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REVIEW

# Progress in understanding *Pseudocercospora* banana pathogens and the development of resistant *Musa* germplasm

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Banana and plantain (*Musa* spp.) are important food crops in tropical and subtropical regions of the world where they generate millions of dollars annually to both subsistence farmers and exporters. Since 1902, *Pseudocercospora* banana pathogens, *Pseudocercospora fijiensis*, *P. musae* and *P. eumusae*, have emerged as major production constraints to banana and plantain. Despite concerted efforts to counter these pathogens, they have continued to negatively impact banana yield. In this review, the economic importance, distribution and the interactions between *Pseudocercospora* banana pathogens and *Musa* species are discussed. Interactions are further scrutinized in the light of an emerging climate change scenario and efforts towards the development of resistant banana germplasm are discussed. Finally, some of the opportunities and gaps in knowledge that could be exploited to further understanding of this ubiquitous pathosystem are highlighted.

*Keywords:* banana, climate change, effectors, genetic improvement, *Pseudocercospora* banana pathogens

## Importance of *Musa* spp.

Edible bananas are native to Southeast Asia and are a result of hybridization events involving two wild, seeded species, *Musa acuminata* (A genome) and *Musa balbisiana* (B genome). However, a few cultivars are thought to have originated from hybridization of *Musa schizocarpa* (S genome) with either *M. acuminata* or *M. balbisiana*. One clone is thought to have resulted from hybridization between *M. balbisiana* and *Musa textilis* (T genome; Simmonds, 1962; D'Hont *et al.*, 2000). Banana and plantains are important food crops in tropical and subtropical regions where they are grown in nearly 120 countries on about 10 million hectares. They are estimated to yield 144 megatonnes (Mt) annually (Food & Agriculture Organization of the United Nations (FAO), 2014). The 10 leading banana-producing countries are India 28.28 Mt, China Mainland 11.64 Mt, Uganda 11.23 Mt, Phillipines 9.45 Mt, Ecuador 8.24 Mt, Brazil 7.65 Mt, Indonesia 6.34 Mt, Colombia 5.27 Mt, Cameroon 4.94 Mt and Tanzania 4.08 Mt (FAO, 2014). Worldwide, bananas are ranked sixth among the staple food crops after rice, wheat, maize, cassava and potato (Food & Agriculture Organization of the United Nations, 2014). Over 85% of banana is produced by smallholder farmers for food, nutritional security and as a source of income, while the rest of the production (approximately 16.5 Mt per year) is primarily dominated by cultivar Cavendish that is targeted for the export market, generating approximately $13.2 billion in earnings annually (Food & Agriculture Organization of the United Nations, 2014).

## Impact of *Pseudocercospora* Banana Pathogens

Production of banana and plantain worldwide is constrained by various pests and diseases. One of these major disease complexes results from three fungi belonging to the genus *Pseudocercospora* (previously named *Mycosphaerella*) that are closely related phylogenetically. These three fungi are *P. fijiensis* (sexual morph: *Mycosphaerella fijiensis*), the causal agent of black leaf streak diseases/black Sigatoka, *P. musae* (sexual morph: *Mycosphaerella musicola*), that causes yellow Sigatoka and *P. eumusae* (sexual morph: *Mycosphaerella eumusae*), the causal agent of eumusae leaf spot (Stover, 1980; Carlier *et al.*, 2000a; Marín *et al.*, 2003; Arzanlou *et al.*,

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2007; Zandjanakou-Tachin *et al.*, 2009). *Pseudocercospora* banana pathogens are hemibiotrophic. Once the spores of these pathogens are disseminated to the banana leaf surface and environmental conditions are conducive for pathogen growth, they germinate and penetrate leaf tissues through stomatal openings to colonize the intercellular spaces within mesophyll and pallisade cell layers (Jones, 2000). Symptom expression occurs 7–14 days after infection and is manifested by streaks on leaves that later turn necrotic, thus significantly reducing the leaf's ability to photosynthesize (Churchill, 2011). This may ultimately lead to 35–100% yield loss, depending on the cultivar, environmental conditions and level of infection (Marín *et al.*, 2003; Churchill, 2011). These losses are reflected by poorly filled fruits, smaller bunches and reduced bunch weight (Chillet *et al.*, 2014). In addition, severe infections induce physiological changes that result in heightened climacteric rise ($CO_2$ and $C_2H_4$ production) that leads to reduced green life (Castelana *et al.*, 2012; Chillet *et al.*, 2014); this is associated with premature and uneven fruit ripening, reduced pulp colour clarity and altered fruit taste. The pathogen-induced early fruit ripening results in farmers harvesting the fruit before maturity to avoid fruit spoilage before reaching the market (Jones, 2000; Marín *et al.*, 2003; Pérez-Vicente, 2012; Chillet *et al.*, 2014). Infection by *Pseudocercospora* banana pathogens also triggers the production of hormones such as abscisic acid that are known to play a role in the plant abiotic stress response. Similarly, foliar stress from these pathogens triggers anthocyanin synthesis which results in changes in pulp colour from white to grey or red (Dixon *et al.*, 1994; Chaiker-Scott, 1999; Zhang *et al.*, 2006; Gagné *et al.*, 2011; Chillet *et al.*, 2014).

## Management of *Pseudocercospora* Banana Pathogens

Application of combinations of systemic and contact fungicides is effective against *Pseudocercospora* banana pathogens. However, this practice results in an increase in the cost of production by 25–30% (i.e. from \$1000 $ha^{-1}$ up to \$1800 $ha^{-1}$). In total these chemical control efforts cost \$550 million annually worldwide (Churchill, 2011). The use of chemicals effective against *P. fijiensis* is known to encourage the pathogen to develop resistance against these fungicides (Rodriguez *et al.*, 2016). Another concern associated with chemical applications is the environmental risk posed to those working and living near banana plantations (de Bellaire *et al.*, 2010; Churchill, 2011). Other non-chemical disease management approaches, such as the use of biological control agents, organic farming, cultural practices and phytosanitary legislation, have not been widely adopted as they are often laborious and require specialized equipment that might be prohibitive in developing countries (Marín *et al.*, 2003; Jimenez *et al.*, 2007; Churchill, 2011). The majority of banana and plantain producers in sub-Saharan Africa are small-scale farmers with limited income hence particularly vulnerable to impacts of *Pseudocercospora* banana pathogens.

## Distribution of *Pseudocercospora* Banana Pathogens and Predicted Climate Change Effects

Early reports show that *P. musae* was the first pathogen of the *Pseudocercospora* species to be identified as a pest to banana in the Java Islands in 1902 and it rapidly spread to other banana-growing areas causing mild to severe symptoms. Later, in 1963, *P. fijiensis* was reported in the Sigatoka district of the Fiji islands, giving the disease the name 'Sigatoka' by which it is commonly known. However, it is believed that *P. fijiensis* may have existed long before this initial documentation and probably coexisted with *P. musae* or may have been misidentified for the same pathogen (Meredith & Lawrence, 1970; Stover & Dickson, 1976; Jones, 2000). In less than four decades from its initial documentation, *P. fijiensis* has spread to attain global distribution in most banana-growing regions, earning the reputation of the most problematic disease of bananas in Africa, Asia, the Pacific Islands and the Americas (Stover, 1980; Stover & Simmonds, 1987). *Pseudocercospora eumusae* is the latest *Pseudocercospora* species to be recognized as a banana production constraint and since its first identification in Southeast Asia in the 1990s, the disease has since been reported in South Asia, Sri Lanka, Mauritius, Malaysia, Vietnam and Nigeria (Carlier *et al.*, 2000b; Crous & Mourichon, 2002; Zandjanakou-Tachin *et al.*, 2009). In Africa, there are no reports of *P. eumusae* in major banana-growing areas except Nigeria (Tushemereirwe *et al.*, 1993; Zandjanakou-Tachin *et al.*, 2009). However, distribution of this pathogen is suspected to be more widespread than currently reported but may be undetected due to its similarity of symptoms as well as coexistence with *P. fijiensis* and *P. musae*. The lack of comprehensive information calls for more robust epidemiology studies on this pathogen in major banana-growing areas, especially in Africa where limited management efforts are employed.

Traditionally, *Pseudocercospora* banana pathogens were separated by altitudinal and climatic gradients; for instance, *P. musae* was restricted to higher altitudes with cooler temperatures (Tushemereirwe *et al.*, 1993; Carlier *et al.*, 2000b; Jones, 2009). On the other hand, *P. fijiensis* was more prevalent in lower, warmer areas with higher rainfall (Tushemereirwe *et al.*, 1993; Arzanlou *et al.*, 2007; Churchill, 2011). However, there appears to be an adaptation shift towards higher altitude areas by *P. fijiensis* and in some cases it has replaced *P. musae* to become the dominant *Pseudocercospora* banana pathogen (Jones, 2000; Arzanlou *et al.*, 2007). This displacement has been gradual, therefore suggesting an evolutionary adaptation in response to either a changing climate or host fitness (Jones, 2000). Growth, reproduction, dispersal and survival of *Pseudocercospora* banana pathogens are highly dependent on prevailing

environmental conditions, primarily humidity, temperature and precipitation (Chakraborty & Newton, 2011; Churchill, 2011). Changes in climatic variables can often lead to shifts in the relative importance of diseases by altering their distribution in such a manner that previous habitats become more or less favourable for individual pathogens. These shifts are often gradual as the pathogens slowly adapt to new environments to avoid displacement (Chakraborty *et al.*, 2000; Jones, 2000; Chakraborty, 2013). It has often been predicted that these ecological changes may affect virulence of plant pathogens and therefore impact negatively on plant quality and yield (Garrett *et al.*, 2006, 2011; Chakraborty & Newton, 2011; Luck *et al.*, 2011; Savary *et al.*, 2011; Jones & Barbetti, 2012; Juroszek & Von Tiedemann, 2013).

Predictions of the effects of climatic change on banana and *P. fijiensis* have also been made. For instance, areas suitable for banana production are suggested to increase by 50% by the year 2070 as a result of increased annual temperatures that will make conditions more favourable for banana in higher altitudes. Thus, increases in banana cultivation area will increase fruit yield in the subtropics and in the tropical highlands (Calberto *et al.*, 2015). However, the higher temperatures will potentially result in higher water demand, projected to increase by 12–15%. This high water demand may lead to enhanced irrigation to mitigate against water deficiency. Further, higher temperatures may threaten other perennial crops such as coffee and cocoa that are often intercropped with banana, possibly forcing farmers to abandon them and, in the long run, abandon banana. It has also been predicted that increased temperatures will increase incidence and severity of *Pseudocercospora* banana pathogens as it is likely to accelerate spore germination. However, some suggest this may not be as great a concern because leaf wetness plays a bigger role than temperature on disease severity; this is associated with rainfall distribution that may not be affected by climate change (Ghini *et al.*, 2007; Calberto *et al.*, 2015). In addition, new banana habitats may emerge, resulting in habitat shifts of *Pseudocercospora* banana pathogens. These climatic change models highlight the effect of habitat suitability on pathogen survival rather than the effect on pathogen development or host response to pathogens (Elad & Pertot, 2014).

Given the polycyclic nature and sexual reproduction of the *Pseudocercospora* banana pathogens, it is likely that they will benefit from a prolonged host growing season occasioned by global warming (Calberto *et al.*, 2015); the pathogen will probably achieve more generations per season, which in turn will result in increased disease severity. In addition, changes in climate have also been shown to favour sexual reproduction in fungi, which increases the potential for emergence of new pathotypes (Legler *et al.*, 2012). The genetic diversity among and within *Pseudocercospora* banana pathogens is considerable and has often resulted in emergence of resistance to existing chemical formulations used to control them (Rodriguez *et al.*, 2016). Therefore, it is expected that these difficulties will be further exacerbated in scenarios where humidity and temperature are changing.

Although it is difficult to discriminate between the effect of temperature on the effectiveness of host resistance mechanisms and its effect on pathogen virulence, it has been documented that host–pathogen gene pairs related to resistance respond differently to different temperature ranges (Browder & Eversmeyer, 1986; Olson *et al.*, 1990; Newton & Young, 1996; Chakraborty *et al.*, 2000; Garrett *et al.*, 2006; Luck *et al.*, 2011; Elad & Pertot, 2014). These changes in temperature resulting from climate change may be perceived by banana plants as a form of abiotic stress. Abiotic stresses are known to alter gene expression profiles of plants (Zhang *et al.*, 2006); however, it is still not clear whether these changes will result in reduced or increased manifestation of *Pseudocercospora* banana diseases. Increased infection by the *Pseudocercospora* banana pathogens has been reported to illicit abiotic-like responses in banana by enhancing anthocyanin biosynthesis that in turn results in alteration in fruit colour (Chillet *et al.*, 2014). The effect of climate change on the profile or quantity of small fungal molecules called effectors, which are known to aid in pathogen infection, also remains unknown (Vleeshouwers & Oliver, 2014). Recently, transcriptomic and bioinformatic analysis identified such factors in *Pseudocercospora* banana pathogens (Arango-Isaza *et al.*, 2016; Noar & Daub, 2016a,b). It is possible that climatic changes will not only affect optimal conditions for infection but may also influence host specificity and success of disease management strategies (Elad & Pertot, 2014). To date, the optimum environmental conditions for *P. eumusae* are unclear, although it has been reported to survive in similar environmental conditions as *P. fijiensis* (Carlier *et al.*, 2000a). More detailed research is needed to elucidate clearly how changes in climate will affect the distribution and virulence of *Pseudocercospora* banana pathogens. In addition, it is imperative to understand how these changes will influence pathogenicity of the more than 20 *Pseudocercospora* spp. reported to colonize banana but whose economic importance on banana is yet to be determined (Arzanlou *et al.*, 2008). In fact, because the effects of climatic change remain predictive, elaborate surveillance and management programmes targeting *Pseudocercospora* banana pathogens could be the most cost-effective option to reduce future impacts (Hulme, 2017).

## Advances in Genomics of *Pseudocercospora* Banana Pathogens

Efforts towards sequencing *Pseudocercospora* banana pathogens were initiated in 2003 and first focused on *P. fijiensis*, using the genome of *Zymoseptoria tritici* (previously named *Mycosphaerella graminicola*) as a reference. By 2004, a draft of the *P. fijiensis* genome had been assembled and the size estimated to be 74.1 Mb. The then newly sequenced genome was found to be 87% larger than *Z. tritici* due to a considerable number of repetitive sequences (Goodwin *et al.*, 2004). Later, two idiomorph

genes, *mat1-1* and *mat1-2*, were isolated in *P. fijiensis* using long-range PCR, providing insight into the possible evolution of *Pseudocercospora* species (Conde-Ferráez *et al.*, 2007). Additional studies have concentrated on genomic sequence annotation and isolation of useful markers (Yang & Zhong, 2008; Ferreira *et al.*, 2009). These efforts have resulted in the identification of gene homologues associated with *P. fijiensis* virulence (Cho *et al.*, 2008; De Wit *et al.*, 2009; Stergiopoulos *et al.*, 2010). Later, the genomes of *P. fijiensis* isolates CIRAD86 and CIRAD139 were sequenced using Sanger and Illumina Hi Seq technologies, and have provided more detailed information on genomic structure and content, as well as the functionality of several genes in the pathogen (Ohm *et al.*, 2012; Arango-Isaza *et al.*, 2016). These studies have clearly demonstrated genome expansion in *P. fijiensis* by the presence of repetitive sequences; such expansion may possibly be a result of horizontal gene transfer from banana or from other fungi colonizing banana (Friesen *et al.*, 2006; Arzanlou *et al.*, 2008; Ma *et al.*, 2010; Santana *et al.*, 2012; Czislowski *et al.*, 2017). Comparative genomic analysis between *P. fijiensis* and *P. eumusae* has led to the suggestion that the two pathogens share a common ancestor and could have possibly emerged as a result of radiative evolution (Arango-Isaza *et al.*, 2016). More importantly, these studies have determined that the gene content of virulent *P. fijiensis* and *P. eumusae* display complementary expansions and contractions in gene families involved in metabolism and enzymatic degradation (Chang *et al.*, 2016). These findings point to a possibility of convergent evolution that resulted in variation of the virulence of the pathogens. One important paradigm would be to understand how the gene expansions and increase in repetitive sequences increase virulence of fungal pathogens and, by extension, host resistance (Möller & Stukenbrock, 2017). The analysis of repetitive sequences in *P. fijiensis* suggests that they play an important role in evolution of this pathogen through modification of gene structure or expression, resulting in new strains of the pathogen (Santana *et al.*, 2012). Recently, transcriptome sequencing of *P. fijiensis* during association with banana and on media identified key pathogenicity genes that are up-regulated during plant infection (Noar & Daub, 2016a). Furthermore, a bioinfomatic approach to predict polyketide synthase (*PKS*) gene clusters from *P. fijiensis* found that the fungus contained three *PKS* genes that were also present in *P. musae* and *P. eumusae* but absent in other fungal species. Further comparison to *P. musae* and *P. eumusae PKS* gene clusters showed that many of the *PKS* genes were similar to those of *P. fijiensis*, with a single gene exception that encoded a unique compound (Noar & Daub, 2016b). An analysis of several isolates of *P. fijiensis* has also revealed allelic variation in some of the effector-coding sequences (Stergiopoulos *et al.*, 2014). These findings further strengthen the radiative evolution theory for the *Pseudocercospora* banana pathogens (Arango-Isaza *et al.*, 2016). With this information and knowledge of gene expansions in these pathogens, gene validation studies could be further intensified to gain more insight on gene function in the *Pseudocercospora* banana pathogens (Santana *et al.*, 2012). Indeed, bioinformatics analysis of the repetitive sequences has revealed that gene expansions and increases in repetitive sequences lead to emergence of new strains/pathotypes of *P. fijiensis* (Santana *et al.*, 2012; Möller & Stukenbrock, 2017). To some extent these genomic expansions could be responsible for several *PKS* genes that are only found in the *Pseudocercospora* banana pathogens and no other fungal genera, making it an even more complex pathosystem (Noar & Daub, 2016b).

## Genetic Transformation of *Pseudocercospora* spp: A Tool to Understand Genomics of *Pseudocercospora* Banana Pathogens

Currently, several transformation systems for *P. fijiensis*, *P. musae* and *P. eumusae* using polyethylene glycol, *Agrobacterium tumefaciens* and underwater shock waves exist (Balint-Kurti *et al.*, 2001; Donzelli & Churchill, 2003; Portal *et al.*, 2012; Escobar-Tovar *et al.*, 2015; Onyilo *et al.*, 2017). These transformation protocols have led to better understanding of the infection process of *Pseudocercospora* banana pathogens in both susceptible and resistant banana germplasm through the use of tagged fluorescent markers. Furthermore, it has been demonstrated that the pathogens are hemibiotrophic (Balint-Kurti *et al.*, 2001; Portal *et al.*, 2012; Escobar-Tovar *et al.*, 2015). Recently, Onyilo *et al.* (2017) demonstrated that silencing of a gene (*PfHog1*) responsible for the *P. fijiensis* mitogen-activated protein kinase known as *P. fijiensis* high osmolarity glycerol significantly suppressed growth of *P. fijiensis* on potato dextrose agar media supplemented with 1 M NaCl. This indicated that *PfHog1* regulates osmotic stress and plays a critical role in osmotic stress regulation. In addition, the *PfHog1*-silenced mutants displayed reduced virulence as well as growth rate through reduced mycelial density (Onyilo *et al.*, 2017). Although gene knockout or silencing approaches are not routine in these fungi, this study sets a solid foundation for further exploration in that area. Other tools such as gene overexpression as well as genome editing may also be feasible with the genomic information that is now available for *P. fijiensis*, *P. eumusae* and *P. musae*. The genomes of these three pathogens have lineage-specific transposable element expansions that may have played an important role during species divergence. Furthermore, it may be useful to apply reverse genetic studies to elucidate gene function in the *Pseudocercospora* banana pathogens; this may reveal new factors that are important for the virulence of the pathogens when colonizing banana (Arango-Isaza *et al.*, 2016; Chang *et al.*, 2016).

## Identification and Validation of Effector Proteins and Other Virulence Factors of *Pseudocercospora* Banana Pathogens

Plant pathogens secrete a wide range of small molecules (microRNAs, metabolites and toxins) and proteins

referred to as effectors that, depending on the host lifestyle, result in triggered resistance or susceptibility responses (Weiberg *et al.*, 2013; Vleeshouwers & Oliver, 2014; Arango-Isaza *et al.*, 2016). Effector-triggered immunity is a result of interaction between pathogen virulence factors and their cognate resistance genes in the host (Vleeshouwers & Oliver, 2014). While the importance of *Pseudocercospora* species as banana pathogens has been known for a long time, the repertoire of effector proteins involved in pathogenicity and their functional characterization is only now being understood (Noar & Daub, 2016a,b). It is suspected that microRNAs, metabolites and toxins play effector roles in virulence of *Pseudocercospora* banana pathogens, although this has not been clearly demonstrated (Weiberg *et al.*, 2013). Few effector proteins that are functional orthologues of the much studied *Cladosporium fulvum* have been identified in the genome of *P. musae* and *P. fijiensis* (Stergiopoulos *et al.*, 2010; Passos *et al.*, 2013; Arango-Isaza *et al.*, 2016; Noar & Daub, 2016b). Notably, a higher number of effectors have been identified in *P. fijiensis* than other related fungi such as *M. graminicola*, *P. eumusae*, *P. musae* and *C. fulvum* (Cho *et al.*, 2008; Arango-Isaza *et al.*, 2016; Friesen, 2016; Noar & Daub, 2016a). Such findings, coupled with the current understanding that the three pathogens have different strategies for manipulating the host immune system, have great implications especially for the development of *Musa* germplasm with resistance to *Pseudocercospora* banana pathogens. Recently, transcriptomic and bioinformatic analyses have identified genes that code for molecules that aid *Pseudocercospora* banana pathogens to successfully infect banana, including PKS and proteins involved in metabolism and enzymatic degradation (Chang *et al.*, 2016; Noar & Daub, 2016a,b). Bioinformatic analysis has also demonstrated that some of the effectors are unique to the three *Pseudocercospora* banana pathogens while one is only present in *P. fijiensis* and not in any known fungal pathogen (Noar & Daub, 2016b). Recently, a purified protein of the putative gene *PfAvr4* from *P. fijiensis* successfully illicited a hypersensitive reaction in a resistant banana genotype but not in the susceptible cultivar, hence cementing the importance of such molecules in virulence of the pathogen and their potential use in germplasm resistance screening (Arango-Isaza *et al.*, 2016).

Understanding the types of effectors produced by the pathogens and their connection to virulence as well as host response will aid in the development of new management strategies against these pathogens. Effector-assisted breeding can facilitate the discovery of resistance genes and can be used to determine the durability of the resistance genes by evaluating their ability to recognize genetic variants of effector protein genes in a pathogen population (Vleeshouwers & Oliver, 2014). This approach may be more feasible in developing resistance to *Pseudocercospora* banana pathogens that exhibit a great deal of plasticity. Effectors have been exploited in resistance breeding and resistance gene deployment in various pathosystems such as *Parastagonospora nodorum* and *Pyrenophora tritici-repentis* on wheat and *Phytophthora infestans* on potato (Vleeshouwers & Oliver, 2014). Recent work by Arango-Isaza *et al.* (2016) has demonstrated that using effector proteins may provide a rapid screening method for resistance to *Pseudocercospora* banana pathogens that may culminate in early deployment/release of germplasm. It is understood that *P. musae* and *P. eumusae* employ similar mechanisms for host attack, thus, a similar approach of resistance identification is likely to be applicable (Noar & Daub, 2016a,b). Genomic comparisons of various pathogens with different virulence abilities may also help to explain why *P. fijiensis* and *P. eumusae* have managed to coexist while *P. musae* appears to have been displaced by *P. fijiensis* (Carlier *et al.*, 2000a; Gauhl *et al.*, 2000; Arzanlou *et al.*, 2007; Zandjanakou-Tachin *et al.*, 2009). It may be interesting to understand how virulence factors from these different but related fungi affect their coexistence.

## Understanding the Host Response to *Pseudocercospora* Banana Pathogens and Development of Resistant *Musa* germplasm

Development of *Musa* germplasm with resistance to *Pseudocercospora* banana pathogens has been a focus of several breeding programmes since 1922 (Jones, 2000). Results from the breeding efforts have shown that *Pseudocercospora* banana pathogens appear to have evolved different virulence abilities against banana, sometimes leading to breakdown in resistance in well-characterized germplasm (Fullerton & Olsen, 1995; Escobar-Tovar *et al.*, 2015). These reports confirm that major gene resistance, as proposed in the gene-for-gene resistance model by Flor (1946), is unlikely to be a long-term solution against *Pseudocercospora* banana pathogens. Ortiz & Vuylsteke (1994) proposed that resistance to *P. fijiensis* is a result of one recessive allele ($bs_1$) and two independent resistant additive alleles ($bsr_2$ and $bsr_3$). Later, Craenen & Ortiz (1997) demonstrated that intralocus interaction in the $bs_1$ locus apparently regulates the appearance of symptoms on the leaf surface whereas the additive effect and intralocus interaction of the *bsr* locus affects disease development in banana.

Several studies have determined that the biochemical reactions that occur in other plant species during invasion by pathogens also occur in banana when infected by *Pseudocercospora* pathogens (Dhakshinamoorthy *et al.*, 2014; Hölscher *et al.*, 2014; Vaganan *et al.*, 2014; Wang *et al.*, 2015). These include strengthening of physical barriers, such as lignification, the hypersensitive response, and the production of compounds such as phytoanticipins, phenols, phenylphenalenones, peroxidase, phenylalanine ammonia-lyase, β-1,3-glucanase and hydrogen peroxide (Hoss *et al.*, 2000; Otálvaro *et al.*, 2007; Cruz-Cruz *et al.*, 2010; Cavalcante *et al.*, 2011; Torres *et al.*, 2012; Sanchez-García *et al.*, 2013; Hidalgo *et al.*, 2016). Specific changes in gene expression profiles have also been detected only in resistant cultivars. For instance, in

Calcutta 4, high expression of several genes associated with pathogenesis-related (PR) proteins has been recorded during infection with *P. fijiensis* (Portal *et al.*, 2012; Rodriguez *et al.*, 2016). Furthermore, an analysis of the banana genome has identified genes encoding putative proteins associated with plant response to invasion by pathogens and their effectors (D'Hont *et al.*, 2012). The early gene expression pattern of Calcutta 4 in response to infection by *P. fijiensis* was determined using microarray chip and quantitative real-time PCR, which also established that genes coding for peroxidase, pathogenesis-related 4 (PR-4), PR-10, phenylalanine ammonia-lyase and disease response 1 were up-regulated during early infection with the pathogen (Rodriguez *et al.*, 2016). Concurrently, using the cDNA suppression subtractive hybridization technique, different pathways such as glycolysis/gluconeogenesis and genes encoding zinc finger domains, metallothioneins and putative disease resistance protein RGA1 were shown to play a role in the early resistance response against *M. fijiensis* in Calcutta 4 (Timm *et al.*, 2016). A recent study established that genes coding for ethylene responsive factor, flavin-containing monoxygenase, serine glyoxylate lipoxygenase and metallothionein were up-regulated when the resistant cultivar Manoranjitham was infected with *P. eumusae* (Saravanakumar *et al.*, 2016). Furthermore, other innate host factors such as stomatal density and pseudostem waxiness have been proposed to play a role in resistance to *P. fijiensis* (Craenen & Ortiz, 1997). It is important to note that true or complete resistance to *P. fijiensis* has not been shown to exist, although some level of resistance or tolerance has been clearly demonstrated (Craenen & Ortiz, 1997).

As observed in the studies above, different approaches as well as cultivars are used in determining host resistance responses against *Pseudocercospora* banana pathogens. However, it is clear that resistant and susceptible cultivars respond differently to infection by these pathogens. These gene response studies are therefore helpful on two fronts. First, the gene expression profiles could be used in early selection of resistant candidates through artificial infection by the pathogens followed by gene expression profiling of the target genes. This approach could save time and funds used in maintaining fields during selection of resistant parents under natural infection. Secondly, the genes identified to confer resistance against these pathogens could be cloned into overexpression vectors and transformed into banana. In the latter approach, the transgenic banana produced could be screened against *Pseudocercospora* banana pathogens and, if found to be resistant, deployed for wider cultivation. However, the acceptance of this approach may face barriers in countries that have restrictions on genetically engineered crops.

The major banana breeding programmes worldwide are mainly clustered in Africa, Asia and the Americas. In Africa, the International Institute of Tropical Agriculture (IITA) in Nigeria, Uganda and Tanzania, the National Agricultural Research Organization (NARO) of Uganda, the Centre Africain de Recherches sur Bananiers et Plantains (CARBAP) in Cameroon and the Centre National de Recherche Agronomique (CNRA) in Côte d'Ivoire have responsibility for banana improvement. In Asia, the Chinese Academy of Tropical Agricultural Sciences (CATAS) and the Guangdong Academy of Agricultural Sciences (GDASS) in the Peoples' Rebublic of China, the Indonesian Fruits Research Institute (ITFRI) in Indonesia and the ICR-National Research Centre for Banana (NRCB) in Tamil Nadu, India perform similar functions on locally adapted varieties. In the Americas, breeding is carried out by the Empresa Brasileira de Pesquisa Agropecuária–Mandioca e Fruticultura Tropical (EMBRAPA-CNPMF) in Brazil, the Centre de Coopération Internationale en Recherche Agronomique pour le Dévelopement – Département des productions fruitières et horticoles (CIRAD-FLHOR) in Guadeloupe, and the Fundación Hondureña de Investigación Agrícola (FHIA) in Honduras that absorbed the United Fruits Company.

At IITA, the evaluation of several subspecies of wild *M. acuminata* (subsp. *banksii*, *burmannica*, *malaccensis*, *microcarpa*) against *P. fijienis* has shown that partial resistance (whereby disease symptoms develop but do not progress beyond the streak stage) was more durable than a gene-for-gene hypersensitive-like reaction (Craenen & Ortiz, 1997; Carlier *et al.*, 2000b). Even with this information, the complexities of breeding *Musa* spp., including its sterility, polyploid nature, low seed germination and narrow genetic basis, call for the need to integrate genomic tools into banana breeding programmes (Swennen & Vuylsteke, 1993). When investigating *Pseudocercospora* banana pathogens, Ferreira *et al.* (2004) used RAPD markers to characterize banana diploids (AA) with contrasting responses to *P. fijiensis* and *P. musae*. In addition, methylation-sensitive amplification polymorphism (MSAP) markers were found that were associated with resistance to *P. fijiensis* toxins and could be useful in selection of germplasm with resistance to the pathogen (Ortiz & Vuylsteke, 1995; Gimenez *et al.*, 2006; Lin *et al.*, 2010). To date, the genomes of two banana progenitors, *M. acuminata* subsp. *malaccensis* (A genome) and *M. balbisiana* (B genome), and another wild banana, *M. itinerans*, have been sequenced with the resulting sequences revealing genome evolution peculiarities of Musaceae (D'Hont *et al.*, 2012; Davey *et al.*, 2013; Čížková *et al.*, 2015; Christelová *et al.*, 2016). This information could shed more light on *Musa* spp. evolution and inherent disease resistance factors. In parallel, several studies have also demonstrated the utility of various classes of DNA markers in *Musa* spp. (Ude *et al.*, 2002; Nwakanma *et al.*, 2003; Buhariwalla *et al.*, 2005; Teo *et al.*, 2005; Heslop-Harrison & Schwarzacher, 2007; Garcia *et al.*, 2010; Saraswathi *et al.*, 2011; Ravishankar *et al.*, 2013; Brown *et al.*, 2017). Currently, a form of marker-selection known as genomic selection is being developed for use in generating appropriate breeding models for the improvement of banana (Nyine *et al.*, 2017).

Validation of markers linked to resistance to *Pseudocercospora* banana pathogens will facilitate faster gene introgression and marker-assisted selection (MAS) that will accelerate the germplasm selection process. Furthermore, the markers can also identify gene targets whose function could be validated by transforming *Musa* species with RNA interference (RNAi) constructs targeting specific genes. Already, RNAi mechanisms have been demonstrated in *Musa in vitro* using the reporter gene for β-glucuronidase (*GUS*; Dang *et al.*, 2014). Finally, some of these studies have illustrated the great genetic diversity in existing *Musa* spp., which could be used in selection of germplasm for genetic improvement of banana against *Pseudocercospora* banana pathogens, especially in programmes that need to expand the *Musa* genetic pool (Christelová *et al.*, 2016; Janssens *et al.*, 2016).

Despite the difficulties in breeding banana highlighted earlier, significant progress has been achieved by different programmes in the generation and delivery of *P. fijiensis*-resistant cultivars to farmers (Swennen & Vuylsteke, 1993). First, given the inherent need to distribute clean planting materials in sufficient quantities, the International *Musa* Transit Center (ITC) in Belgium was formed with the intention of sanitizing, multiplying and distributing genetic stocks from around the world. ITC provides an invaluable resource to breeding programmes and farmers as they can provide resistant germplasm. For example, *P. fijiensis*-resistant materials from the FHIA programme are now being grown in many countries worldwide, including Uganda, Tanzania, Ghana, Nigeria and Kenya (Tenkouano & Swennen, 2004). Similarly, *P. fijiensis*-resistant diploids from FHIA are being used in several breeding programmes including those at IITA, EMBRAPA, CIRAD and CARBAP. The breeding programme at IITA has also generated several improved *P. fijiensis*-resistant plantain hybrids, known as PITAs, and cooking banana hybrids called BITAs now available in several countries including Nigeria, Uganda, Cameroon, the Ivory Coast and Ghana (Tenkouano & Swennen, 2004; Tenkouano *et al.*, 2011). IITA, in collaboration with NARO-Uganda, has developed several East-African Highland cooking banana hybrids (generally referred to as NARITAs). Some of these NARITA hybrids have been tested in Uganda by IITA and NARO and the most promising ones have already been released to farmers (Ortiz, 2015). EMBRAPA-CNPMF also developed Pome and Silk dessert banana hybrids that are now being evaluated for *P. fijiensis* resistance in Nigeria and Uganda by IITA (EMBRAPA-CNPMF, unpublished data). If confirmed and accepted, they could be an alternative to the existing *P. fijiensis*-susceptible dessert banana in East and West Africa. The CIRAD and CARBAP breeding programmes have also developed *P. fijiensis*-resistant plantain and dessert banana hybrids, respectively. These successes highlight the important role that integration of banana breeding with molecular tools will play in the development of germplasm resistant to the *Pseudocercospora* banana pathogens. In addition, they emphasize the benefits that accrue when materials are shared among breeding programmes.

## The Role of Genetic Transformation in Development of *Pseudocercospora*-resistant Banana Germplasm

As discussed earlier, classical banana breeding faces considerable bottlenecks, especially caused by infertility and the limited variation in resistance in existing germplasm. These challenges have delayed deployment/release of *Pseudocercospora*-resistant banana germplasm to farmers (Swennen & Vuylsteke, 1993). Therefore, genetic transformation offers a platform where such challenges can be circumvented. A number of studies have generated transgenic plants and evaluated them for resistance to *Pseudocercospora in vitro*, in the screenhouse and under limited field conditions. For example, Cammue *et al.* (1993) demonstrated antifungal activity towards *P. fijiensis* on detached leaves from plants transformed with several genes. In their investigations, Remy *et al.* (1998) used the antifungal gene *Dm-AMP1* from *Dahlia merckii* under the control of CaMV 35S promoter, a super-promoter or a maize ubiquitin promoter. The CaMV 35S promoter and super-promoter each resulted in an over 6-fold increase in gene expression in the transformed leaves and provided resistance to *P. fijiensis* under screenhouse conditions. In another study, magainin, an antimicrobial peptide isolated from the skin of the African clawed frog *Xenopus laevis*, was transformed into banana using two constructs targeting delivery of the peptide into the cytoplasm or the extracellular spaces. The resistance to *P. musae* was measured as reduced lesion area in detached leaf assays. The study established that the lesion area on transgenic leaves was 40% for those from plants transformed with a construct targeting the intracellular region and ranged from 18% to 58% for those from the construct targeting the extracellular region (Chakrabarti *et al.*, 2003). In another study, Cavendish banana cv. Grand Naine was transformed with a cassette expressing an endochitinase gene (*En-42*) from *Trichoderma harzianum* together with the grape stilbene synthase (*StSy*) gene under the control of the 35S promoter and the inducible PR-10 promoter, respectively (Vishnevetsky *et al.*, 2011). The superoxide dismutase gene *Cu,Zn-SOD* from tomato, under control of the ubiquitin promoter, was also added to this cassette to improve scavenging of free radicals generated during fungal attack. Results from 4 years of field trials against *P. fijiensis* showed that, out of the 12 transgenic lines tested, six lines showed reduced disease symptoms while two lines exhibited significantly reduced symptoms with no change in fruit quality or yield. Kosky *et al.* (2010) simultaneously transformed banana with an antifungal glucanase gene and an antifungal protein, AP24 osmotin. From 25 transformants, only one showed significantly low foliar *P. fijiensis* symptoms compared to the control under field conditions. Kovács *et al.* (2013) transformed banana with either of two chitinase genes from rice. The

transgenic lines developed had a transgene copy number of 1–5 copies per line with single transgene copy lines accounting for 25% of all transgenic lines. The group reported a considerable decrease in necrotic leaf area, of 73–94%, at 63 days post-inoculation of detached leaves.

In most studies only a single location, screening technique or isolate is used in germplasm evaluation. This could be limiting as there are numerous isolates of the main *Pseudocercospora* banana pathogens whose diversity should be included in the screening process. It is also important that the copy numbers of the transgene be known before evaluation, as it has a significant influence on the level of symptom expression (Kovács *et al.*, 2013). With the emergence of increasingly more efficient banana transformation protocols, it is likely that genetic engineering will play an even greater role in banana genetic improvement in the near future (Sagi *et al.*, 1995; Ganapathi *et al.*, 2001; Khanna *et al.*, 2004; Liu *et al.*, 2017). To date, the transgenic studies have focused primarily on gene overexpression, but it is also possible that host-induced gene silencing, also referred to as host-triggered RNA interference (Hamilton & Baulcombe, 1999), may play a significant role in the development of transgenic banana with resistance to *Pseudocercospora* banana pathogens. This technology has shown utility against various phytopathogenic fungi such as *Fusarium verticillioides* (Tinoco *et al.*, 2010), *F. graminearum* (Koch *et al.*, 2013), *Puccinia striiformis* (Yin *et al.*, 2011), *Blumeria graminis* (Nowara *et al.*, 2010) and *Aspergillus flavus* (Masanga *et al.*, 2015; Thakare *et al.*, 2017). RNA interference mechanisms have already been demonstrated in both banana and *P. fijiensis* albeit *in vitro* (Mumbanza *et al.*, 2013; Dang *et al.*, 2014; Onyilo *et al.*, 2017). Horizontal gene transfer is also suspected to have occurred between *P. fijiensis* or *P. eumusae* and *Musa* spp. leading to gene expansions (Friesen *et al.*, 2006; Ma *et al.*, 2010; Santana *et al.*, 2012; Czislowski *et al.*, 2017); thus, it may be possible to use such horizontal gene transfer, the mechanism of which is currently unknown, to deliver RNAi molecules into the pathogen from transformed hosts. Although an increase in the development of transgenic banana resistant to the three important *Pseudocercospora* banana pathogens is envisaged, the acceptability of genetically transformed banana still faces resistance from anti-GMO groups and banana-importing countries. This may affect funding of transgenic research and the rapid development and deployment of transgenic products, especially to small-scale farmers. Nevertheless, countries need to put in place clear legislation and policies on research and adoption of genetically modified crops as a starting point. Finally, with the emerging cisgenic technologies, such as clustered regularly interspaced short palindromic repeat (CRISPR) technology (Sander & Joung, 2014), RNA-guided targeted genome editing could be used as an additional platform for the development of *Psuedocercospora*-resistant banana exhibiting good quality characteristics. The latter has proved to be a stumbling block in newly developed hybrids. Genome editing has an advantage over transgenic approaches as the resulting plants are not subject to the stringent regulations governing genetically engineered crops in many countries.

## Conclusions, Way Forward and Future Prospects

In this genomic era a wealth of tools such as sequencing technologies, bioinformatics, transcriptomics and proteomic analysis will continue to play an important role in the multifaceted path of development and testing of high-yielding banana varieties with resistance to *Pseudocercospora* banana pathogens. Although some breeding programmes have already developed high-yielding *P. fijiensis*-resistant hybrids, there is still a need to evaluate and identify additional sources with potentially higher levels of resistance from under-used wild *Musa* spp. The currently available hybrids were developed to provide resistance against *P. fijiensis* but their response to *P. eumusae* and *P. musae* and the durability of this resistance in the face of frequently evolving pathotypes have not been evaluated. There is also a need to consider breeding for resistance against *P. eumusae* and *P. musae*. Finally, although detached leaf assays, screenhouse artificial inoculations and field evaluations have been used for evaluation of resistance against *Pseudocercospora* banana pathogens, the methods have been selectively applied by different groups. In fact, no study has applied multiple resistance screening approaches to improve efficiency and reliability of selection. This has sometimes led to contradicting results that can mislead a screening programme into discarding a potential cultivar. Even where field evaluations have been conducted, the results could be suspect if only a single location has been used. Refining the procedures on how to apply these evaluation tools may lead to better understanding of the host × pathogen × environmental interaction that will ultimately result in development of resistant and durable cultivars.

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