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Pathogen profile

Moniliophthora perniciosa, the causal agent of witches' broom disease of cacao: what's new from this old foe?

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SUMMARY

Moniliophthora perniciosa (= *Crinipellis perniciosa*) causes one of the three main fungal diseases of *Theobroma cacao* (cacao), the source of chocolate. This pathogen causes Witches' broom disease (WBD) and has brought about severe economic losses in all of the cacao-growing regions to which it has spread with yield reductions that range from 50 to 90%. Cacao production in South America reflects the severity of this pathogen, as the yields in most of the infected regions have not returned to pre-outbreak levels, even with the introduction of resistant varieties. In this review we give a brief historical account and summarize the current state of knowledge focusing on developments in the areas of systematics, fungal physiology, biochemistry, genomics and gene expression in an attempt to highlight this disease. *Moniliophthora perniciosa* is a hemibiotrophic fungus with two distinct growth phases. The ability to culture a biotrophic-like phase *in vitro* along with new findings derived from the nearly complete genome and expression studies clearly show that these different fungal growth phases function under distinct metabolic parameters. These new findings have greatly improved our understanding of this fungal/host interaction and we may be at the crossroads of understanding how hemibiotrophic fungal plant pathogens cause disease in other crops.

Historical summary of WBD: The first WDB symptoms appear to have been described in the diaries of Alexandre Rodrigues Ferreira (described as *lagartão*; meaning big lizard) from his observations of cacao trees in 1785 and 1787 in Amazonia, which is consistent with the generally accepted idea that *M. perniciosa*, like its main host *T. cacao*, evolved in this region. The disease subsequently arrived in Surinam in 1895. WBD moved rapidly, spreading to Guyana in 1906, Ecuador in 1918, Trinidad in 1928, Colombia in 1929 and Grenada in 1948. In each case, cacao production was catastrophically affected with yield reductions of 50–90%. After the arrival of *M. perniciosa* in Bahia in 1989, Brazil went from being the world's 3rd largest producer of cacao (347 000 tonnes in 1988–1990; *c.* 15% of the total world production at that time) to a net importer (141 000 tonnes in 1998–2000). Fortunately for chocolate lovers, other regions of the world such as West Africa and South East Asia have not yet been affected by this disease and have expanded production to meet growing world demand (predicted to reach 3 700 000 tonnes by 2010).

Classification: *Moniliophthora perniciosa* (Stahel) Aime & Phillips-Mora: super-kingdom Eukaryota; kingdom Fungi; phylum Basidiomycota; subphylum Agaricomycotina; class Agaricomycetes; subclass Agaricomycetidae; order Agaricales; family Marasmiaceae; genus *Moniliophthora*.

Useful websites: www.lge.ibi.unicamp.br/vassoura/, nt.ars-grin.gov/taxadescriptions/keys/TrichodermaIndex.cfm, www.worldcocoafoundation.org/info-center/research-updates.asp, www.ars.usda.gov/ba/psi/spcl

INTRODUCTION

A fungus by any other name

The causal agent of Witches' broom disease (WBD), *Moniliophthora perniciosa* (Stahel) Aime & Phillips-Mora, is a hemibiotrophic fungal pathogen of cacao, *Theobroma cacao* L. (Malvaceae) (Fig. 1A,B). Along with frosty pod rot (FPR) and black pod rot

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Fig. 1 WDB disease in the field. (A) An infected cacao grove in Bahia, Brazil. (B) An uninfected cacao grove in Bahia, Brazil. (C) Infected tree with WBD symptoms of dry brooms, parthenocarpic fruits and an infected pod. The ovals with the white dashed lines represent dry brooms. The ovals with the solid white lines show parthenocarpic fruits. The white arrow points to a diseased pod. (D) Infected tree with WBD symptoms with a single dry broom. The oval with the dashed white line encircles the dry broom.

(BPR), WBD is one of the three most important diseases of this crop in the Western Hemisphere (Evans, 1980; Purdy and Schmidt, 1996). This fungus was first described as *Marasmius perniciosus* Stahel, but in 1942 Singer transferred it to *Crinipellis* as *C. perniciosa* (Stahel) Singer. It was known under this name until 2005, when Aime and Phillips-Mora (2005) used DNA sequencing to demonstrate that it was very closely related to the FPR pathogen, *M. roreri* (Cif.) H.C. Evans *et al.* (2003), and only distantly related to the type species of *Crinipellis*. The formation of hybrids (sterile and unstable but intermediate in morphology with clamp connections) between *M. roreri* and *M. perniciosa* attests to the relatedness of these two species (Griffith *et al.*, 2003). These two species now form a separate lineage of Marasmiaceae that includes several members of *Crinipellis* Section *Iopodinae*, which formerly comprised the pink/purple-pigmented members of the genus *Crinipellis* (Aime and Phillips-Mora, 2005; M.C. Aime unpublished data; G.W. Griffith, unpublished data). In addition to causing WBD on cacao, *M. perniciosa* is also capable of causing disease on several members of the Solanaceae (Bastos and Evans, 1985) and other unrelated tropical hosts (Griffith and Hedger, 1994a; Griffith *et al.*, 2003; Resende *et al.*, 2000). *Moniliophthora perniciosa* is believed to have originated and coevolved with host plants in the Upper Amazon River basin on the eastern side of the Andes (Purdy and Schmidt, 1996), while the origin and evolution of *M. roreri* is believed to be in central or north-eastern Colombia (Phillips-Mora *et al.*, 2007).

DISEASE PROGRESSION

WBD of cacao begins with the production of basidiospores from small pink basidiomata (mushrooms) formed on previously infected plant tissues, including pods and affected vegetative tissue. Airborne dispersal of the basidiospores primarily occurs during nocturnal periods. Under high humidity (Frias *et al.*, 1991), the basidiospores have the ability to infect any meristematic cacao tissues including shoots, flowers and young developing fruits (Evans, 1980). Infection through stomatal openings by basidiospore germ tubes has been reported on several occasions (Frias *et al.*, 1991; Sreenivasan and Dabydeen, 1989; Suarez, 1977), with Frias *et al.* (1991) also observing infection through the bases of damaged trichomes on unhardened flushes. At this stage in the disease cycle, the density of fungal mycelium in the plant is very low (Orchard and Hardwick, 1988; Penman *et al.*, 2000) and the fungus is in the biotrophic growth phase, which is recognized by

the production of wider convoluted intercellular hyphae that lack clamp connections (Evans, 1980).

Early observation of the nuclear status of this mycelium in cacao meristems suggested that it was uninucleated (Calle *et al.*, 1982), although Griffith and Hedger (1994c) found this to be much more variable (up to ten nuclei per cell) in biotrophic mycelium formed in dual culture with potato callus. During the biotrophic phase, the fungal/host interaction produces the distinctive symptoms of WBD: hypertrophy and hyperplasia of the tissues distal to the infection site (Holliday, 1980), loss of apical dominance, proliferation of auxiliary shoots, and the formation of abnormal stems resulting in a broom-like structure called a green broom. Infection of the cauliform flowers results in the formation of cushion brooms or small parthenocarpic fruits (Fig. 1C). After 1–2 months of developmental alterations, necrosis and death of the infected tissues occurs distal to the original infection site, thus forming the structure called a dry broom (Evans, 1980; Lawrence *et al.*, 1991) (Fig. 1C,D). None of these necrotic tissues form abscission layers so the infected necrotic tissues remain attached. Sometime during or before the death of the infected tissues, the fungus proliferates and colonizes necrotic or dead host cells and undergoes a morphological change with hyphae becoming narrower and less convoluted, with clamp connections and regular binucleated (dikaryotic) mycelium, known as the saprotrophic phase. The exact timing, mechanisms and signalling factors involved in the developmental alterations of both the plant and the fungus remain unknown. Following alternating wet and dry periods (Almeida *et al.*, 1997), basidiocarp production (Fig. 2A,C)

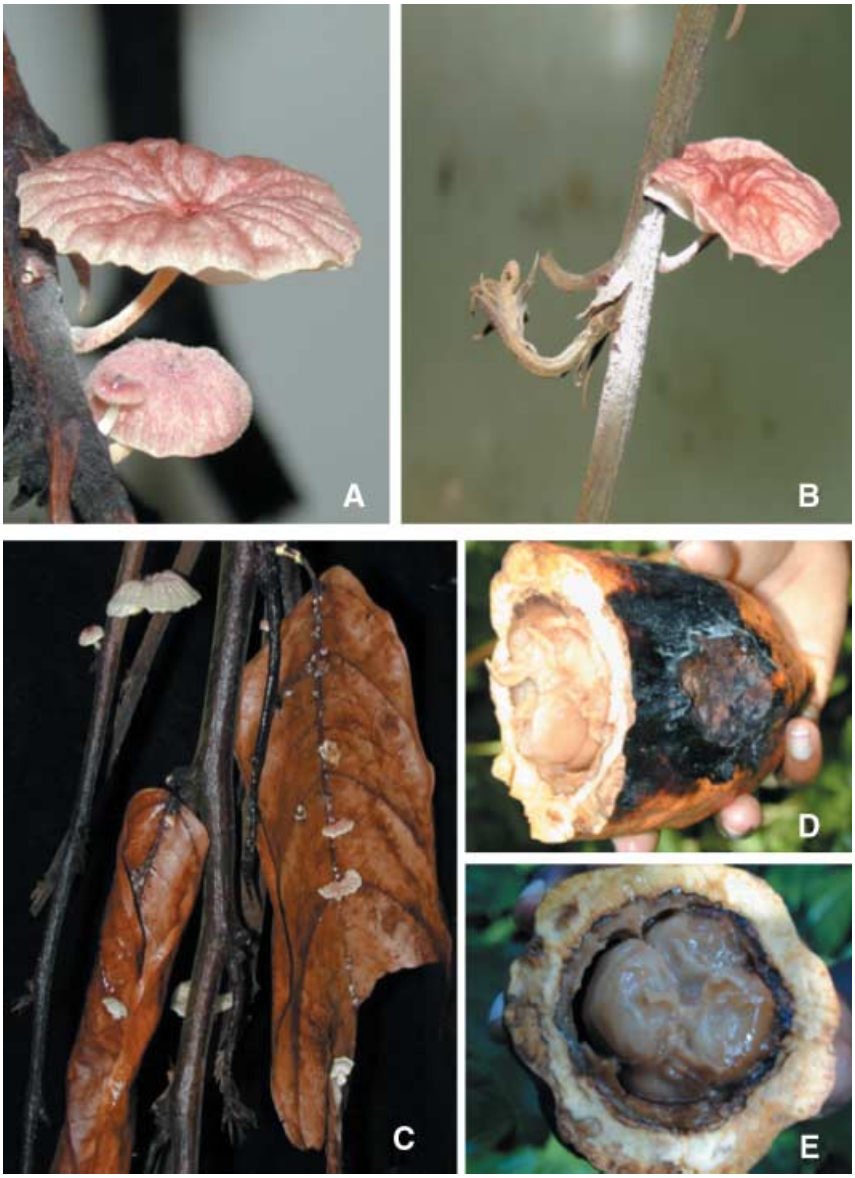

Fig. 2 Basidiocarps and pod rots resulting from *M. perniciosa* infection of cacao. (A) Basidiocarps on a dry broom at various development stages. (B) A basidiocarp on a dry broom after spore release. The white arrow shows the accumulation of the spores on the dry broom. (C) Basidiocarp development on a dry broom in the field. (D) Pod rot caused by *M. perniciosa*. Longitudinal view. (E) Pod rot caused by *M. perniciosa*. Transverse view.

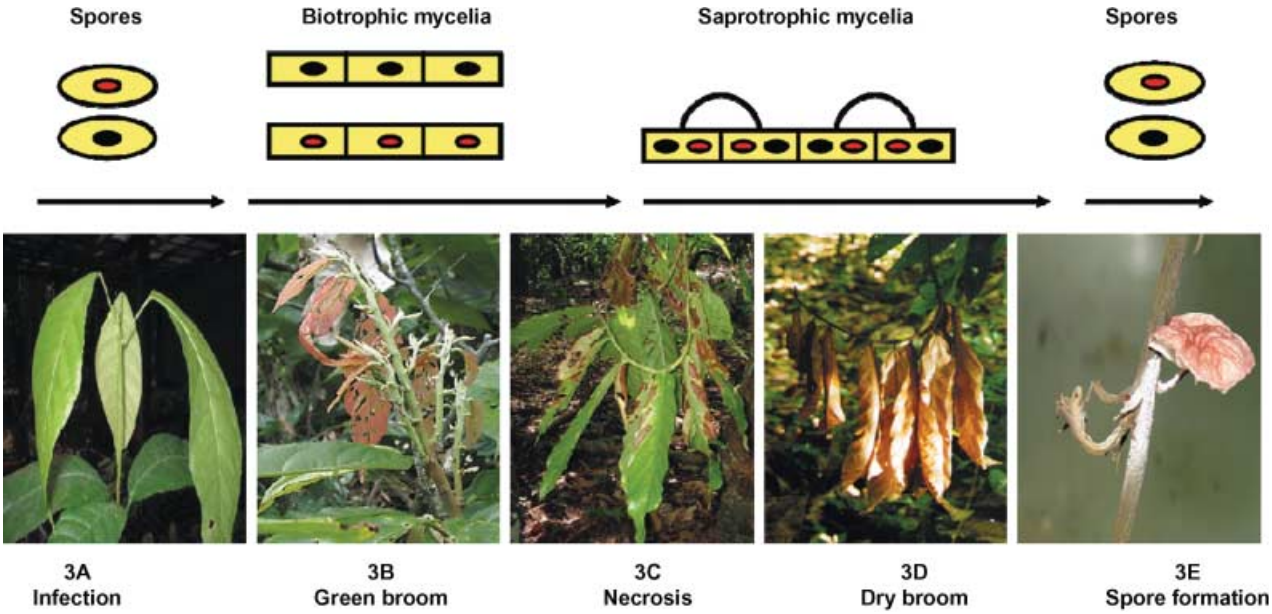

Fig. 3 Disease progression (left to right). The images of the fungal stage above represent the progression of the fungus from the biotrophic phase to the saprotrophic phase and the typical mycelial structures and nuclei number at each symptomatic stage shown below. (A) Infection by penetration through stomatal opening. (B) Growth of the green broom. (C) Necrosis of infected tissues. (D) Dry broom. (E) Basidiocarp and spore formation.

and subsequent spore formation (Fig. 2B) can occur on any infected necrotic tissue, thus completing the disease cycle. Basidiocarp formation and spore production from a single dry broom can occur repeatedly over a period of several years. One additional feature of the biotrophic phase that has been observed in senescing green brooms (Suarez, 1977), in leaf trichomes (Sreenivasan, 1991) and in potato callus dual culture (Griffith and Hedger, 1994c) is the formation of chlamydospores. The role of these thick-walled intercalary spores in WBD is not known but they may represent a dormant phase following host infection. The life cycle with images of the fungal stages at the various host symptom stages are depicted in Fig. 3.

Moniliophthora perniciosa is also responsible for one of the main pod rotting diseases of cacao, previously know as Crinipellis pod rot (Maddison *et al.*, 1995) (Fig. 2D,E). *Theobroma cacao* produces large thick pods that contain 30–40 seeds that require approximately 6 months to reach maturity. The developing fruits are susceptible to infection throughout their growth and young pods will abort if infected during the first few weeks. As the pod grows, infection progresses, eventually causing a watery rot on mature pods with the complete loss of the seeds, while later infections of more mature pods will result in a partial loss of the seeds. This has become especially important in Brazil, as most of the cacao clones with resistance to apical meristem infections are still susceptible to this pod rot.

Theobroma cacao is typically grown in an environmentally friendly production system resulting in the conservation of large regions of tropical rainforest as a shade canopy for the understory growth of cacao. This typically helps to preserve habitat for numerous animal and bird species found in these regions (Rice and Greenberg, 2000). With the production losses associated with WBD, tropical landowners are forced to convert their land to other production systems that normally require the destruction of the forest cover. Therefore, WBD not only affects the supply of cacao but also has a major impact on the conservation of tropical environments where cacao is grown.

M. PERNICIOSA SPECIES COMPLEX AND BIOTYPES

The species *M. perniciosa* consists of a number of geographically separated populations that infect a broad range of different hosts. This species was first subdivided into three different varieties (var. *perniciosa*, var. *ecuatoriensis* and var. *citriniceps*) according to morphological characters of the basidiocarp (Pegler, 1978). However, plant pathologists, using data based on breeding biology and host specificity, currently classify *M. perniciosa* into four different biotypes (C, H, L and S) (Table 1). The C-biotype infects species of *Theobroma* and the closely related genus *Herrania* (Malvaceae). The S-biotype infects a diverse range of hosts within the Solanaceae (Bastos and Evans, 1985), while the H-biotype, which has recently been identified, infects *Heteropterys acutifolia* (Malpighiaceae) (Griffith *et al.*, 2003; Resende *et al.*, 2000). Isolates of the L-biotype are found on liana vines and plant debris, especially of the species *Arrabidaea verrucosa* (Standl.) A.H. Gentry (Bignoniaceae) (Evans, 1978; Griffith and Hedger, 1994a,b;

Table 1 Summary of the pathogenicity of the biotypes and their genetic diversity.

	Host	Genetic diversity
Biotype C	*Theobroma* and *Herrania* spp. (Malvaceae)	numerous isolates have been found*
Biotype H	*Heteropterys acutifola* (Malpighiaceae)	reclassified into a new *Crinipellis* species
Biotype L	*Arrabidaea* spp. (Bignoniaceae)	Extreme diversity
Biotype S	*Solanum* spp. (Solanaceae)	numerous isolates have been found*

*Indicates that isolates tend to have a geographical orientation and most genetic diversity is associated with isolates from different geographical origins.

Hedger *et al.*, 1987). Griffith and Hedger (1994b) found that basidiocarps often formed on the bark of apparently healthy vines with only a single report of broom-like symptoms (H. C. Evans, personal communication). Furthermore, Griffith and Hedger (1994c) found that L-biotype basidiospores were able to form the characteristic biotrophic mycelium associated with broom tissues in dual culture with potato callus. The L-biotype exhibits an outcrossing reproductive strategy (bifactorial heterothallism; the most common outcrossing mechanism in the Agaricomycotina), which contrasts with the primary homothallism of the C-, S- and H-biotypes (Griffith and Hedger, 1994b). Consistent with its outcrossing breeding strategy, levels of local genetic diversity for L-biotype populations are much higher than for other biotypes (Griffith, 2004; Griffith and Hedger, 1994b).

A B-biotype isolate was once described infecting a plantation of *Bixa orellana* that was adjacent to a cacao farm in Bahia, Brazil (Bastos and Andebrhan, 1986; Griffith and Hedger, 1994a). The authors reported that this isolate was unable to complete its life cycle on *B. orellana* (Bastos and Andebrhan, 1986) and the isolate was later proven to be genetically identical to isolates of the C-biotype found in Bahia (Andebrhan and Furtek, 1994). Therefore, we do not consider the B-biotype as a distinct subgroup of this species.

Phylogenetic analysis was undertaken by de Arruda *et al.* (2005) to determine the species connection with regards to the C-, S- and H-biotypes. These authors compared the three biotypes by combining morphological data (light and scanning electron microscopy of reproductive structures) with genetic variation data shown in RFLP patterns and sequence analysis of the internal transcribed spacer (ITS) region of the rDNA. They found that the H-biotype could be distinguished from the other two biotypes at the species level and proposed the new species *Crinipellis brasiliensis* (de Arruda *et al.*, 2005). Phylogenetic analyses to compare the whole spectrum of biotypes and determine the extent of the speciation process have not been conducted.

GENETIC DIVERSITY AND PATHOGENICITY OF THE C-BIOTYPE

The genetic variability of C-biotype isolates from the cacao-producing region of Bahia, Brazil, has been evaluated with various molecular markers with the goal of measuring the extent of host specificity and genetic variability in different geographical regions. RAPD genotyping revealed that C-biotype isolates from Bahia grouped together with B and S isolates found nearby, thus suggesting a possible 'jump' of isolates to alternative host plants (Andebrhan and Furtek, 1994). However, a more detailed RAPD analysis performed in 1999 by the same group showed that isolates from Bahia formed two distinct groups, with some isolates grouping with C-biotypes from the Amazon, but not with the S- or B-biotypes (Andebrhan *et al.*, 1999). These two groups appeared to give creditability to the reports of two independent introductions into the State of Bahia, leading the authors to conclude that WBD in Bahia was introduced from Amazonian isolates. Subsequent molecular studies have utilized: RFLP analyses of the mitochondrial DNA, ITS and the intergenic spacer (IGS) region of the rDNA (de Arruda *et al.*, 2003a); ERIC-PCR genomic fingerprinting (de Arruda *et al.*, 2003b); AFLP analysis using nine pairs of primers (Ploetz *et al.*, 2005); and, most recently, microsatellite analysis coupled with electrophoretic karyotyping (Rincones *et al.*, 2003, 2006). All of these studies have confirmed the host specificity of the different biotypes, due to the fact that isolates from different biotypes fail to group together independent of geographical proximity. Moreover, these studies have shown that the genetic variability of the C-biotype isolates of *M. perniciosa* is greater in the Amazon when compared with the State of Bahia, with the latter region showing only two main genotypic groups, thus adding support to the hypothesis of two independent introductions (Andebrhan *et al.*, 1999). Furthermore, C-biotype isolates have been shown to form clonal populations that correlate with geographical proximity (Ploetz *et al.*, 2005; Rincones *et al.*, 2006), which is consistent with the non-outcrossing breeding strategy exhibited by the C-biotype (Griffith and Hedger, 1994c). Nevertheless, in spite of the clonal population structure of this biotype, pathogen populations have broken the resistance of cacao trees after a few generations (Bartley, 1986). Moreover, genetic variability was observed at the chromosomal level in several C-biotype isolates from Bahia 15 years after their introduction (Rincones *et al.*, 2006). These authors also observed multiple copies of transposable elements throughout the genome of *M. perniciosa* and proposed that the chromosomal rearrangements observed could be caused by ectopic recombination or through the activation of these elements (Rincones *et al.*, 2006). Studies are now being conducted to characterize the numerous transposable elements found in the genome of *M. perniciosa*.

It is possible that the formation of heterokaryons could occur in meristems infected by genetically different basidiospores prior to development of the binucleate clamped mycelium in dead brooms. Griffith (1989) reported the occurrence of numerous somatic compatibility groupings (SCGs) among C-biotype isolates from Napo Province, Ecuador, in contrast to coastal Ecuador where only a single SCG was identified. However, when Shaw and Vandenbon (2007) conducted dual inoculation of cacao meristems with isolates from Brazil and Trinidad, they found that the basidiocarps that were formed on the resulting dry brooms produced basidiospores that did not germinate.

DISEASE CONTROL

Phytosanitation and chemical control

Management of cacao diseases including WBD (Evans and Prior, 1987; Fulton, 1989; Purdy and Schmidt, 1996; Wheeler and Suárez, 1993) has received considerable attention from the beginning of the twentieth century. Broadly, there are four major strategies that may be adopted: phytosanitation, chemical control, genetic resistance and biological control. Phytosanitation, i.e. the removal and destruction of diseased plant parts, has been shown to reduce pod loss and delay disease epiphytotics of WBD (Rudgard and Butler, 1987; Soberanis *et al.*, 1999). However, this strategy is tedious and expensive, and in one study it was shown that 95% removal was required to achieve 50% reduction in pod loss (Rudgard and Butler, 1987).

Although widely studied (Achicanoy and Buritica, 1982; Cronshaw, 1979; Evans and Prior, 1987; Laker, 1991; Laker and Ram, 1992), chemical control of WBD or FPR with protectants and systemic fungicides is not a routine practice in cacao production because of the high costs and risks associated with cacao bean contamination and environmental health. Furthermore, much of the research concerning the use of fungicides for the control of cacao diseases has not been formally published.

Host resistance to WBD

Genetic resistance to WBD exists in a number of cacao accessions, both in wild cacao and in farmers' selections (Bartley, 1986; Marita *et al.*, 2001; Pires *et al.*, 1999; Pound, 1943; Umaharan *et al.*, 2004). Among the various resistant germplasm, two Peruvian accessions, 'SCA6' and 'SCA12', were found to have high resistance and have thus have been most extensively used in breeding programmes SCA = scavino (Bartley, 2001; Laker, 1990; Pound, 1943). At least 300 improved varieties in the International Cacao Germplasm Database have their direct parental contribution from these two SCA clones. SCA clone resistance appeared to be durable based on observation in Trinidad over 50 years (Bartley, 2001; Laker, 1990). However, a strong location effect was also observed in SCA clones and their derived progenies. Clones identified as resistant in Brazil and Trinidad were susceptible in Ecuador (Bartley, 2001). It has been hypothesized that different populations of *M. perniciosa* are responsible for the variation (Wheeler and Mepsted, 1988), suggesting that breeding for WBD resistance should take into account geographical variations within the pathogen.

Major efforts are underway to develop and use techniques associated with marker-assisted selection (MAS) and quantitative trait loci (QTL) mapping to accelerate the development of disease-resistant cacao clones (Brown *et al.*, 2005). A qualitative host–pathogen interaction was reported in *M. perniciosa* isolates from different producing regions (Shaw and Vandenbon, 2007). The segregation pattern of 'SCA-6 × Amalonado' in the same study also suggested that the resistance was caused by a single recessive major gene, which is homozygous in SCA-6. The observed 'vertical resistance' was compatible with the result of linkage mapping, which suggested a monogenic resistance in SCA-6. A major QTL was identified on chromosome 9 near the SSR locus mTcCIR35, using the mapping population derived from 'SCA-6 × Amelonado'. This QTL explained up to 51% of the phenotypic variance for resistance, while a secondary minor QTL was detected on chromosome 1, near a resistance gene analogue locus (Brown *et al.*, 2005; Faleiro *et al.*, 2006). A BAC library from 'Scavina 6' was constructed (Clement *et al.*, 2004) for map-based cloning of this major gene. The major QTL was also reported in another mapping progeny derived from 'SCA-6 × ICS-1', which explained 35% of the phenotypic resistance to WBD (Queiroz *et al.*, 2003).

In addition to the SCA clones, resistance to WBD exists in other germplasm groups, mostly in the Forastero cacao (Bartley, 1986; Marita *et al.*, 2001; Thévenin *et al.*, 2006). Also, a number of farmer selections from Peru, Ecuador and Brazil (Pound, 1938, 1943; Pires *et al.*, 1999; Arevalo-Gardini, personal communication) have resistance to WBD. For example, 'Refractario' cacao were selected during the 1920s from the putative resistant trees in the coastal valley of Ecuador after years of WBD infection (Pound, 1938, 1943). Exploration of resistance from other germplasm groups will expand the genetic background of WBD resistance and a combination of the different resistances will probably offer a more durable and stable resistance to WBD.

NEW ADVANCES

Biocontrol

Theobroma cacao, which is found in tropical rainforests of Central and South America (Wood and Lass, 2001), carries a portion of this region's diverse microbial community in endophytic associations (Arnold, 1999; Arnold *et al.*, 2003; Crozier *et al.*, 2006; Evans *et al.*, 2003; Holmes *et al.*, 2004; Rubini *et al.*, 2005; Samuels *et al.*, 2006). Endophytic microbial species inhabit

different plant tissues including roots, trunks, stems, leaves, flowers and fruit and have been shown to limit disease in other tropical trees (Arnold, 1999; Arnold *et al*., 2003). Mejía *et al*. (2007) observed a reduction in pod loss due to BPR and reduced sporulation of *M. roreri* when spores of leaf endophytes were sprayed onto trees in Panama. It is this diverse microbial community association that offers hope for new WBD control measures through biocontrol strategies.

Among the endophytic fungi associated with cacao are many species of *Trichoderma* (Evans *et al*., 2003; Holmes *et al*., 2004; Samuels *et al*., 2000). Control of plant pathogens by *Trichoderma* spp. occurs through several mechanisms including antibiosis, induced resistance, niche exclusion and mycoparasitism (Chet *et al*., 1998; Harman *et al*., 2004; Howell, 1998, 2003). Several new *Trichoderma* species have been isolated from cacao, including *T. stromaticum*, which parasitizes the saprotrophic mycelium and basidiocarps of *M. perniciosa* (Bastos, 1996; Samuels *et al*., 2000). Commercial formulations of *T. stromaticum* have been developed in Brazil and although promising, these have shown variable performance to date. In most cacao production areas, rainfall totals and temperature maximums range between 1300 and 3000 mm and 30 and 33 °C (Wood and Lass, 2001). These conditions are ideal for WBD development, but are not necessarily ideal for optimizing biocontrol efficacy. For example, Sanogo *et al*. (2002) found that *T. stromaticum* required high humidity (100%) and temperatures below 30 °C for optimum growth and colonization of *M. perniciosa*. Holmes *et al*. (2004) found that cacao pods could be endophytically colonized by *T. ovalisporum* if the humidity around the pods was maintained after the biocontrol treatment. These results indicate that more information is needed regarding the relationship between the cacao forest environment and the establishment and survival of biocontrol agents.

Several lines of evidence indicate that certain *Trichoderma* species have significant potential for biocontrol of plant diseases in the plant canopy (Elad, 2000; Elad *et al*., 1996; Holmes *et al*., 2004; Samuels *et al*., 2000; Wilson, 1997). The endophytic capabilities of particular *Trichoderma* species have been confirmed in aerial cacao tissues including stems and leaves (Arnold, 1999; Arnold *et al*., 2003; Holmes *et al*., 2004). Researchers at INIAP in Ecuador have recently shown the abilities of *Trichoderma* species to colonize cacao flower cushions and flowers over several weeks to months (C. Suarez, personal communication). Furthermore, several *Trichoderma* species have shown the ability to enhance fruit set but have not shown long-lasting pod protection (P. Hebbar, personal communication). If significant populations of the biocontrol agent could be established on or in specific targeted tissues, the potential for persistent disease control with limited reapplication exists.

Related studies have demonstrated an interaction between some of the tested *Trichoderma* species and *T. cacao* seedlings that altered gene expression in both organisms (Bailey *et al*., 2006). However it is unclear, at this time, whether these alterations can provide functional resistance to cacao diseases.

Disease physiology

Biochemical analysis of *T. cacao* shoots during the infection and development of WBD have been reported by Scarpari *et al*. (2005). A systematic analysis of the changes in the contents of soluble sugars, amino acids, alkaloids, ethylene, phenolics, tannins, flavonoids, pigments, malondialdehyde (MDA), glycerol and fatty acids in cacao shoots during the development of WBD was conducted in an attempt to reveal the chemical alterations in the infected plant. Coordinated biochemical alterations were found in the infected tissues that correspond with alterations in the content of soluble sugars (sucrose, glucose and fructose), asparagine and alkaloids (caffeine and theobromine), ethylene and tannins. Ethylene and tannin levels were shown to increase prior to symptom development and decline with the senescence of the green brooms. MDA and glycerol concentrations were higher in infected shoots and were associated with changes in fatty acid compositions. Alterations in the contents of chlorophyll a and b were also found throughout the development of the disease while carotenoid and xanthophyll levels dropped in the infected tissue with symptom development. It was hypothesized that ethylene levels played a key role in broom development while some of the other observed biochemical alterations were directly associated with ethylene synthesis and/or may be important for the modification effect on the infected tissues (Scarpari *et al*., 2005). Furthermore, the infected tissue appeared to be under intense oxidative stress, which was indicated by the increase in lipid peroxidation.

In a related study, infected cacao seedlings were shown to undergo programmed cell death (PCD). Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) analysis showed PCD in infected meristems, with a significantly higher number of cells showing nuclear DNA fragmentation as the disease progressed (Ceita *et al*., 2007). In susceptible cacao tissues this group observed a higher amount of calcium oxalate crystals (COC) than in uninfected tissues. However, when infected with *M. perniciosa*, these plants showed an initial increase in COC followed by a drastic decrease, which appeared to be correlated with an increase in H_2O_2 production in the same regions. Hydrogen peroxide production was associated with increased gene expression of germin oxalate oxidase (G-OXO) and decreased expression of the ascorbate peroxidase (APX) genes in infected tissues. WBD-resistant plant material showed the opposite reaction to COC and H_2O_2 levels as well as to G-OXO and APX expression. The authors concluded that H_2O_2 levels are derived from COC and that these higher concentrations trigger PCD in infected plant tissues. They also hypothesized that the release of available nutrients during PCD may have an effect on the phase

conversion of the fungus. Further analysis of the COC connection to WBD by do Rio *et al.* (2008) found that the biotrophic phase of *M. perniciosa* produces COC. A putative fungal gene that encodes for oxaloacetate acetylhydrolase (*oah*), which catalyses the hydrolysis of oxaloacetate to oxalate and acetate, was found to be expressed in the biotrophic growth phase of the fungus (do Rio *et al.*, 2008).

Biotrophic phase

Carbon source analysis revealed that *M. perniciosa* could be successfully grown on glycerol as the sole carbon source (Meinhardt *et al.*, 2006). Due to the fact that glycerol was found in high concentrations during the formation of the green broom (Scarpari *et al.*, 2005), it was hypothesized that glycerol was a possible carbon source that could sustain the biotrophic phase of the fungus. Experiments with germinating spores of *M. perniciosa* revealed that glycerol could maintain the fungus in a slow-growing biotrophic-like stage with a single nucleus per cell and no clamp connections for up to 2 months (Meinhardt *et al.*, 2006). While nuclear condition and a lack of clamp connections were the initial criteria used to identify the biotrophic-like mycelium from saprotrophic mycelium, DNA analysis was used to confirm the determination. The biotrophic-like mycelia were found to be unstable in the presence of other carbon sources and the mycelium rapidly transformed into the saprotrophic phase. Numerous attempts were made to infect plants with the biotrophic-like mycelium; however, no successful infections of cacao plant tissues were accomplished. This new growth technique has facilitated the isolation of RNA and DNA from biotrophic-like mycelia, which has been utilized to construct cDNA libraries and to conduct expression analyses by microarrays and real-time RT-PCR. The ability to grow both phases of this fungus *in vitro* is an extremely important development and allows this pathosystem to be utilized as a model system for understanding hemibiotrophic processes in fungal plant pathogens.

Genomic analysis

Due to the great socio-economic impact of WBD in the State of Bahia, Brazil, the WBD Genome Project sequencing consortium was established in late 2000. An initial draft sequence corresponding to 1.5–2× coverage of the estimated total genome size of 30–40 Mb (Rincones *et al.*, 2003, 2006) was completed in 2006. This draft sequence contained a total of 94 613 reads of genomic DNA, totalling 75 Mbp (> 20 quality according to PHRED). This initial draft sequence manuscript detailing the genome information will be released in 2008 and those sequences will be made publicly available at that time. Initial comparative analysis findings show that *M. perniciosa* is closer to *Laccaria bicolor* in sequence identity than it is to *Coprinus cinereus*.

The *M. perniciosa* sequencing consortium was joined by the USDA and Penn State University in 2007 with the addition of 245 Mbp obtained through 454 pyrosequencing. These new sequences are being used to develop an *M. perniciosa* × *M. roreri* comparative genome project that will explore the relatedness and function of these pathogens. The findings so far support the new formation of a separate lineage of Marasmiaceae with a high percentage of the *M. perniciosa* sequences showing high identity to *M. roreri* sequences. This comparative genome project will be completed in 2008 and the sequences will be placed into the public domain shortly thereafter.

In addition, the mitochondrial genome of *M. perniciosa* was completely sequenced as part of this project and has been deposited in GenBank (accession number AY376688). With a total size of 109 103 bp, it is the largest fungal mitochondrial genome sequenced so far. It contains the 14 typical mitochondrial genes plus *rps3*, two rRNA genes and 26 tRNA genes. The large genome size is related to a number of hypothetical genes and, of particular interest, a linear plasmid-like sequence (Griffiths *et al.*, 1990). This plasmid sequence was not detected in an independent form and it seems to have been incorporated recently in a stable manner into the mitochondrial genome of *M. perniciosa.* Another important finding related to this mitochondrial genome is the fact that *M. perniciosa* undergoes senescence in culture. As the fungus is cultured each successive plate shows signs of slower growth along with sectoring, whereby eventually the fungus stops growing and can no longer be subcultured. *M. perniciosa* senescence seems to be associated with a decline in the content of mitochondrial DNA per cell but is not the result of the movement and subsequent gene disruption of the plasmid (Formighieri *et al.*, 2008).

Pathogenicity factors

Analyses of the *M. perniciosa* genome sequences have led to the identification of three putative necrosis and ethylene-inducing protein (MpNEP) genes that were designated MpNEP1, 2 and 3 (Garcia *et al.*, 2007). These are the first genes of their type reported for a basidiomycete and all three genes are located on chromosome 6. MpNEP1 and 2 are able to induce necrosis and ethylene emission in tobacco and cacao leaves and have high sequence similarity. The MpNEP3 sequence is incomplete and located in tandem with MpNEP1. Expression analysis revealed that MpNEP1 is expressed not only in saprotrophic mycelium but also in biotrophic hyphae. The MpNEP1 protein is an oligomer in solution and is inactivated at high temperatures. MpNEP2 protein is a monomer at low concentration (< 40 μM) in solution that is predominantly expressed in biotrophic mycelia and can regain its necrosis activity after boiling. These findings suggest that similar NEPs can have dissimilar physical characteristics and possibly different or complementary roles during disease development.

The NEP proteins appear to have a secondary structure that makes it similar to the cupin protein family, a family that contains the germin oxalate oxidases, which were previously mentioned with regard to COC and PCD. Additional studies are currently underway to determine specifically what role these proteins have in the WBD pathosystem.

In addition to MpNEPs, a second family of necrosis-inducing proteins was found in *M. perniciosa* based on a survey of the genome. At least five sequences encoding putative proteins similar to cerato-platanin-like proteins (first discovered in the pathogen *Ceratocystis fimbriata*) were found in the genome sequences. One of the *M. perniciosa* cerato-platanin genes (MpCP1) was expressed *in vitro* and proved to have necrosis-inducing ability in tobacco and cacao leaves. The protein forms a dimer in solution and is able to recover necrotic activity after heat treatment. Transcription analysis *ex planta* showed that MpCP1 is expressed at much higher levels in the biotrophic-like mycelium than in the saprotrophic mycelium. The necrosis profile induced by this protein in tobacco and cacao leaves was different from that caused by the MpNEPs. The MPCP1 was able to diffuse across a leaf's veins while MpNEPs were contained between the veins. Furthermore, a mixture of MpCP1 with MpNEP2 led to a synergistic necrosis effect, which is similar to that observed in naturally infected plants (G. Zaparolli *et al*., unpublished data).

Besides known pathogenicity genes, genome analyses revealed the presence of genes involved in gibberellic acid synthesis. A detailed literature search revealed independent confirmation of this by Bastos and Andebrhan (1981), who identified the production of gibberellic acid in the basidiospores of *C. perniciosa* and correlated it with excessive flushing and abnormal leaf development symptoms. The gibberellin biosynthetic pathway in *M. perniciosa* is currently being investigated by gene complementation analysis in *Fusarium fujikuroi* in collaboration with the University of Münster, Germany.

Transcriptome

In addition to genomic sequence data, four full-length-enriched cDNA libraries obtained under different physiological and culture conditions of *M. perniciosa* have been constructed: (1) biotrophic mycelium (Meinhardt *et al*., 2006), (2) saprotrophic mycelium grown in the presence of cacao extract, (3) saprotrophic mycelium grown in glucose and (4) basidiocarp tissue, plus an additional subtracted cDNA library enriched for saprotrophic mycelium transcripts induced by cacao extract (subtracted with cDNA of the saprotrophic phase grown in glucose) (Rincones *et al*., 2008). In total, 10 165 ESTs have been sequenced from these five cDNA libraries, from which 5800 were accepted and clustered into 652 contigs and 2478 singlets that could represent up to 31.3% of the total number of genes estimated for this fungus. Manual annotation of 3130 clusters points to several genes involved in fungal pathogenesis and show that the different fungal phases use divergent metabolic pathways.

The transcriptome of *M. perniciosa* was also studied using microarrays. A total of 2304 fragments obtained from the genomic DNA libraries of the Witches' Broom Genome Project were selected based on their sequence similarity to pathogenicity genes of other pathogens and analysed with regard to their differential gene expression between the two mycelial phases (biotrophic and saprotrophic) of the life cycle of this fungus (Rincones *et al*., 2008).

Together, the combined results show that the two mycelial stages differ primarily in how they metabolize carbon, with glycolysis and the malate shunt being repressed in the biotrophic mycelium. Moreover, the biotrophic mycelium presents induced transcripts indicative of amino acid starvation, such as various tRNA ligases. Transcripts characterized in other pathogens as pathogenicity factors, such as glyoxal oxidase 1, ceratoplatanin, several proteases and peroxidases, were up-regulated in the biotrophic mycelium. These analyses of the differential gene expression between the infective and saprotrophic stages of a hemibiotrophic fungal pathogen are the first of their kind and represent a significant advance toward understanding the molecular basis underlying the progression of WBD of cacao (Rincones *et al*., 2008).

WHAT WE DON'T KNOW AND WOULD LIKE TO!

Although major advances have been have been made in the last few years, our present knowledge of WBD still contains many gaps.

We have almost no knowledge regarding the infection process and the disease progression of the pod rot disease caused by *M. perniciosa*. This pod rot disease interaction leads to the majority of the direct production losses associated with this pathogen.

Although we have some basic information on the genetic diversity of *M. perniciosa* we do not know how this relates to the disease interaction on the known resistant tree lines that have been developed to date. Furthermore, no detailed information exists concerning the mapping of the clonal populations for the fungus across the production region. With this combined information a selective planting strategy could be implemented that would allow the planting of only tree lines that are resistant to the existing fungal populations in a given area, which could possibly help prevent resistance breakdown.

CLOSING REMARKS

The occurrence of WBD in South America has caused significant socioeconomic upheaval (Silva, 1987; Cuatrecasas, 1964; Went, 1904; Barros, 1979; Baker and Holiday, 1957; Pound, 1943; FAO, 2003) and the possibility of further spread to other continents is

a matter of grave concern to the chocolate industry. Although we have made major progress in our understanding of this pathosystem we still have much to learn. Increased international shipments of commodities and the expansion of international air travel have increased the risk of spread. Given the high dependence of the economies of Western African countries on cacao exports and the catastrophic consequences that could occur should WBD reach them, knowledge is urgently needed to assess and minimize such risks. Proactive programmes are required to prevent the establishment of these diseases should they reach Africa, through the education of regional farmers to recognize disease symptoms quickly and regional action plans to mount a quick response to protect crop production on larger and smaller scale farms.

Quantitative methodologies developed for assessing the risk of *M. perniciosa* could also be applied to other diseases of cacao such as FPR or to the movement of cacao diseases from Africa to South America as in the case of BPR caused by *Phytophthora megakarya* or cacao swollen shoot virus.

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