traits in different environmental conditions [4]. Moreover, some plant pathogenic bacteria have been found to modulate their behavior in a cell density manner by utilizing some signal molecules that are produced by their plant hosts [5, 8, 27, 28].

The LuxI/R QS (depicted in **Figure 1**) has been extensively studied in a large number of Gram-negative plant pathogenic bacteria. This system regulates expression of various genes (i.e. for example see [9–11, 26, 29–34]). Bacteria encode one or more AHL synthases and one or more protein receptor molecules (discussed below). It was thought that in mixed populations, each bacterial species detects and responds to its specific AHL molecule [3]. However, there is evidence of inter-specific signaling that is the basis of the detection of AHL production by *Chromobacterium violaceum* 026 bio reporter [7]. Notably, the LuxI/R QS in plant pathogenic bacteria is species specific, for example, QS target genes differ in different *Pectobacterium* QS systems [12, 35] and the SolI/R QS plays no role in pathogenicity of *Ralstonia* [36, 37].

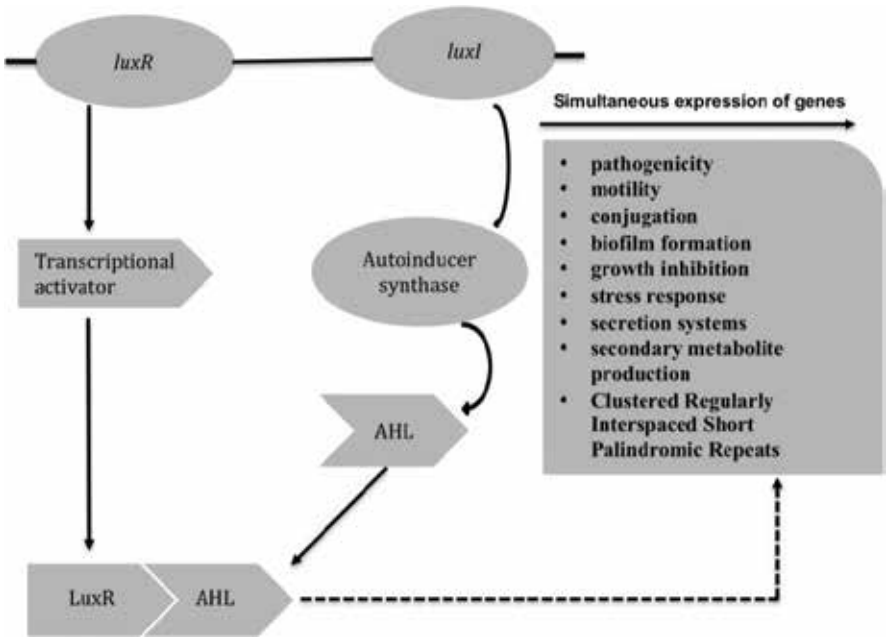

Figure 1. A schematic presentation of the LuxI/R quorum sensing system in Gram-negative bacteria. LuxI encodes an acyl homoserine lactone (AHL) synthase whereas LuxR encodes a protein receptor molecule. The AHL binds to the protein receptor molecule, the complex then binds to specific promoters and trigger multiple gene expression. The traits that are regulated by this system include pathogenicity, production of secondary metabolites, motility, secretion systems, stress response, conjugation, growth inhibition, biofilm formation and clustered regularly interspaced short Palindromic repeats (CRISPR-Cas).

2.1. QS systems in *Pectobacterium carotovorum* and *Pectobacterium atrosepticum*

Plant pathogenic bacteria belonging to the genus *Pectobacterium* cause soft rot and blackleg disease in economically important plants. The best studied *Pectobacterium* species include *carotovorum* subsp. *carotovorum (Pcc),* subsp. *brasiliense (Pcb)* and *Pa. Pectobacteria* are often called brute force pathogens due to their mode of host infection [26] i.e. the release of plant cell wall degrading enzymes (PCWDE) such as pectinases, polygalacturonases and cellulases that rupture the plant tissues during infection and cause rotting [38]. The precise timing for release of PCWDE is crucial for a successful infection [31]. Plant cell wall hydrolyzing enzymes help degrade the cell wall barrier in plants and thus facilitate entrance and spread of a pathogen in the host tissue.

Pectobacterium strains encode one LuxI homolog and two or more LuxR homologs. In *Pcc* there are three LuxR homologs, namely, CarR [39], ExpR [12] and VirR [35]. Each LuxR homolog in *Pectobacterium* plays an essential role. In contrast to CarR that regulates synthesis of antibiotics, the ExpR and VirR are involved in regulation of production of PCWDE [12, 35]. *Pectobacterium* spp. produces either one or two major AHL compounds and minute amounts of other AHL molecules depending on the species and strain [40]. The AHL synthases in *Pectobacterium* strains include the ExpI in *Pcc* SCC3193 [13, 14], *Pcc* SCR1193 [30], *Pcc* SCR11043 [15, 41], the AhlI in *Pcc* EC153 and *Pcc* 71 [15, 16, 35] and the CarI in *Pcc* ATCC390048 [35].

Secretion systems in bacteria are essential for transportation of effectors that are important for pathogenicity [42]. A transcriptomics study showed that AHL dependent QS regulate 26% of the entire transcriptome in *Pa* [26], representing the largest QS regulon in a plant pathogenic bacterium. In addition, it regulates Type 3 secretion system (T3SS) and a Type 6 secretion system (T6SS) in *Pa* [26]. The T6SS has been shown to transport proteins directly to target organism through direct cell–cell contact [43] and this secretion system has been implicated in bacterial competition [44]. The LuxI/R QS also regulates Type 1 (T1SS) and Type 2 (T2SS) secretion systems in *Pa* that are responsible for secretion of PCWDE [26]. In *Pcb*, QS is important for pathogenicity, production of PCWDE and cell aggregation in xylem tissues [17].

In *Pectobacterium* spp., QS regulates production of an antibiotic called carbapenem [39, 45]. Antibiotics give *Pcc* a competitive advantage over other bacteria coexisting during infection [46]. It is important to note that QS regulates motility in *Pcc* [11] and *Pcb* [17] and a cluster of genes for amino acids metabolism i.e. *ilvGMEDA*, *ilvIH*, *ilvBN* and *leuABCD*, signal transduction and lipid metabolism in *Pa* [18] Furthermore, the *xylAB* and *xylFGH* operons for xylose/xylulose metabolism as well as genes for anaerobic formate metabolism and operons for assimilation of hydrogen (*hyp* and *hyb*) are influenced by QS in *Pa* [18]. Notably, QS could help strike a balance in metabolism and nutrient acquisition by individual cells thus ensuring co-operative group activity (i.e. see [47]). Thus, QS regulation of metabolic processes is important for efficient utilization of resources by bacteria in a population.

2.2. QS in *Erwinia amylovora*

In some plant pathogenic bacteria, one LuxI/R QS system is encoded in the genome, for example the EamI/R in *E. amylovora* [34, 48]. *Erwinia amylovora* is a destructive plant pathogen that causes fire blight disease. The AHL-dependent QS system in *E. amylovora* regulates pathogenicity, exopolysaccharides production and tolerance to oxidative stress in this bacterium [34]. It is noteworthy that a unique QS system, namely LuxS was reported in this bacterium. Initial reports suggested that LuxS is restricted to metabolism and is not important for QS in *E. amylovora* [49, 50]. This bacterium is the only plant pathogenic bacterium in which the involvement of LuxS/autoinducer 2 QS signaling has been shown to regulate pathogenicity and pathogenicity traits [51]. However, contradictory reports on the role of the autoinducer 2 signaling in *E. amylovora* leaves gaps on the information available for QS in this plant pathogen. Determination of the entire QS regulon/s of this bacterium could help bring a better understanding of its QS systems.

2.3. QS in *Pantoea stewartii* subsp. *stewartii*

Another example of a plant pathogenic bacteria that encode more than one LuxR homolog, is *P. stewartii subsp. stewartii.* This bacterium encodes the *EsaR* [19, 52] and an additional LuxR homolog, the *sdiA* [53, 54]. Furthermore, one LuxI homolog that was designated as EsaI is encoded in this bacterium [19, 52]. The AHL QS regulates transcription of other transcriptional regulators for example the **r**egulation of **c**apsule **s**ynthesis A (RcsA) and **L**ys**R** **h**omolog **A** (LrhA), which influences exopolysaccharides (EPS) production and motility, respectively in *P. stewartii subsp. stewartii* [55]. The QS regulon in *P. stewartii subsp. stewartii* represents almost 8% of the entire genome [33]. A transcriptome study showed that QS regulates several stress

response genes in *P. stewartii subsp. stewartii* (see [33, 56]). The universal stress protein (Usp) is important for bacterial survival in adverse environmental conditions, for examples of such conditions, see [57]. Importantly, QS regulates EPS production in *P. stewartii* subsp. *stewartii* [52]. This EPS, also called sterwartan, plays a role in cell attachment and is an important constituent of biofilms in this bacterium [52]. In some plant pathogens, biofilm formation is a direct pathogenicity factor. In Stewart's wilt disease, biofilms clog the xylem vessels causing the wilt [52].

2.4. QS in *Dickeya dadantii* and *D. solani*

An AHL-dependent QS system, namely ExpI/R, is encoded in the genomes of *Dickeya solani* and *D. dadantii*. There are differences in the role played by the ExpI/R system in pathogenicity of different strains of *Dickeya.* For example, this system regulates production of protease and motility (swarming and swimming) in *D. solani* strains [58]. On the other hand, it plays no role in production of cell wall degrading enzymes, motility and pathogenicity of *D. dadantii* 3937 [59, 60]. Contrary, the ExpI/R was found to regulate pathogenicity in *D. dadantii* 3937 on potato tubers [58]. Furthermore, it was showed that the strength of AHL QS systems is strain specific in *Dickeya* spp., i.e. the effects of ExpI/R mutation were more pronounced in *D. solani* than in *D. dadantii* [20] indicating that this system regulates pathogenicity in *D. dadantii*. Together these findings may suggest that the regulation of pathogenicity by ExpI/R QS system in *D. dadantii* is host specific, strain specific and/or could be dependent on the experimental conditions used. Nonetheless, this leaves unanswered questions.

A QS system that differs from all QS systems described thus far in plant pathogenic bacteria was identified in *Dickeya* spp. This unique system (schematic presentation in **Figure 2**) makes use of **v**irulence **f**actor **m**odulating (*vfm*) [21]. This QS system directly regulates pathogenesis factors including production of PCWDEs in *Dickeya dadantii* and *Dickeya solani* [20, 21]. Mutation and characterization of *vfmA*, *vfmE*, *vfmH*, *vfmI* and *vfmK* suggested that all *vfm* gene transcripts are important for regulation of pathogenicity in *D. solani* [20]. Furthermore, there are variations in the degree of regulation of pathogenicity factors by VFM system in different *Dickeya* strains [20]. The VFM system is repressed by PecS, a global regulator of pathogenicity in *Dickeya* spp. [61] while ExpI/R and VFM QS systems do not work in synergy in modulating QS dependent traits [55]. Certainly, elucidation of the VFM QS regulon could help uncover many aspects of this QS system in *Dickeya* spp. that are not yet understood.

2.5. QS in *Pseudomonas syringae* subsp. *syringae* (Pss)

Pseudomonas syringae encodes a single LuxI homolog, designated AhlI [62] and four LuxR homologs, namely, AhlR [32], SalA, SyrF and SyrG [9, 29]. The AhlI/R QS system in *Pseudomonas syringae* subsp. *syringae* (*Pss*) is subject to modulation by other regulatory proteins. For example, **A**HL and **e**piphytic **f**itness **r**egulator (AefR), a novel regulatory protein and GacA influence the transcription of the AHL synthase gene, *ahlI* in *Pss* [63]. Most QS regulated processes in *Pss* are associated with epiphytic fitness and plant infection [32]. In addition, QS regulates motility in *P. syringae* [32]. Notably, in *Pss*, alginate production is regulated by the AhlI/R system that is in turn influenced by the GacS/GacA two component system [64].

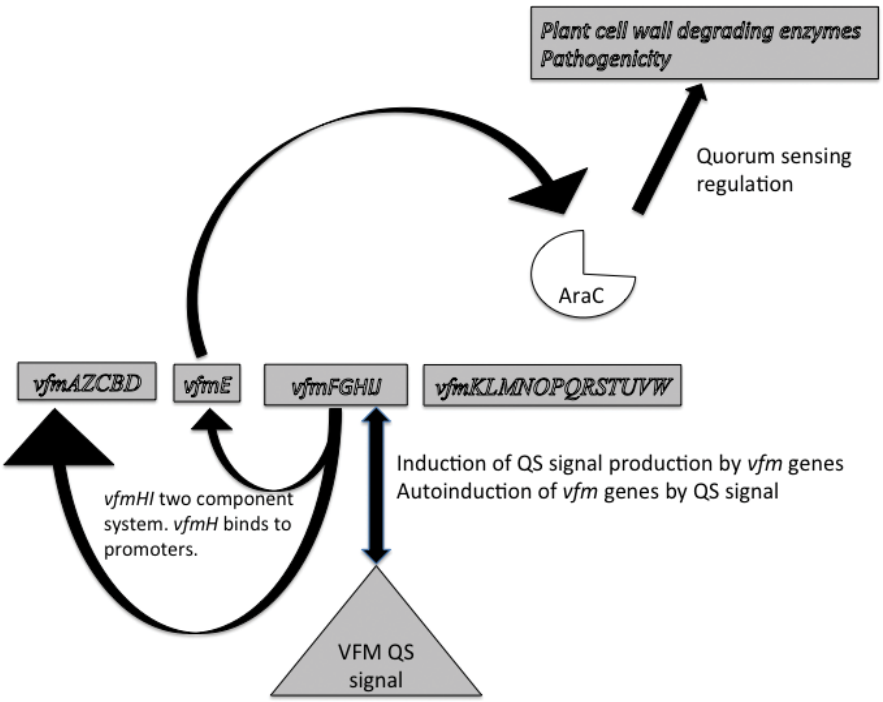

Figure 2. A schematic presentation of the VFM quorum sensing system in *Dickeya* spp. this QS system is made up of four gene transcripts, *vfmAZCBD*, *vfmE*, *vfmFGHIJ*, and *vfmKLMNOPQRSTUVW*. The *vfm* genes encode for the VFM QS signal, production of this QS signal results in auto induction of *vfm* genes. The *vfmHI* genes make up a two-component system where *vfmI* is a histidine kinase and VfmH binds to promoters of the *vfmAZCBD* and *vfmE* kinase. This results in activation of production of a transcriptional activator, AraC (encoded by *vfmE*) and regulation of specific phenotypes. Phenotypes regulated by the VFM QS include production of plant cell wall degrading enzymes and pathogenicity.

In *Pss*, the production of syringomycin and syringopeptin is regulated by LuxR homologs SalA and SyrF and SyrG, respectively [9, 29]. Phytotoxins produced by *P. syringae* cause chlorosis in plants and attenuated the pathogenicity of this bacterium [65–67]. Contrary to other plant pathogenic bacteria, the LuxI/R QS regulon of *Pss* was found to be very small, it is made up of about nine genes [68, 69] both in planta and *in vitro*. The AhlI/R QS regulon is composed of genes important for pyruvate metabolism and response to stress [68].

2.6. QS in *Agrobacterium tumefaciens*

Some plant pathogenic bacteria encode more than one LuxI homologs that are paired with their cognate LuxR. Typical examples include the TraI/TraR and TraI2/TraR2 in *A. tumefaciens*. Interestingly, the AHL QS system in *A. tumefaciens* differs from the model QS system based on *Vibrio* spp. This AHL dependent QS system is encoded on the Ti plasmid of *A. tumefaciens*. Quorum sensing regulates expression of type 4 secretion system (T4SS) [70] as well as conjugation [70, 71] and amplification of Ti plasmid in *A. tumefaciens* [72]. The TraI/R system depends on the expression of TraM whose transcription is indirectly regulated by QS. TraM binds to TraR forming an inactive complex, this helps prevent plasmid transfer before the optimal

cell densities for QS are reached [73]. A second QS system named TraI2/TraR2 was identified in *A. tumefaciens* [74]. This system also makes use of a TraR2 inactivator called TraM2. This second QS system in *A. tumefaciens* was postulated to play a redundant role in conjugation and replication of the Ti plasmid. The QS regulon was identified in *A. tumefaciens* strain P4, though this strain is non-pathogenic and outside the scope of this chapter, it is noteworthy that the QS regulon in this bacterium was found to constitute 32 genes [70]. Most genes in the QS regulon were those associated with conjugative transfer.

2.7. QS in *Ralstonia solanacearum/R. pseudosolanacearum*

Another plant pathogenic bacterium with two LuxI/R homologs is *R. solanacearum/R. pseudosolanacearum.* One of these LuxI/R homologs, the SolI/R system has been characterized. The SolI/R system is required for production of C6-HSL and C8-HSL in *R. solanacearum/R. pseudosolanacearum.* However, this QS system does not influence pathogenicity traits [36, 37]. To date, only three genes, *aidA, lecM* and *aidC,* have been reported to be influenced by *solI/R* [37]. The *aidA* and *aidC* genes encode proteins that have not yet been functionally characterized while *lecM* encode a mannose-fucose binding lectin. The physiological role of the LuxI/R QS systems in *R. solanacearum/R. pseudosolanacearum* still needs further investigation.

As the list of bacteria that employ QS for signaling increases, so does the list of new QS signaling systems. For example, *Ralstonia solanacearum/R. pseudosolanacearum* makes use of **ph**enotype **c**onversion (Phc) regulatory system [75] for signaling (simplified schematic diagram depicted in **Figure 3**). Phc is a LysR type transcriptional regular that makes use of 3-OH palmitic acid methyl ester (3-OH PAME) or methyl 3-hydroxypalmitate (3-OH MAME) (depending on the *R. solanacearum/R. pseudosolanacearum* strain) as a signal molecule [37, 76]. This system regulates pathogenicity traits such as exoenzyme production, exopolysaccharide synthesis [77], motility [78], siderophore production [79], production of phytotoxic ralstonins [80] and aryl furanones [81]. The aryl furanones are directly involved in QS signaling [81], biofilm formation [22] and pathogenicity [82]. The Phc in *R. solanacearum/R. pseudosolanacearum* regulates the expression of AHL dependent QS system, SolI/R mentioned above [75]. The Phc QS regulon in *R. solanacearum/R. pseudosolanacearum* constitutes a total of 620 (12% of the whole genome) genes [83]. Transcriptome profiling showed that this system influenced many genes associated with various metabolic pathways, transport systems, growth, several adhesins, attachment, dispersal and morphology of bacterial cells [83].

2.8. *Burkholderia glumae* quorum sensing

Bacteria belonging to the genus *Burkholderia* are not listed in the top 10 plant pathogenic bacteria. *Burkholderia glumae* was included in this chapter due to interesting findings in its QS regulon, an addition to the list of traits regulated by LuxI/R. *Burkholderia* spp. are characterized by multiple AHLs QS systems and additional LuxR homologs [84]. *Burkholderia glumae* causes grain rot in rice and inflicts serious yield losses internationally [85]. Within *Burkholderia* the LuxI/R QS system has been best studied in *B. glumae*, where this system has been named the TofI/R system [23]. The AHL QS regulon of three QS systems namely BGI1, BGI2 and BGI3 in *B. glumae* constituted 11.5% of the whole transcriptome [86]. Also of note, is the QS regulation of flagella biosynthesis and

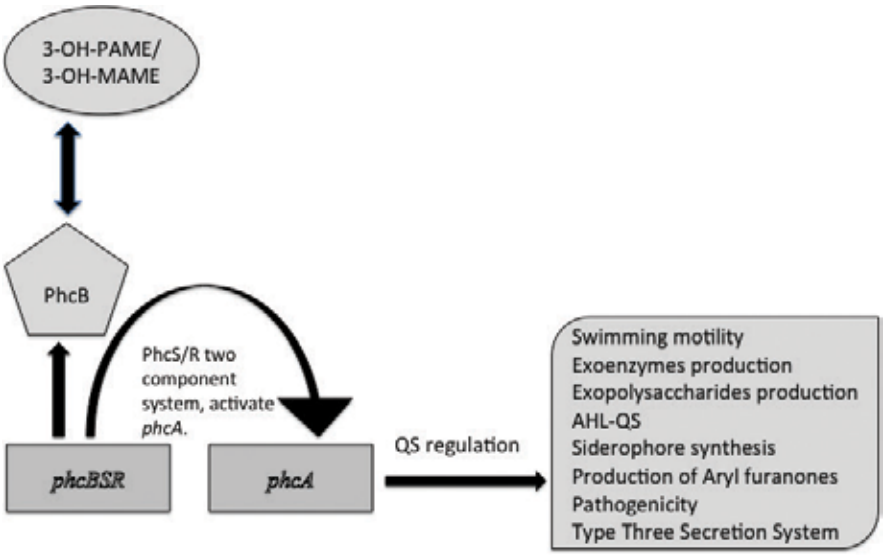

Figure 3. The phenotype conversion (Phc) quorum sensing system is encoded by the *phcA* and the *phcBSR* operon. The PhcB is a putative methyltransferase that catalysis the production of the QS signal molecule, 3-OH-PAME or 3-OH-MAME depending on strain. The PhcS/PhcR constitute a two-component system where PhcS, a histidine kinase phosphorylates PhcR. The phosphorylated PhcR responds to the presence of the QS signal and derepress PhcA resulting in elevated levels of functional PhcA at high cell densities. Phenotypes regulated by this system include swimming motility, exoenzymes production, exopolysaccharides production, acyl homoserine lactone dependent quorum sensing, siderophore synthesis, production of aryl furanones, pathogenicity and type three secretion system (T3SS).

swarming motility in *B. glumae* [87–89] and QS regulation of toxin biosynthesis, the phytotoxic toxoflavin [86, 90] an important pathogenicity factor in *B. glumae* [91]. Quorum sensing also regulates the Usp in *B. glumae* [92]. The Usp in *B. glumae* is important for surviving adverse temperatures [92]. Quorum sensing has also been reported to regulate metabolic pathways, for example, in *B. glumae* BG1. A transcriptome analysis *in vitro* showed that about 40% of the QS regulon in *B. glumae* BG1 is made up of genes for metabolic activities [86]. In addition, transcriptome analysis showed for the first time that QS influences the (CRISPR-Cas) associated proteins in *B. glumae* BG1 [86]. Given the biological role of the CRISPR-Cas system (see [93–99]), it is thus not surprising that this system has been found to be regulated by QS in a plant pathogenic bacterium.

2.9. QS in *Xanthomonas oryzae pv. oryzae*

Xanthomonas oryzae pv. *oryzae (Xoo)* causes bacterial leaf blight, one of the most destructive diseases, in rice. This bacterium does not produce AHLs. Its genome encodes a LuxR homolog called OryR [5]. The OryR protein has been found to impact pathogenicity of this pathogen [27]. Unlike LuxR proteins in other bacteria, the OryR does not bind to AHLs but binds to a yet to be identified diffusible plant molecule that acts as a QS signal [5]. The production of these plant signal molecules increases when a plant is infected. A schematic presentation of interkingdom QS signaling is shown in **Figure 4**. A transcriptomic study showed that OryR regulates 330 genes in *Xoo*, the majority of which influenced flagella and motility [100]. This is essential for movement, spread, colonization of host tissues and pathogenicity. Like in other LuxR that are without their LuxI (discussed below), the OryR regulates proline–imino-peptidase (*pip*) expression [100].

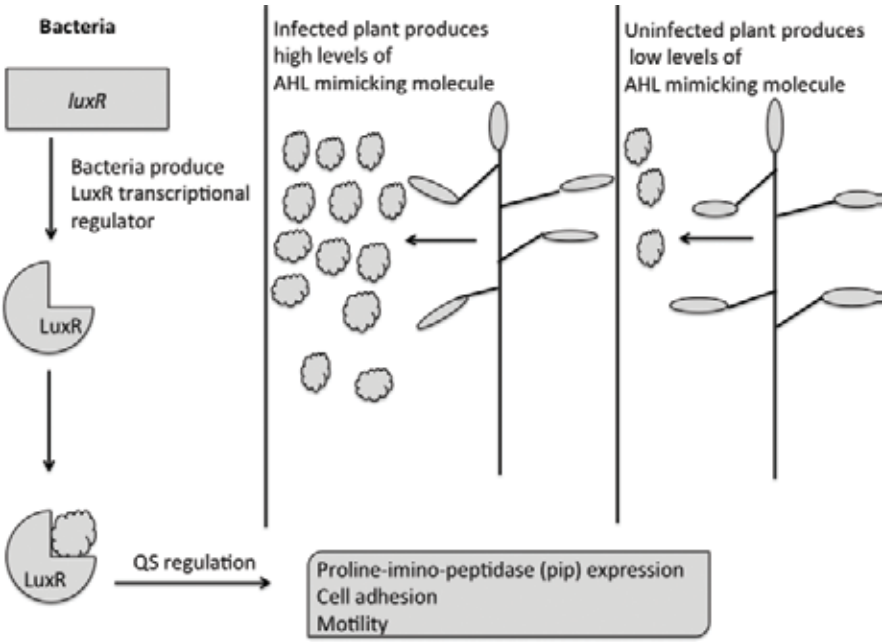

Figure 4. The LuxR in some plant pathogenic bacteria respond to acyl homoserine lactone (AHL) mimicking molecules produced by plants. The production of AHL mimicking molecules increases when a plant gets infected. These molecules bind to the LuxR in bacteria and trigger quorum sensing gene regulation of proline-imino-peptidase (pip) expression, cell adhesion and motility.

The second QS system in *Xoo* is the *DSF (*cis-11-methyl-2-dodecenoic acid) dependent QS [101]. The genes for biosynthesis and signaling of DSF are encoded on the **r**egulation of **p**athogenicity **f**actors (*rpfABCDEFG*) genes and the major catalyst in DSF production is RpfF [102]. The Rpf elements involved in DSF signaling are those that are part of the two- component system RpfCG. In this QS system, the DFS QS modulates the levels of second messenger cyclic di-GMP (see **Figure 5** for a schematic diagram of this QS system). At low cell density, the RpfG is inactive, the cyclic di-GMP levels are high while RpfC binds to RpfF and reduces its catalytic activity. Consequently, at low cell densities the cyclic di-GMP binds to the transcriptional activator, a cyclic di-GMP effector also called Clp and renders it inactive. At high cell densities, RpfC detaches from RpfF, the unbound RpfF then catalysis the production of more DFS signals, these signals then bind to RpfC. The RpfG is phosphorylated at high cell densities, it then binds and inhibits enzymes that synthesize cyclic di-GTP resulting in a decrease in cyclic di-GMP levels. The cyclic di-GMP detaches from Clp resulting in activation of the transcriptional activator, Clp [103]. Moreover, the DSF QS system in *Xoo* was found to be activated by the plant hormone, salicylic acid [104], indicating an involvement of interkingdom signaling in this QS system during plant infection.

In *Xoo*, the DSF QS system produces three distinct molecules i.e. DSF, BDSF (*cis*-2-dodecenoic acid) and *CDSF* (*cis*-11-methyldodeca-2,5-dienoic acid) [105]. The three DSF QS molecules are produced differentially during exponential growth, with BDSF production occurring ahead of the other two. The three DSF molecules influence production of EPS and exoenzymes in *Xoo*, however, CDSF is less active compared to the other two. In addition, the synthesis of the different DSF molecules varies depending on nutrients available, for example, DSF dominates

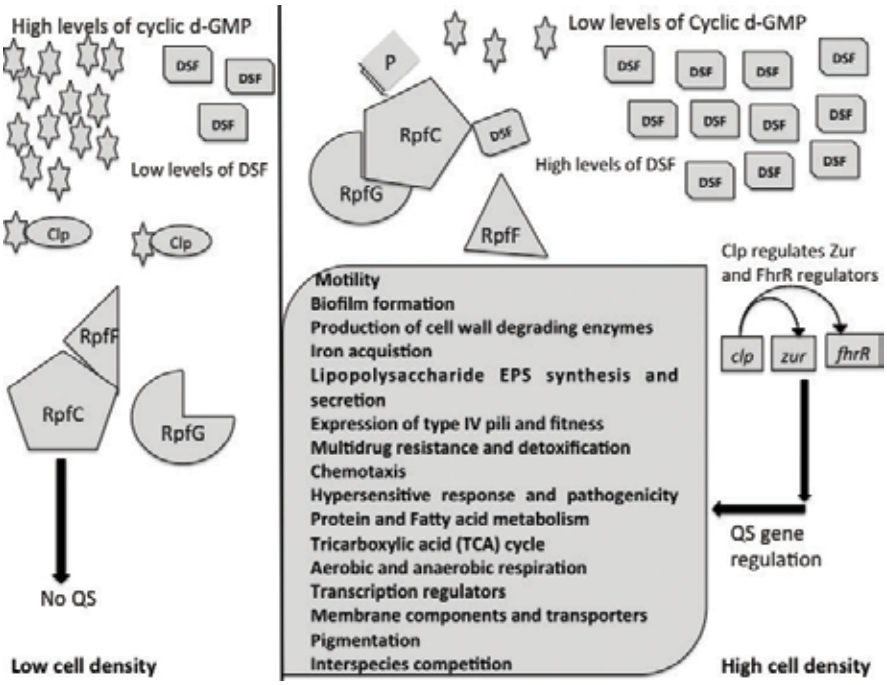

Figure 5. Schematic presentation of the DSF quorum sensing (QS) system showing the modulation of cyclic di-GMP. At low cell densities, the cyclic di-GMP levels are high, they bind to the transcription factor and prevent quorum sensing. The RpfF acts as an enzyme for synthesis of QS signal. At low densities, RpfF binds to RpfC and its catalytic activity is reduced. At high cell densities, RpfF is unbound, it is activated and produces the DSF which then binds to the phosphorylated RpfC. Phosphorylation of RpfC leads to a reduction in the levels of cyclic di-GMP which then detaches from the transcription factor leading to QS gene regulation. The phenotypes influenced by this QS system include motility, biofilm formation, production of plant cell wall degrading enzymes, iron acquisition, lipopolysaccharide/exopolysaccharides (EPS) synthesis and secretion, expression of type IV pili and fitness. Other traits regulated include chemotaxis, multidrug resistance and detoxification, pathogenicity, metabolism, transport, interspecies competition and pigmentation.

in nutrient rich medium whilst in poor nutrients BDSF dominates. One other trait regulated by DSF is iron acquisition in *Xoo* [6]. In addition to the mentioned QS systems, *Xoo* also harbors the Diffusible factor (DF) QS signaling, the autoinducer for this system was characterized as 3-hydroxybenzoic acid (3-HBA) [106]. The DF in *Xoo* regulates the synthesis of the yellow pigments, xanthomonadins that help protect the bacteria from photodamage. The production of both DSF and DF in *Xoo* are activated by the plant hormone, salicylic acid [104] implicating these QS systems in interkingdom signaling.

2.10. QS in *Xanthomonas campestris* pv. *campestris*

The QS systems in *Xanthomonas campestris* include the DSF and DF (3-hydroxybenzoic acid (3-HBA)) [105]. The enzyme involved in DF synthesis (XanB2) has not been identified [106]. The DSF and DF QS systems regulate the exopolysaccharide, xanthan, production. Other traits regulated by the DSF system in *X. campestris* include production of extra cellular enzymes, glucan production and biofilm formation [107, 108] and fitness advantage in interspecies competition [109].

Like the DSF system in *Xoo,* the DSF system in *X. campestris* was found to produce multiple DSF molecules i.e. DSF, BDSF, CDSF and the newly identified IDSF (*cis*-10-methyl-2-dodecenoic acid) [102, 109, 110]. However, the levels of IDSF reported in this bacterium [102] are not sufficiently high enough to have any regulatory effect. On the other hand, the DF system regulates EPS synthesis and production of a yellow pigment, xanthomonadin that acts as a shield against ultra violet (uv) light and thus contributes to epiphytic fitness and pathogenicity of *X. campestris* [111]. The DSF QS in *Xcc* regulates important pathogenicity factors in this bacterium, for example, xanthan and glucan have been shown to suppress the host's innate immune defense, possible through inhibition of callose deposition [24, 112] .

In *Xcc*, EPS production is co regulated by DSF QS and the RavS/RavR two component system [10]. In this pathogen, the DSF QS mutants were impaired in pathogenicity [108] and in fitness, for example, in the ability to cope in iron limiting environments [6, 10]. The regulation of different pathogenicity factors by different QS systems in different bacteria, coupled with differences in QS regulated processes, further emphasizes the specificity of QS systems in bacteria. The DSF QS regulon in *Xanthomonas campestris* pv. *campestris* has been identified and is made up of 165 genes of which 10 of them are hypothetical proteins [10]. The regulon represents 12 functional categories that include extracellular enzymes, lipopolysaccharide and EPS synthesis and secretion. In addition, multidrug resistance and detoxification, flagellum biosynthesis, motility and chemotaxis, hypersensitive response and pathogenicity (Hrp) system are regulated by DSF in this pathogen. Other factors regulated include iron uptake, protein metabolism, tricarboxylic acid (TCA) cycle, aerobic and anaerobic respiration, transcription regulators, membrane components and transporters, and fatty acid metabolism [10].

Another LuxR homolog that does not bind to AHLs is the XccR in *X. campestris*. The AHL synthase gene is absent in this bacterial species. The LuxR homolog found in *X. campestris* binds to yet to be identified molecules produced by the plant and regulates the proline–imino-peptidase (*pip)* gene, a pathogenicity factor in this bacterium [8]. In the absence of AHL mimicking molecules produced by plants, the XccR is repressed by a negative regulator, XerR [113]. The plant derived molecules interact with the repressor, XerR resulting in de repression of XccR. Such QS highlights an interesting inter-kingdom signaling between a plant and its pathogen.

2.11. QS in *Xanthomonas axonopodis*

In *X. axonopodis pv. glycines*, a bacterium that causes bacterial pustules on soybean, one LuxR homolog called XagR was found. Similarly to the other LuxR homologs that are without their cognate LuxI synthase in *Xanthomonas spp.,* XagR binds to signal molecules produced by the host resulting in QS regulation. The XagR regulates proline-imino-peptidase (*pip*) expression, cell adhesion, motility and pathogenicity [28]. XagR regulation of *pip* is not host specific, induction of *pip* expression was observed in soybean, rice and cabbage [28].

2.12. QS in *Xylella fastidiosa*

The complete genome sequence of *Xylella fastidiosa* revealed that this bacterium lacks an AHL synthase gene. This bacterium makes use of DSF for signaling [114], the QS regulated processes includes motility, biofilm formation, pathogenicity [115] and biosynthesis of DSF [116]. However,

the DSF QS system in *Xylella* is not the same as the DSF in *Xanthomonas* spp. The *Xylella* DSF signals have been characterized as cis-2-tetradecenoic acid (XfDSF1) and 2-cis-hexadecanoic acid (XfDSF2), [117, 118]. Whilst in *Xylella*, mutation of the DSF signaling results in up regulation of pathogenicity genes [114], production of cell wall degrading enzymes and expression of type IV pili in the mutants, the opposite happens in DSF QS mutants in *Xanthomonas* spp. [6].

3. Progress in understanding interkingdom QS

As noted in the discussion above, some plant pathogenic bacteria encode LuxR homologs that are capable of 'eavesdropping' by utilizing AHL mimicking low molecular weight compounds that are produced by plants. In place of the LuxI, the LuxR homologs in plant pathogenic bacteria are in most oftenly in close proximity to the *pip* gene [119]. The *pip* harbors an inverted repeat unit similar to *luxI* and is directly involved in pathogenicity, hence its biological role merits further investigation. Over the past decade, researchers have attempted to investigate these LuxR proteins especially on deciphering their role in QS signaling. The binding motifs of these LuxR homologs is unique and distinct from the conventional LuxR homolog, they lack one or two of the several conserved regions required for AHL binding [5, 8, 119]. The AHL binding domain of these proteins are substituted by methionine and tryptophan in the conserved region allowing specificity for binding to plant derived molecules [119]. The orthologs of these LuxR proteins are also encoded on the genomes of AHL producing bacteria including *Pseudomonas syringae* [8]. Consequently, questions arise, do these LuxR homologs bind to the AHL mimicking compounds and function in a similar way in the AHL producing and non AHL producing bacteria? In addition, the AHL mimicking molecules produced by plants still need to be characterized.

4. Conclusions

A variety of bacterial species are increasingly becoming resistant to the antimicrobial agents that are currently in use [120]. Resistance to streptomycin in plant pathogenic bacteria was reported within a decade of its use in controlling plant infections and diseases [121]. Research efforts are now focusing on alternative bacterial control strategies. The discovery of the involvement of QS in the regulation of bacterial virulence has led to escalated research efforts towards discovering possible biological control measures that target QS systems. The main advantage of control measures that target QS systems, though not yet scientifically proven, is that they are less prone to selective pressure [122].

For an effective application of QS inhibition as a biological antimicrobial measure, a better understanding of the genes influenced by QS is crucial. Latest technology including research tools such as RNA-Seq has made it possible for whole transcriptome investigations to be conducted. In addition, targeted mutation and characterization of mutants has helped in unveiling the biological significance of specific genes in bacteria, the complexity of bacterial transcriptomes and thus regulation of gene expression. Nonetheless, as additional experimental and analytical tools become available, the critical role of bacterial QS to plant pathogenesis will undoubtedly become much clearer.

The literature cited in this chapter reflects on QS and its role in influencing pathogenicity and pathogenicity-associated traits in Gram-negative plant pathogenic bacteria. The different QS systems, the extent of those QS regulons that have been elucidated as well as the different signaling molecules employed by plant pathogenic bacteria have been explored. This chapter highlights interesting similarities and differences of QS systems and the diversity of QS signal molecules utilized by plant pathogenic bacteria. Understanding QS regulation in plant pathogenic bacteria could provide useful tools for control and management of bacterial plant diseases.

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Conflict of interest

Authors declare no conflict of interest.

Author details

Siphathele Sibanda[1,2], Lucy Novungayo Moleleki[1], Divine Yufetar Shyntum[1] and Teresa Ann Coutinho[1,2]*

*Address all correspondence to: teresa.coutinho@fabi.up.ac.za

1 Department of Microbiology, Faculty of Natural and Agricultural Sciences, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa

2 Faculty of Natural and Agricultural Sciences, Centre for Microbial Ecology and Genomics (CMEG), University of Pretoria, Pretoria, Republic of South Africa

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Section 3

Advances in Viral Plant Pathology

Chapter 5

Leaf Curl Disease: A Significant Constraint in the Production of Tomato in India

Pradeep Kumar and Manish Kumar

Additional information is available at the end of the chapter

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Abstract

Tomato (*Lycopersicon esculentum Mill.*) is one of the most economically important vegetable crops in the world. Among the major biotic constraints, virus-associated Tomato leaf curl disease (ToLCD) is a major limiting factor affecting its cultivation and yield. Different symptoms associated with disease are reported such as leaf curling, puckering of leaves, vein yellowing, stunting, excessive branching, from pale yellowing to deep yellowing, and small leaves. The genus *Begomovirus* is a circular single-stranded DNA virus which is exclusively being transmitted by whitefly (*Bemisia tabaci*) in a persistent circulative manner. Most of the begomovirus species are monopartite (having DNA-A molecule only), except few species, which are bipartite (having DNA-A and DNA-B as the genomic component). No absolute effective control measures of the disease could be developed so far, except resistance, management of insect vectors, and altering the dates of sowing to avoid peaks of insect vector population. This chapter reports an account of history, symptoms, transmission, genome organization, distribution, and management of Tomato leaf curl disease.

Keywords: *Begomovirus*, leaf curl, AAP, IAP, betasatellite, DNA-A, DNA-B

1. Introduction

Tomato (*Lycopersicon esculentum Mill.*) is one of the most economically important vegetable crops in the world. The total area of tomato cultivation in the world is 4.582 mha with production of 150.51 MT, and China, India, the USA, Italy, Turkey, and Egypt are major tomato-growing countries in the world.

India is ranked second in area and production after China. The tomato crop covers a total area of 0.879 mha with a production of 18.22 m.t. Andhra Pradesh, Karnataka, Orissa, Maharashtra, West Bengal, Bihar, Gujarat, Chhattisgarh, Tamil Nadu, and Jharkhand are leading states [27].

2. Leaf curl disease

Several biotic and abiotic factors are the major constraints in production of tomato in India. Among the biotic constraints, different viral diseases cause significant impact in tomato production (**Table 1**). Among these viral diseases, tomato leaf curl disease (ToLCD) is devastating and causes significant yield loss under severe conditions. In India, Tomato leaf curl disease (ToLCD) was first reported by Vasudeva in 1948 from Northern India and Sam Raj in 1950 from Central India. The virus can cause infection at any stage of growth and development of plants. Muniyappa et al. [8] reported that yield in summer-planted tomato is less (6.4–52.2%) as compared to winter-planted crops (52.5–100%). Disease incidence, severity, and losses occurred due to this disease depend on the time of infection and is reported to range between 17.6% and 99.7% [1, 2]. Shashti and Singh [3] reported 92.3% loss when infection occurred at 30 days after transplanting. The yield reductions were 94.9, 90.0, 78.0, and 10.8% when plants got infected in 2, 4, 6, and 10 weeks after planting [3]. The disease is caused by different species (**Table 1**) having circular single-stranded DNA (ssDNA), of the

Monopartite and originally reported from tomato				
S. No	Name of the virus species	Acronym	Locality	Year of report
1	*Tomato leaf curl Kerala virus*	ToLKeV	Kerala	2011
2	*Tomato leaf curl Ranchi virus*	ToLCRnV	Ranchi	2011
3	*Tomato leaf curl Patna virus*	ToLCPaV	Patna	2010
4	*Tomato leaf curl Rajasthan virus*	ToLCRV	Rajasthan	2011
5	*Tomato leaf curl Pune virus*	ToLCPuV	Pune	2011
6	*Tomato leaf curl Bangalore virus*	ToLCBV	Bangalore	2000
7	*Tomato leaf curl Karnataka virus*	ToLCKV	Karnataka	2002
8	*Tomato leaf curl Joydebpur virus*	ToLCJoV	Joydebpur	2013
Bipartite and originally reported from tomato				
9	*Tomato leaf curl New Delhi virus*	ToLCNDV	New Delhi	1993
10	*Tomato leaf curl Palampur virus*	ToLCPalV	Palampur	2008
11	*Tomato leaf curl Gujarat virus*	ToLCGV	Gujarat	2003
Monopartite and originally reported from tomato				
12	*Ageratum enation virus*	AEV	Pantnagar	2013
13	*Cotton leaf curl Burewala virus*	CLCBV	Bihar	2013
14	*Tobacco curly shoot virus*	TbCSV	Pantnagar	2013

Table 1. Begomovirus species associated with tomato leaf curl disease in India.

genus *Begomovirus*, family *Geminiviridae* [4, 5]. This chapter summarizes the work carried out on leaf curl of tomato in India.

3. Symptomatology

The symptoms of leaf curl disease are very complex, and the typical symptoms include leaf curling, puckering of leaves, vein yellowing, stunting, excessive branching, from pale yellowing to deep yellowing, and smalling of leaves [6]. Apart from this, it also causes the extreme distortion of leaves, stunting of plants, and premature drop of flower and fruits. Singh and Lal, in 1964, observed that in some genotypes, it causes green vein banding, twisting, and green enation on the under surface of the leaf, upward rolling of margin, and islands of golden colors scattered amidst the normal green tissue [7]. The type of symptom produced is dependent on the genotype cultivated and the developmental stage at which the infection occurs. At cellular level, structural changes have been observed like hypertrophy of nucleus and accumulation of dark granules and the aggregate of virus-like particles in the cytoplasm [8] (**Figure 1**).

Figure 1. Showing leaf curl symptom in tomato crop.

4. Transmission of the virus

Very rich information is available regarding the transmission of the leaf curl viruses of tomato since its discovery. The virus is whitefly transmitted and was demonstrated to occur in several hosts by Vasudeva et al. as early as in 1948. As per the observation of his group, they found

that in winter crop the symptoms appeared 25 days post-inoculation, whereas in summer crop, it took only 15 days. Since the virus characterization was accomplished in the last decade of the twentieth century, it could be presumed that detailed studies on the virus from Southern India may represent the data for monopartite begomovirus mainly ToLCBaV and in Northern India it may include ToLCNDV. The transmission efficiency of the virus depends on the season and the prevailing temperature of the geographical location. Butter et al. [1] reported 100% transmission of virus, the virus with 10 whiteflies/plant at the temperature ranging from 33 to 39°C [1]. Muniyappa et al. [9] described that geographically a different isolate of whitefly behaves differently for acquisition access period (AAP) and inoculation access period (IAP), and he reported minimum 10-min AAP (acquisition access period) and 20-minute IAP (inoculation access period), respectively, for ToLCBaV [9]. It has also recently been reported that ToLCNDV, ToLCGuV, and ToLCKaV are transmissible through sap [10–13]. The begomoviruses causing tomato leaf curl disease have a wide host range affecting various dicotyledonous plants belonging to different families. Host range of the viruses has been determined by graft/whitefly transmission, agroinoculation/biolistic delivery of viral genome into tomato plants, or by detecting the viruses in naturally infected plants using specific primers or probes to virus species.

5. Begomovirus species and its genome organization

The begomoviruses (genus *Begomovirus*, family *Geminiviridae*) constitute the largest group of plant viruses causing devastating crop diseases in India. About 16% of gemini viruses are recorded worldwide occur in India. Currently, 322 begomovirus species have officially been accepted by International Committee on Taxonomy of Viruses (ICTV) from all over the world causing infection in different crops—out of them 82 are reported from India. Among them, around 19 species of begomovirus have been shown to cause leaf curl disease in tomato (**Table 1**). Although the diseases were observed during the mid-twentieth century, the etiology of the disease as a begomovirus was confirmed in the last decade of the twentieth century [14]. Subsequently, based on nucleotide sequence similarity of DNA-A (<89% earlier, and <91% now) genome, different species have been identified. Two species namely *Tomato leaf curl New Delhi virus* and *Tomato leaf curl Palampur virus* predominantly distributed in Northern India and one species namely *Tomato leaf curl Bangalore virus* is dominant in Southern India [9, 14, 15].

The genus *Begomovirus* is a circular single-stranded DNA virus which is exclusively being transmitted by whitefly (*Bemisia tabaci*) in a persistent circulative manner [16]. Most of the begomovirus species are monopartite (having DNA-A molecule only) except few species, which are bipartite (having DNA-A and DNA-B as genomic component). However, in monopartite species, in addition to DNA-A molecule, betasatellite DNA is also present which is almost half of DNA-A component. The DNA-A of both mono- and bipartite species coding for six open reading frames (ORF), two in sense orientation namely AV1 and AV2 and four in complementary orientation namely AC1, AC2, AC3, and AC4, mainly are involved in virus replication and transmission. On the other hand, DNA-B of bipartite species coding for two ORF one in sense orientation namely BV1 and another in complementary orientation namely

BC1 is mainly involved in virus movement within the host [17, 18]. Betasatellite of monopartite species carries only one ORF, which is coded for BC1 protein in complementary sense. ToLCNDV is also found associated with alphasatellites (**Figure 2**).

It has been reported earlier that tomato-infecting begomoviruses have bipartite genome in North India, while monopartite genome in South India [18]. Association of betasatellite DNA molecule with the ToLCD occurring in several places of India was reported [19].

In India, the population of tomato leaf curl viruses is so diverse, and it was shown after coat protein analysis of the 29 infected tomato samples. Based on these analysis, five clusters (with less than 88% similarity among them) were observed among the population, whereas four of them represented the tomato leaf curl viruses. Out of five, one cluster showed 89% similarity with Croton yellow vein mosaic virus [20]. As of now, so many closely related tomato-infecting begomovirus have been cloned and sequenced from India.

Figure 2. Genome organization of begomovirus associated with leaf curl disease of tomato.

6. Management

Management of viruses is difficult as viruses are systemic in nature, highly variable or diverse, insect vectors, and so on. No effective control measures of the ToLCVs associated with tomato have been developed so far for successful management except resistance, management of insect vectors, and altering the dates of sowing to avoid peaks of insect vector population. Management of ToLCV could be achieved by following various management practices.

a. **Crop rotation** can be utilized for management of disease spread by naturally breaking the life cycles of insect vectors, disease, and weeds. Rotating to nonhost crops prevents the buildup of large populations of the insect vector and also establishes host-free periods.

b. **Introducing a host-free period** may delay infection in tomato by reduced whitefly population due to unavailability of proper host, which ultimately leads to lower rates of virus transmission?

c. Careful monitoring of **sequential plantings** should be done for virus management. Avoid sowing of tomato close to already infected fields. Synchronized planting should be followed to avoid initial inoculum from tomato plants.

d. **The source and use of crop transplants** are also important in reducing or delaying infection. Early infection of susceptible seedlings should be monitored prior to transplanting. Nurseries should produce seedlings for commercial distribution in insect-proof environment or under net cover to minimize infestation by the vector and subsequent virus transmission prior to transplanting. **Roguing**, or immediate removal of infected individual plants, may assist in delaying virus spread once the infected material is immediately destroyed and not left to compost near adjacent, developing fields.

e. Reflective plastic mulches, yellow plastic mulch, and whitefly-proof screens can be employed to reduce the incidence of ToLCV-infected tomatoes.

f. **Biological control**: biopesticides, a mass-produced agent manufactured from a living microorganism or a natural product, may offer a solution to disease control through introduction of predators and parasitoids of the vector. Biopesticides based on microbials such as Beauveria bassiana (effective on nymphs and adults) or Paecilomyces fumosoroseus, Green lacewings, ladybirds, minute pirate bugs, big-eyed bugs, and damsel bugs and Encarsia bimaculata [21] based on microbials such as Beauveria bassiana (effective on nymphs and adults) or Paecilomyces fumosoroseus. Green lacewings, ladybirds, minute pirate bugs, big-eyed bugs, damsel bugs and the parasitoid, Encarsia bimaculata [21]. Encarsia formosa is one of the most efficient and studied bioagents of *B. tabaci*. In a recent study, [27] has shown the biological control of ToLCV in tomato by application of chitosan-supplemented formulations of *Pseudomonas sp.* under field conditions. They also observed the higher levels of phenolics, phenylalanine ammonia lyase, peroxidase, and enhanced chitinase activity in rhizobacteria-treated plants (Mishra et al., 2014).

g. **Host resistance**: resistance approach is an easy, more effective approach for control of viral diseases. Three ToLCV-resistant open-pollinated tomato varieties ("**Sankranthi," "Nandi"**

and "Vaibhav") were developed and released officially in 2003–2004 in India. "Gene pyramiding" is combining multiple Ty genes in tomatoes with resistance to several whitefly-transmitted begomoviruses that cause TYLCVD [28]. Until now, six genes (*Ty* genes) derived from different tomato wild species have been identified. Prasanna et al. [22] attempted to combine *Ty-2* and *Ty-3* genes through marker-assisted selection and screened the hybrid lines for resistance to viruses by challenging through agroinoculation of specific, monopartite, and bipartite viruses and found that the lines and hybrids with *Ty-2* were susceptible to ToLCNDV. The *Ty-3* gene showed dosage effect with partial resistance of plants to ToLCNDV in the heterozygotes stage. By pyramiding *Ty-2* and *Ty-3* genes, considerable resistance to ToLCNDV can be achieved. The resistance of some of the tomato genotypes Vaibav, Nandhini, having *Ty-2* gene to ToLCBaV were lost, when these genotypes were individually agroinoculated with ToLCBaV [13] and the cognate betasatellite.

h. **Chemical control**: foliar spray of neem (azadairachtin) and neem plus can kill the eggs, nymphs, and adults of *B. tabaci.* Ethanolic and aqueous extracts of *Annona squamosal, Carlowrightia myriantha, Trichillia arborea, Azadirachta indica*, and *Acalypha gaumeri* are effective against *B. tabaci* population. Neem oil, garlic, and eucalyptus extract give significant results against this disease [23]. The chemical control method is easy and most commonly used approach against the insect pest. A number of insecticides are used. Among them, imidacloprid, acetamiprid, nitenpyram, thiamethoxam, and diafenthiuron give significant results against aphids, whiteflies, and other insect pests [24, 25].

7. Conclusion

Begomoviruses are the most important viral pathogens in the Indian subcontinent. Tomato leaf curl disease is one of the devastating diseases and has been reported to be associated with several begomoviruses, thus making breeding for resistance more challenging. Adding to this, presence of diverse betasatellite increases complexity. Climate change and injudicious use of pesticides ensure the persistence of whitefly throughout the year and pose further challenges for the management of the disease. Besides the viral-induced symptom, symptoms caused by sucking of thrips and whitefly confuse the breeder and increase the difficulty for formulating management strategy. A thorough understanding on variability of the virus complexes and understanding the epidemiology could be an alternative in devising management strategy.

Author details

Pradeep Kumar and Manish Kumar*

*Address all correspondence to: sharma90manish@gmail.com

Division of Plant Pathology, Indian Agricultural Research Institute (IARI), New Delhi, India

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Chapter 6

Plant Defense and Counter Defense by Viruses

Muzafar Ahmad Sheikh

Additional information is available at the end of the chapter

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Abstract

RNA silencing is a robust sequence-specific RNA degradation process triggered by the formation of double-stranded RNA (dsRNA). RNA silencing was first discovered in transgenic plants, where it was termed co-suppression or post-transcriptional gene silencing (PTGS). In plants, it serves as an antiviral defense, and small RNA pathways serve as a defense against viruses and other invading nucleic acids. This chapter focuses on the interactions between host small RNA pathways and viral suppressors of silencing. Invading viruses carry genetic material that controls the host cell's machinery and tricks it into producing proteins and new viruses. Through RNA silencing, plant cells recognize this viral genetic material, remember and copy it so that other cells in the organism can be warned to destroy the virus. All cells in microbes, fungi, plants and mammals employ RNA silencing. However, viruses are known to fight back using RNA silencing suppressors, proteins that inhibit this defense mechanism. RNA silencing suppressors have been reported recently in other forms of pathogens like bacteria and oomycetes, which suggest that these pathogens have this inherent capability of counter defense across various kingdoms. In this chapter, we discuss some of these phenomenal counter defense mechanisms by the viruses.

Keywords: RNA silencing, PTGS, antiviral defense, silencing suppressors

1. Introduction

1.1. The beginning of a story

As a matter of surprise, plant scientists, during the last decade of twentieth century, developed enthusiasm toward mechanism of gene silencing by virtue of plant transformation experiments, in which the introduction of a transgene into genome led to the silencing of transgene and homologous endogene [1, 2]. From these initial studies, plant biologists gained a wealth of information from gene silencing mechanisms and their complex pathways including their

mutual multiserial interactions that they express. Besides, the biologists have made prominent achievements in using RNA silencing as a powerful tool for studying gene expression and crop improvements. RNA silencing (also called as posttranscriptional gene silencing PTGS) refers to a family of gene silencing effects by which the expression of one or more genes is downregulated or entirely suppressed by the introduction of the antisense RNA molecule. The most common and studied representation is RNA interference (RNAi). RNAi is a biological process in which RNA molecules inhibit gene expression or translation by neutralizing targeted mRNA molecules. It also plays a crucial role in defending plants against viruses. Enzymes search double-stranded RNA (dsRNA) that is normally present in cells and digest it into small pieces that render them inefficient to cause disease. The phenomenon of RNAi process can be divided into three steps. First, a long double-stranded RNA (dsRNA) that is introduced into the cell is modified into small RNA duplexes by a ribonuclease III (RNAase III) enzyme known as DICER; second, these duplexes are unwound and one of the strands is advantageously loaded into a protein complex known as the RNA-induced silencing complex, and at this point, RISC binds to an ARGONAUTE (AGO) protein. Third, this complex essentially scans the transcriptome and locates target RNAs. The packed ssRNA called the gRNA (guide RNA) directs an endonuclease that is present in RISC (also known as slicer), now known to be an Argonaute protein to split mRNAs that contain a sequence homologous to the siRNA. The importance of siRNAs in antiviral defense is to direct RISC complex to viral genomic and sub-genomic RNAs, thereby targeting those molecules for destruction (**Figure 1** Courtesy Trends in Microbiology, Vol.16. No.5). Multifarious use of siRNAs as specificity factors has been demonstrated in antiviral defense. The dsRNA sequence source for various RNA and DNA viruses is not known, but it is likely that they could have originated during viral

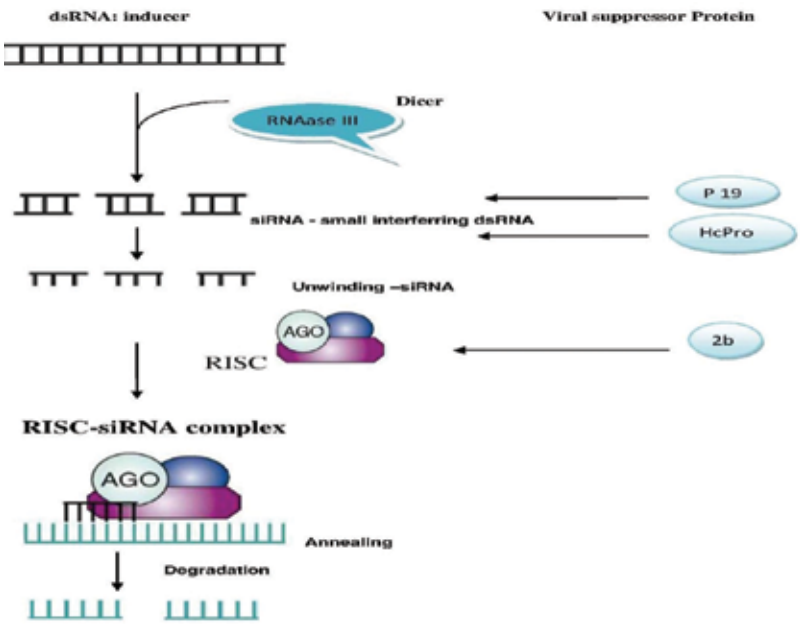

Figure 1. RNA-cleavage activity leading to the degradation of AGO1.

replication and/or from internal pairing of long RNA molecules [3]. RNA silencing is a highly complex system consisting of various proteins and processes [4]. This complexity makes the phenomenon of RNA silencing efficient for endogenous RNA expression during plant development and growth as well as for controlling viral infection. Viruses have adapted a robust mechanism for combating plant defense machinery by expressing suppressor proteins, which are capable of interfering in the process of RNA interference (RNAi) silencing pathway.

Features of RNAi

- Double-stranded RNA (dsRNA) rather than single-stranded antisense RNA is the intrusive agent.
- Silencing can be introduced in various progressive stages of the mechanism.
- RNAi is of high-degree specificity gene silencing mechanism.
- It circumvents the problems caused by knocked out genes in early stages (which could veil desired observations).
- Silencing actions are passed through ages. It means that some of the dsRNA molecules could not silence the genes in parent, but the same ds RNA molecule did silence genes in the offspring because the dsRNA sequence did not match any of the parent genes.

2. Viruses fight back

RNA silencing is predominantly a robust defense mechanism adapted by plants against pathogenic intruders especially viral pathogens. Viral pathogens have the ability to stay by suppressing RNA silencing. Plant viruses have developed tricky measures to overcome the host silencing response. One of these strategies is to overcome host-silencing response by producing proteins that target the signaling steps of RNA silencing [5]. Plants use RNA-silencing mechanism and produce short interfering RNA (SiRNA) molecules in a defense response against viral infection. To counter this defense response, virus produces suppressor proteins that can block the host silencing pathway or interfere with its function in plant cells [6]. The evidence of virus encoding RNA silencing suppressor proteins begins from experiments when silenced transgenes were again activated after virus inoculation. Silencing suppressors have been identified in DNA-containing viruses and from positive-strand RNA viruses [6]. Collectively, the plant viral silencing suppressors are diverse in sequence and evolutionary origin. Being functionally diverse, they target cell autonomous steps and systemic signaling steps of RNA silencing mechanism. Before discussing the specific silencing suppressors, it is important to consider the consequences. At the earliest, there would appear to be a conflict, and later, many viruses encode the protein suppressors that block the signaling steps of RNA silencing. From the pioneer experiments of first viral suppressors of silencing in 1998, the scientists have generated a wealth of information about plant viral proteins that block silencing pathway elucidating their mechanism of action [7, 8]. Virus-encoded RNA-silencing suppressors interfere with various steps of the different silencing pathways and the mechanisms of suppression are being unraveled more and more.

2.1. Suppressor and small RNA function

The prodigy of accumulation of primary siRNAs to considerable level in the presence of some suppressors and that the target RNA degradation blockage indicates that primary siRNAs are not functional. These findings have suggested viral suppressors of RNA silencing bind to small RNA duplexes, after they bind properly to small RNA duplexes, they separate them and prevent their entry into RNA-induced silencing complex or RISC effector complex (**Figure 1**) [9, 10] This separation of small RNA duplexes has been suggested as a usual mode of action for RNA silencing suppression. The suppressors of RNA silencing can also change the biochemical structure of siRNAs, thereby blocking their function. Earlier findings have suggested that plant endogenous small RNA and transgene siRNAs have methylated group at their 3′ termini, this being an HUA ENHANCER$_1$ (HEN$_1$) relying process in their synthesis this step of methylation of viral siRNAs has been shown for DNA or RNA virus-infected plants. It has been demonstrated that many viruses and viral suppressors interfere with both siRNA and/or miRNA methylation [11–13]. Moreover, the virus, alteration of host miRNA accumulation and function is believed to underlie at least some symptoms of plant virus infection [14, 15]. Despite the fact that maximum such research has focused on the role of viral suppressors, a recent study has shown that expression of other viral proteins can also affect miRNA accumulation and function [16]. Previous studies which considered plant viral suppressor's role in transgene induced silencing did not differentiate between primary and secondary siRNAs, and this led to topsy-turvies in the literature about whether a given suppressor did or did not block siRNA production. This ambiguity in results has been purposefully resolved, with the findings that some viral suppressors (i.e., P15 and P25) obstruct accumulation of primary siRNAs, whereas other viral suppressors (i.e., P1/Hc-Pro, P39, P19 siRNAs) leave primary siRNA accumulation unimpaired, [10, 12, 17]. This specialized obstruction in secondary siRNA accumulation might be produced simply by suppressing primary siRNA function.

3. RNA silencing suppressors

Plants defend themselves against viruses by RNA silencing; however, plant viruses spoil this defense machinery by expressing proteins that act as RNA silencing suppressors. Plants react to pathogens using elaborate networks of genetic interactions. Evidential progress has been made in understanding RNA silencing and how viruses counter this apparently ubiquitous antiviral defense. The best example of a viral suppressor that uses host factors that are not direct components of the silencing machinery to block silencing is the HC-Pro suppressor encoded by potyviruses. Two such factors have been reported so far [18, 19], and these will be discussed in more detail subsequently. The first is a calmodulin-like protein called regulator of gene silencing calmodulin-like (rgs-CaM). Tobacco rgs-CaM was identified as an HC-Pro-interacting protein in a yeast two-hybrid screen, and subsequent experiments showed that overexpression of rgs-CaM interfered with virus-induced gene silencing (even in the absence of HC-Pro). Plants encode a large family of calmodulin-like proteins, which are characterized by the presence of a calmodulin domain with either amino-terminal or carboxy-terminal extensions. Experiments to determine if rgs-CaM is required for HC-Pro suppression of

silencing await identification of the *Arabidopsis* homolog of the tobacco gene, which would open up numerous genetic approaches available in that model plant. Many viral protein suppressors of RNA silencing have been described so far, and extensive research was focused on a selection of these below mentioned proteins (suppressors).

3.1. Cucumoviral 2b

The 2b protein of the cucumovirus was recognized as a silencing suppressor at about the same time as P1/HC-Pro of potyviruses. The CMV 2b protein, a nuclear protein that is required for long distance movement of the virus, functions as the silencing suppressor [20]. Viral-suppressor protein 2b interact directly with components of the RNA-induced silencing complex RISC machinery, 2b interact with AGO1, by inhibiting its RNA-cleavage activity leading to the degradation of Argonaute protein AGO1 (**Figure 1**). 2b specifically inhibits AGO1 cleavage activity in RISC reconstitution assessment. In addition, AGO1 recruit's virus-derived small interfering RNAs (siRNAs) *in vivo*, suggesting that AGO1 is a major factor in defense against CMV infection. Viral suppressors of RNA silencing (VSRs) counter act RNAi based viral immunity. Many VSRs proteins have been reported, which play diverse functions in addition to suppressing RNA silencing, like viral replication, movement, coating and pathogenesis. Mostly plant viruses use VSRs as a tool to counter host defense machinery.

3.2. Potexviral P25

The P25 of the potexvirus, Potato virus X (PVX) is one of three cell-to-cell movement proteins (MPs) required for transport of virus from one cell to the next, the effects of P25 on cell autonomous and systemic silencing have been tested. Systemic silencing signal is a P25 – sensitive step and that the signal requires the transgene inducer pathway regardless of whether the inducer is a transgene or a replicating virus [14] However the fact that a viral protein inhibits the pathway leading to systemic signaling strongly implies that the systemic arm of the silencing response is part of the antiviral defense system. Studies have shown that the P25 protein encoded by potato virus X inhibits either the assembly or the function of the effector complexes of antiviral defense. Viruses counter the RNA silencing based counter defense by expressing VSRs. These VSRs are in turn recognized by host as avirulence (avr) factors to induce R- mediated resistance (Plant genomes carry many R genes that recognize specific pathogens and induce resistance against them).

3.3. Helper-component proteinase (HC-Pro)

Helper-component proteinase (a pathogenicity regulator of potyviruses) is a necessary, multifunctional protein of the family *Potyviridae* initially identified as a mediator of synergistic viral disease, acts to suppress the establishment of both transgene-induced and virus-induced gene silencing, and the Hc-Pro protein product is required for suppression. Hc-Pro binds to ds siRNA intermediates and has been suggested to function by sequestering ds siRNAs or by inhibiting their unwinding to ss siRNAs [15, 21] **Figure 1** Courtesy Trends in Microbiology, Vol.16. No.5. HC-Pro in association with p25 or 2b targets intracellular and intercellular silencing respectively. This discovery regarding possible mechanism of silencing suppression was shown by interaction

between P1/HC-Pro of TEV (Tobacco etch virus) and rgs-CaM a tobacco calmodulin like protein [18]. It was demonstrated that rgs-CaM suppresses itself RNA silencing mechanism upon over-expression in the plants points to the role of gene silencing as a natural antiviral defense system in plants and offer different approaches to explain the molecular basis of gene silencing.

3.4. Tombusvirus P19

From the time of its discovery, the Tombusvirus encoded P19 protein (P19) in the late 1980s, the status of this potent suppressor changed from being thought obsolete to its identification a decade later as an important viral pathogenicity factor. A recent study also has confirmed that *Pothos latent virus* (PLV) encode p14 silencing suppressor, although the genome of Tombusvirus is similar to PLV, its suppressor (p14) is smaller than P19 with higher affinity to long dsRNAs. Tombusvirus P19 is a protein encoded by *Tomato bushy stunt virus* and related tombusvirus. Studies have demonstrated that P19 and p14 are RNA silencing suppressors (RSS) in plant cells [22]. P19 was reported to suppress PTGS mainly along the vein tissue and in newly emerging leaves, whereas HC-Pro reversed PTGS in a non-tissue- specific manner [10, 23]. A study confirmed [10, 24] that

- P14 binds to long and short dsRNA including the siRNA duplex.
- P19 is a potent suppressor of PTGS
- P19 is a suppressor of viral induced gene silencing (VIGS), P19 can also fetter to ds siRNA by inhibiting their untwining to ss siRNAs, thereby counter the silencing mechanism.

3.5. V2 suppressor

The suppression of silencing is a key mechanism for successful viral entry The V2 protein of *Tomato yellow leaf curl China virus* (TYLCCNV) was identified as an RNA silencing suppressor by *Agrobacterium*-mediated co-infiltration. The V2 protein could inhibit local RNA silencing [25].

V2 suppressor of *Tomato yellow mosaic virus* binds the coiled-coil protein suppressor of the gene-silencing SGS3 homolog. These reports provide novel insight into the mechanisms developed by viruses to target the defense system of the plant [26]. DNA viruses from the Geminiviridae family encode several proteins namely C2, C4 and V2 which suppress transcriptional and post-transcriptional gene silencing (TGS/PTGS). In Begomovirus, the most abundant genus of this family, three out of six genome-encoded proteins, namely C2, C4 and V2, have been shown to suppress PTGS, with V2 being the potent PTGS suppressor. Beet curly top virus (BCTV), the model species for the Curtovirus genus, is able to infect the widest range of plants among Geminiviruses. In this genus, C2/L2 protein has been described as inhibiting post-transcriptional gene silencing [27].

3.6. P0 protein

The P0 protein of the Polerovirus (Polerovirus is a genus of viruses, in the family Luteoviridae, plants serve as natural hosts) and P1 protein of the Sobemovirus (Sobemovirus is a genus of

viruses, plants serve as natural hosts) suppress the plant's RNA silencing machinery. Here authors [28–30] identified a silencing suppressor protein (SSP), $P0^{PE}$, in the genus *Enamovirus* with only one species *Pea enation mosaic virus*-1 (PEMV-1) and showed that it and the P0s of the Polerovirus *Potato leaf roll virus* and *Cereal yellow dwarf virus* have strong local and systemic SSP activity, while the P1 of genus *Sobemovirus* type species *Southern bean mosaic virus* suppresses systemic silencing. The nuclear localized $P0^{PE}$ has no observable sequence conservation with known SSPs, but proved to be a strong suppressor of local silencing and a moderate suppressor of systemic silencing. Like the P0s from the Polerovirus $P0^{PE}$ destabilizes AGO1 and this action is mediated by an F-box-like domain. Therefore, despite the lack of any sequence similarity, the Poleroviral and Enamoviral SSPs have a conserved mode of action on the RNA silencing mechanism.

4. Conclusion

RNA silencing suppressors (RSSs) are very important factors for virus biology. Virus encodes RSS irrespective of their genome size, for example the geminivirus genome with only 2.7 kb genome size encodes three suppressors (AL2/AC2, AC4 and possibly βC1), whereas CTV with large genome size of 40 kb encodes three RSS. Therefore, the size of the genome is not an indicator of number RSS. Through their evolution, plants and pathogens have adapted and evolved a wide variety of sophisticated strategies to attack, defend, and counterattack. Plants have acquired abilities to sense and defend against invading pathogens by utilizing pre-existing and/or induced barriers to stop infection. In parallel, plant pathogens have evolved diverse ways to counter or overcome host disease resistance. One of the common pathogen strategies involves the production of plant defense suppressors. Viruses evolve rapidly to their host organism and adapt themselves. In contrast, the cellular organisms evolve and adapt to a lesser extent. Keeping in view the fact that viruses possess antiviral defense system suggests that viruses and their host have coevolved. This interdependence among the life forms cannot be fully understood except in an evolutionary frame work. RNAi is a resistance defensive mechanism in plants which targets viral genomes and transcripts to degradation, several findings have revealed viral suppressors that target plant proteins and the possible actions that viruses take during their interference with the defense systems of the host: there remain many unanswered questions for example, the type of proteolysis machinery used by P0 to degrade its plant interactor AGO1 is a matter of debate and the mechanism by which V2 disrupts the RNAi- silencing system of the plant is unknown. The more we dig into the ongoing battle between viruses and their hosts, the more we come to know about the intriguing defense and counter-defense strategies that enable plants and viruses to coexist. To conclude, it can be stated that the interactions between antiviral RNA silencing and the counter measures viruses have evolved to frustrate such process is a continuously evolving action in the continuously evolving microbial world. On the one hand, a very important topic in virology, and on the other hand, a strong starting point for breakthroughs in other fields of research such as functional genomics and development. In an application environment, RNA silencing has allowed us to develop efficient and broad virus resistance in plants, which plays a crucial role to the reliable production of food. RNAi suppression holds the potential of unearthing many unexpected surprises and this promising field is the object of intense investigation.

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Conflict of interest

The author declares that there is no conflict of interest.

Author details

Muzafar Ahmad Sheikh

Address all correspondence to: sheikhmuzafar4@gmail.com

Aligarh Muslim University, Aligarh, India

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Section 4

Advances in Molecular Plant Pathology

Chapter 7

Developing an Online Grapevine Trunk Disease Diagnostic Aid

Mizuho Nita

Additional information is available at the end of the chapter

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Abstract

We face critical challenges in educating growers on wood-canker diseases of grapevines. Unlike other major diseases that appear every year with obvious symptoms (e.g., powdery mildew of grape), wood-canker diseases are often go unnoticed, yet they have more certain and detrimental effects on the plants. Because of this lack of sensational symptom development within a short period, it is difficult for agricultural educators to convince the growers to take urgent action. Modes of delivering extension-related information are changing. Reduced extension education budgets and changes in growers' expectations have led to fewer of the standard, face-to-face meetings between extension agents and growers, and instead, have shifted toward distance-learning style approaches. In order to address these issues, we have developed a new web-based pictorial diagnostic key tool for grapevine trunk diseases. This tool aids users to determine a target grapevine trunk disease using a series of pictorial keys from different tissues of grapevines (leaves, trunks, cluster, etc.). Once enough information is obtained, it will provide the user a list of recommendations for management. This tool covers not only trunk diseases, but also some bacterial diseases and abiotic disorders that are similar in symptom expressions. It is hosted at treeandvinetrunkdiseases.org, and the website is freely available.

Keywords: grapevine, trunk disease, abiotic disorder, diagnostic, application, web

1. Introduction

Various trunk diseases of grapevines affect the grape production around the world [1]. The causal agents of grapevine trunk diseases constitute a complex of fungi mainly belonging to the filamentous Pezizomycotina, which belongs to Ascomycota. Common diseases are

Botryosphaeria canker, Eutypa dieback, Phomopsis dieback, and Esca (also known as Petri disease). Damages caused by these diseases vary in the expression of symptoms. Damages can be made on fruiting structures (spurs, rachis, etc.), trunks and cordons, and/or the vascular system. Infection may result in delaying of fruit ripening and/or overall decline of vines [2]. Since these diseases can affect the trunk of grapevines, which is a semipermanent structure for grape production, the impact of trunk diseases is long-term, and often time, the only remedy is the replacement of infected vines [3].

Unfortunately, field identification of these trunk diseases is challenging because symptoms are very similar to each other. For example, *Botryosphaeria* spp., *Phomopsis viticola*, and *Eutypa lata* can cause dead spurs and cordons. In fact, there was a confusion on the causal agent of grapevine "dead arm" disease where people discussed whether this disease was caused by *Eutypa* or *Phomopsis* [4]. There are some characteristic symptoms such as discoloration of leaves (called tiger striping) of Esca, but it can vary year-to-year [5], or as in symptoms of Eutypa, symptom expression may depend on the environmental factors [6].

Moreover, there are a number of other causes, from biotic factors such as bacterial infection by Pierce's disease pathogen, *Xylella fastidiosa*, to abiotic factors such as winter cold injury that can cause similar symptoms because these factors damage the vascular system of grapevines. Depending on the cause of the problem, management practice will differ; however, extension publications, such as Fact sheets, tend to describe only one or a few diseases. To learn all the possible causes, growers need to read several sources of information. This situation makes more difficult for growers as well as extension and agricultural educators to communicate about diseases and disorders associated with the grapevine trunk.

In order to address this complex issue, with a collaboration of the project PI, Dr. Kendra Baumgartner at USDA/ARS at Davis, CA, USA, we launched a new website (treeandvine-trunkdisease.org) ('trunk disease website' hereafter). This website is designed not only to distribute information on current research topics by a group of researchers but also to provide trunk disease management information that growers can use.

One of the tools that the website offers is a grapevine trunk disease diagnostic application ('trunk disease app' here after). This web-based application is designed to guide users to diagnose grapevine trunk diseases and disorders by providing pictorial keys. Although the name states trunk disease, this application covers not only fungal trunk diseases but also bacterial diseases that can affect the vascular system and abiotic disorders that may cause similar symptoms.

2. Platform for the application

Initially, our idea was to develop a smart device application, such as an app for iOS or Android OS. However, we decided to use a website because of the ease of development and maintenance. A smart device application run better under an appropriate operating system, but it requires us to develop two applications. Also, in order for users to obtain the smart device application, they need to visit the proper application store to download. Depending on the size of the application or the version of the operating system, some users may not be

able to install the app. Moreover, if there is a major update on either of the operating system, the application may require an update. In the worst-case scenario, these operation system updates can make the application entirely useless.

On the other hand, with an application on the website, we can focus our resources on one location. If we keep the coding simple, users do not need to install any application on their devices. Since it is basically a website, any devices with a web browser can access the information. This lack of dependency on the platform helps to reach out more audiences since some growers may not be as technologically perceptive as the others may.

Therefore, we developed our application on a remote web server that hosts our website. The content management system was WordPress (Wordpress.org), and coding was a combination of the HTML and the CSS. Using a template of WordPress that was designed to automatically scale and place menu items based on the platform that was accessing the website, we developed a mobile- (or smartphone-) ready website with ease.

3. Diseases and disorders covered by the application

As noted earlier, although this application has a name associated with grapevine trunk diseases, it handles more than trunk diseases. **Table 1** shows the list of diseases and disorders that are covered by the application.

Type	Common name	Causal agent
Fungal disease	Botryosphaeria canker	*Botryosphaeria* spp.: *B. australis*, *B. dothidea*, *B. lutea*, *B. obtusa*, *B. parva*, *B. rhodina*, and *B. stevensii* [7]
	Esca, Petri disease	*Phaeomoniella chlamydospora*
		Phaeoacremonium minimum
		Other *Phaeoacremonium* species [8]
	Eutypa dieback	*Eutypa lata* [4]
	Phomopsis dieback	*Phomopsis viticola*
Bacterial disease	Crown gall	*Rhizobium vitis* (also known as *Agrobacterium vitis*) [9]
	Pierce's disease	*Xylella fastidiosa* [10]
	North American Grapevine Yellows (NAGY)	Phytoplasma (related to *Candidatus Phytoplasma pruni* [11]
Abiotic disorder	Frost damage	
	Nutrient deficiencies	
	Other physical injuries such as cold and hail damage	

Table 1. Diseases and abiotic disorders that are covered in the trunk disease app.

4. Interface and plug-ins

WordPress theme Parabota (www.cryoutcreations.eu) was chosen as an interface of the website since it is a scalable template that automatically recognizes the operation system of the site visitor, then scales images and rearranges menus. In addition, a plug-in WPPatch (www.wptouch.com) was installed to enhance the experience of mobile phone users. We used a package RPS image gallery (redpixel.com) to add a description (footnote) to each image, place images as columns, and also allow the site visitor to click image to enlarge. The usage of the website is tracked by Google Analytics (www.analytics.google.com).

5. Trunk disease app interface

When a site visitor comes to the trunk disease website (**Figure 1**), which is a portal site for many project pages, including the trunk disease diagnostic app, the user finds the app under the main menu 'Extension.'

The menu opens an introductory page that explains how this application works. Then, the application starts by showing four tissues of grapevine, 'leaves', 'shoots', 'clusters', and 'cross sections and bark' (**Figure 2**).

Once the user clicks the tissues that are showing symptoms, the application displays different types of symptom for the user to select (**Figure 3**).

Then, the application follows up with a series of questions. For example, even if the user chooses leaves as symptomatic tissue, the application shows pictures of other parts of the grapevines (**Figure 4**).

After answering several questions, the application displays the most likely scenario for the symptoms (**Figure 5**).

Figure 1. The SCRI trunk disease project page on a computer screen (treeandvinetrunkdiseases.org).

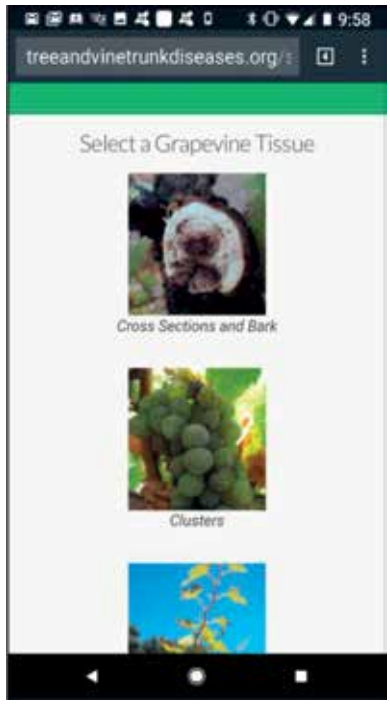

Figure 2. Trunk disease app's opening dialogue on a smartphone screen: Asking users to select a symptomatic tissue.

Figure 3. Trunk disease app on a smartphone screen: app users click or tap the best-described symptom picture to determine the disease in question.

Figure 4. Trunk disease app on a smartphone screen: The app shows multiple pictures to app users for the confirmation.

Figure 5. Trunk disease app on a smartphone screen: A diagnostic screen to display the most likely disease based on the user inputs.

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6. Summary and conclusions

The major issue with grapevine wood-canker diseases and other trunk-related disorders is the difficulty of diagnostics. Many growers overlook the symptoms simply because they are not familiar with these diseases and disorders. In addition, these diseases do not cause immediate damage to the infected vines. However, when we look into the life of a vineyard, the long-term effect of these diseases can be detrimental [12]. One of the issues is the lack of obvious symptom expressions from trunk diseases and disorders. Many of the symptoms are similar to each other, and since many extension education materials often talk about one disease, it is difficult for the growers to cross-examine symptoms to find the diseases that they are dealing with.

In order to address these issues, we have developed a new web-based pictorial diagnostic key tool for grapevine trunk diseases and other disease and disorders that affect the trunk of grapevines. This application is freely available through our project page (treeandvine-trunkdiseases.org). This diagnostic tool aids users to determine a target grapevine trunk disease and disorder with a series of pictorial keys from different tissues of grapevines (leaves, trunks, cluster, etc.). Users will answer several questions by matching symptoms with pictures. Once enough information is obtained, it will provide recommendations for management. In order to appeal to many users, we selected mobile-friendly themes and plug-ins in the website and application.

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Author details

Mizuho Nita

Address all correspondence to: nita24@vt.edu

Alson H. Smith Jr. Agricultural Research and Extension Center, Department of Plant Pathology, Physiology, and Weed Science, Virginia Polytechnic Institute and State University, Winchester, Virginia, USA

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Plant pathology deals mainly with biotic phenomena that interfere with the normal metabolism of plants. Plants have developed mechanisms to deal with pathogenic attacks, while at the same time, pathogens are actively devising ways of overcoming the plant defense systems. Plant pathologists have been advancing their studies from morphological and physiological to now molecular studies at the gene level. There are various approaches for different microorganisms and plants. This makes the study of plant pathology diverse. This book, *Advances in Plant Pathology*, attempts to investigate advances in viral, fungal, bacterial, and other diagnostic molecular approaches in various plants.

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