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Pathogen profile

The coffee leaf rust pathogen *Hemileia vastatrix*: one and a half centuries around the tropics

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SUMMARY

Taxonomy and History: *Hemileia vastatrix* Berk. and Broome (Basidiomycota, Pucciniales) was described in 1869 in eastern Africa and Ceylon as the agent of coffee leaf rust and has spread to all coffee cultivation areas worldwide. Major disease outbreaks in Asia, Africa and America caused and continue to cause severe yield losses, making this the most important disease of Arabica coffee, a cash crop for many tropical and subtropical countries.

Life cycle and Disease symptoms: *Hemileia vastatrix* is a hemicyclic fungus with the urediniosporic life cycle as its most important (if not only) source of inoculum. Chlorotic spots are the first macroscopic symptoms, preceding the differentiation of suprastomatal, bouquet-shaped, orange-coloured uredinia. The disease can cause yield losses of up to 35% and have a polyetic epidemiological impact on subsequent years.

Disease control: Although the use of fungicides is one of the preferred immediate control measures, the use of resistant cultivars is considered to be the most effective and durable disease control strategy. The discovery of 'Híbrido de Timor' provided sources of resistance that have been used in several breeding programmes and that have been proven to be effective and durable, as some have been in use for more than 30 years.

Genetic diversity and Molecular pathogenicity: Although exhibiting limited genetic polymorphism, the very large genome of *H. vastatrix* (*c.* 797 Mbp) conceals great pathological diversity, with more than 50 physiological races. Gene expression studies have revealed a very precocious activation of signalling pathways and production of putative effectors, suggesting that the plant–fungus dialogue starts as early as at the germ tube stage, and have provided clues for the identification of *avr* genes.

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INTRODUCTION

Coffee is the most important agricultural commodity, with an estimated retail value of 70 billion US dollars. It is crucial for the economy of more than 60 countries and is the main source of income for more than 100 million people (Hoffmann, 2014; ICO, 2016). Coffee leaf rust (CLR) causes losses of one to two billion US dollars annually (McCook, 2006) and is one of the main limiting factors of Arabica coffee (*Coffea arabica*) production worldwide. CLR was first recorded by an English explorer in 1861 near Lake Victoria (East Africa) on wild *Coffea* species. The disease symptoms and signs include large orange spore masses on the lower leaf surface, leading to premature leaf fall (Fig. 1). The causal agent was described as *Hemileia vastatrix* Berkeley and Broome (1869). Soon after its first report, the disease wiped out coffee cultivation from Ceylon (Sri Lanka), with devastating social and economic consequences (Morris, 1880). Since this sudden and devastating outbreak, CLR has become one of the most well-known diseases in the history of plant pathology.

The two main cultivated coffee species, *C. canephora* (Robusta coffee) and *C. arabica*, account, on average, for 40% and 60%, respectively, of the world's coffee production (ICO, 2016). *Coffea arabica* is native to the relatively dry and high-altitude areas of Ethiopia and northern Kenya and its genetic pool is considered to have low diversity (Steiger *et al.*, 2002). Arabica coffee was domesticated in Yemen, and its cultivation subsequently spread to Asia, America and other parts of Africa. Severe genetic bottlenecks during its domestication have narrowed even further the genetic diversity of the crop: it is believed that a single coffee plant from the Botanical Garden of Amsterdam was one of the progenitors of most of the current coffee cultivars. These genetic bottlenecks were particularly relevant for rust response traits, as its domestication in Yemen, the driest coffee cultivation area in

Fig. 1 Coffee leaf rust symptoms and signs. (A) Chlorotic spots and urediniosporic sori on the lower leaf surface. (B) Severe defoliation in plants at the front as a result of disease, contrasting with resistant plants elsewhere in the field.

the world, led to the absence of selection pressure towards rust resistance (Rodrigues *et al.*, 1975). Coffee germplasm disseminated from Yemen was most probably free of rust. Further selection and adaptation to other regions and climates in different parts of Asia and America during the 17th and 18th centuries occurred in the absence of the pathogen, but nonetheless under disease-favourable conditions. The 19th century epidemic in Ceylon was the outcome of such genetic, biological and agronomic circumstances (McCook, 2006; McCook and Vandermeer, 2015). Since then, rust has spread to most coffee-growing countries worldwide, first from 1870 to 1920 through the coffee zones of the Indian Ocean Basin and the Pacific, second reaching the Africa Atlantic countries in the 1950s and 1960s, and, finally, crossing the Atlantic Ocean (Muller, 1971), presumably carried by wind currents (Bowden *et al.*, 1971), spreading throughout South and Central America during the 1970s and 1980s. In the second half of the 20th century, the identification and characterization of 'Híbrido de Timor' (HDT) populations provided the basis for a breeding programme that enabled the release of rust-resistant cultivars in different coffee-growing countries, including the Americas (Rodrigues *et al.*, 1975; Silva *et al.*, 2006). Recently CLR has regained notoriety because of a severe and widespread epidemic throughout Central America, Colombia, Peru and Ecuador, as a result of the convergence of several agronomic, climatic and economic factors (Avelino *et al.*, 2015; Cressey, 2013; Rozo *et al.*, 2012). Yield losses were up to 35%, with a direct impact on the income and livelihood of hundreds of thousands of farmers and labourers.

TAXONOMY AND PHYLOGENY

The genus *Hemileia* is a member of the phylum Basidiomycota, class Pucciniomycetes, order Pucciniales (rust fungi). It comprises 42 species occurring mainly in tropical to sub-tropical regions of Africa and Asia, mostly on uncultivated Rubiaceae and Apocynaceae plants (Ritschel, 2005). *Hemileia vastatrix* is the type species of the genus. As described by Berkeley and Broome (1869) and Ward (1889), *H. vastatrix* urediniospores are reniform, 28–36 × 18–28 μm; the urediniospore wall is hyaline, strongly warted on the convex face, smooth on the straight or concave face, and 1 μm thick; teliospores are spherical, subglobose to napiform, 20–28 μm in diameter; the teliospore wall is hyaline, smooth and 1 μm thick. *Hemileia vastatrix* can be distinguished from *H. coffeicola*, as the latter produces sori scattered throughout the leaf and presents urediniospores with fewer but larger spines. Both rusts have *C. arabica* and other *Coffea* species as hosts, but *H. coffeicola* is of low economic importance and is geographically confined (Ritschel, 2005).

The genus *Hemileia* is distinguished from other rust genera by the unique combination of three morphological features: suprastomatal bouquet-shaped sori; ovoid to reniform urediniospores with a smooth ventral side and a delicately to coarsely echinulated convex dorsal side; and angular-globose to very irregular teliospores (Ritschel, 2005). Molecular evidence consistently places the genus *Hemileia* among the more basal phylogenetic group of the Pucciniales (Aime, 2006; Grasso *et al.*, 2006; McTaggart *et al.*, 2016; Silva *et al.*, 2015a; Wingfield *et al.*, 2004). *Hemileia* is presently included in the Mikronegeriaceae (Aime, 2006; McTaggart *et al.*, 2016), a family which probably represents the most ancient rust lineages and diverged at *c.* 91–96 million years ago. These findings challenge the old notion that ancestral rusts are harboured by phylogenetically ancestral hosts and support the view that the ancestors to the extant rusts may have been tropical short-cycled species that evolved on angiosperms (McTaggart *et al.*, 2016).

LIFE CYCLE AND INFECTION PROCESS

Hemileia vastatrix is a hemicyclic fungus producing urediniospores, teliospores and basidiospores, whereas pycniospores and aeciospores are not known. Urediniospores and teliospores are produced in the same sorus, but at different times. Urediniospores are dikaryotic and represent the asexual cycle, re-infecting the leaves whenever environmental conditions are favourable. Teliospores occur rarely and germinate *in situ*, producing a promycelium from which four basidiospores are formed (Chinnappa and Sreenivasan, 1965; Coutinho *et al.*, 1995; Fernandes RC *et al.*, 2009; Rodrigues *et al.*, 1980). Basidiospores cannot infect coffee, but no other host plant has been identified (Kushalappa and Eskes, 1989; Rodrigues *et al.*, 1980). However, several reports have proposed that *H. vastatrix* could be depicted as a primitive autoecious rust lacking pycnial and aecial stages (Hennen and Figueiredo, 1984), with urediniospores functionally acting as teliospores (Carvalho *et al.*, 2011; Rajendren, 1967; Rodrigues *et al.*, 1980). On the other hand, *H. vastatrix* may have lost the ability to

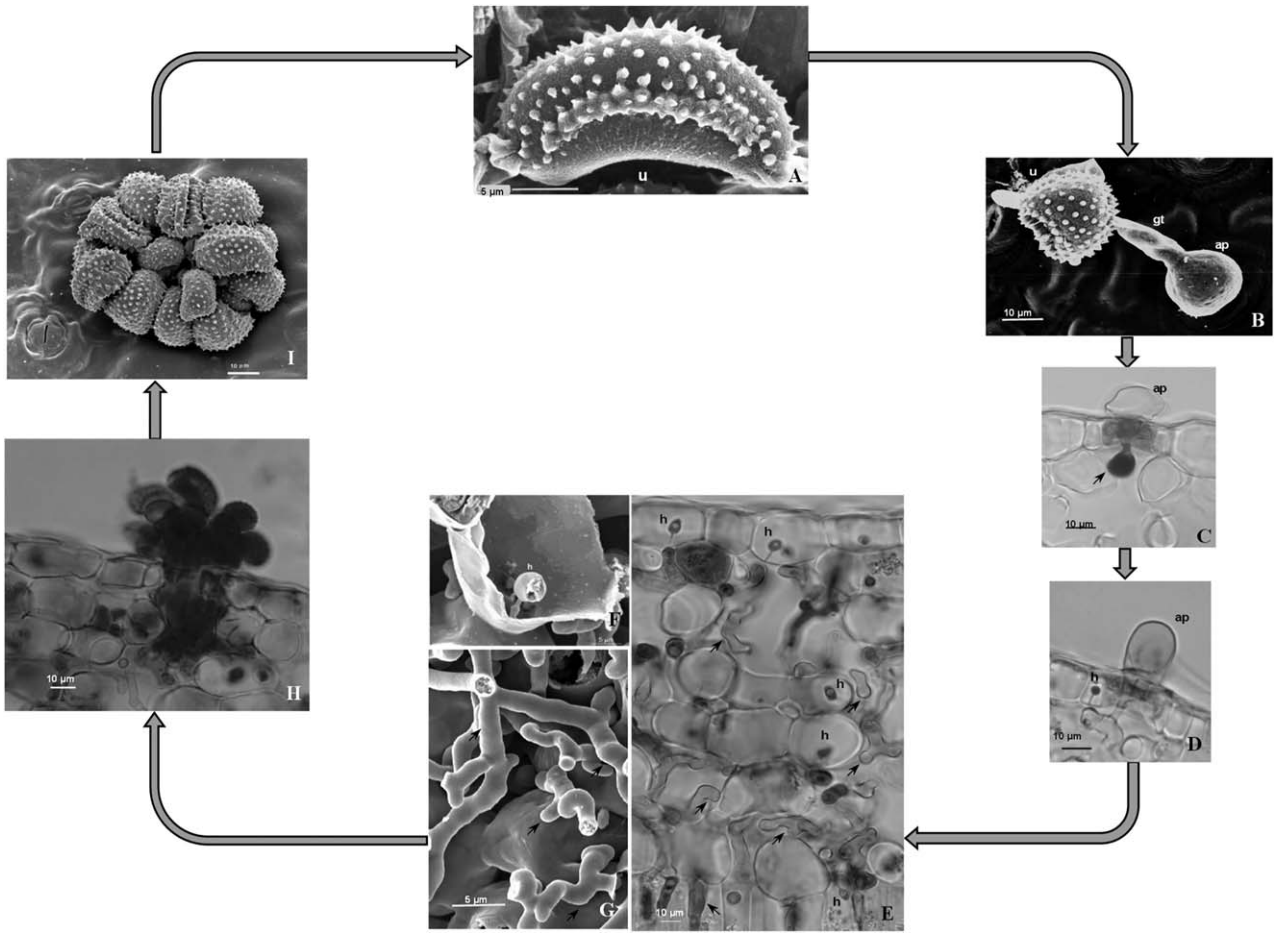

Fig. 2 *Hemileia vastatrix* infection process. (A) Urediniospore (u), scanning electron microscopy (SEM). (B) Germinated urediniospore (u) with germ tube (gt) and appressorium (ap) over stomata on the lower surface of the coffee leaf, 17 h after inoculation (hai), SEM. (C) Appressorium (ap) over stomata and penetration hypha (arrow), 24 hai, light microscopy (LM). (D) Appressorium (ap) over stomata and intercellular hypha with an haustorium (h) within a subsidiary cell, 48 hai, LM. (E) Intercellular hyphae (arrows) and haustoria (h) within epidermal and mesophyll cells, 20 days after inoculation (dai), LM. (F) Haustorium (h) within a spongy parenchyma cell, 20 dai, SEM. (G) Intercellular hyphae (arrows) in the spongy parenchyma, 20 dai, SEM. (H) Urediniosporic sorum protruding through the stomata in a bouquet shape, 21 dai, LM. (I) Urediniosporic sorum, 21 dai, SEM.

produce sexual spores during evolution since adaptation to survival would have mostly been achieved in the uredinial stage. The prevalence of uredinial stages in short-cycled rust species is considered an adaptation to the short growing seasons and heterogeneous vegetation landscapes in the tropics (Berndt, 2012).

The initiation of *H. vastatrix* infection on coffee leaves, like other rust fungi, involves specific events, including adhesion to the host surface, urediniospore germination, appressorium formation over stomata, penetration and inter- and intracellular colonization (Fig. 2).

Adhesion to the host, an essential step in the successful establishment of pathogenesis, prevents fungal displacement and is critical for the correct sensing of topographic signals involved in thigmotropic responses and for the differentiation and function of the appressoria (Braun and Howard, 1994). In *H. vastatrix*, the involvement of esterases has been demonstrated in urediniospore adhesion and appressoria differentiation, both *in planta* and *in vitro* (Azinheira *et al.*, 2007).

Urediniospore germination requires water and is optimal at about 24°C. After appressorium formation, the fungus penetrates the host through the stomata, forming a penetration hypha that grows into the substomatal chamber (Fig. 2A–C). This hypha produces two thick lateral branches, resembling an anchor, a unique trait of *H. vastatrix*. Each lateral branch of the anchor differentiates into a haustorial mother cell (HMC) that gives rise to a haustorium, which primarily infects the stomatal subsidiary cells (Fig. 2D), another unique feature of *H. vastatrix*. The fungus continues to grow, forming more intercellular hyphae, including HMCs, and a large number of haustoria in the cells of the spongy and palisade parenchyma and even of the upper epidermis (Fig. 2E–G). At

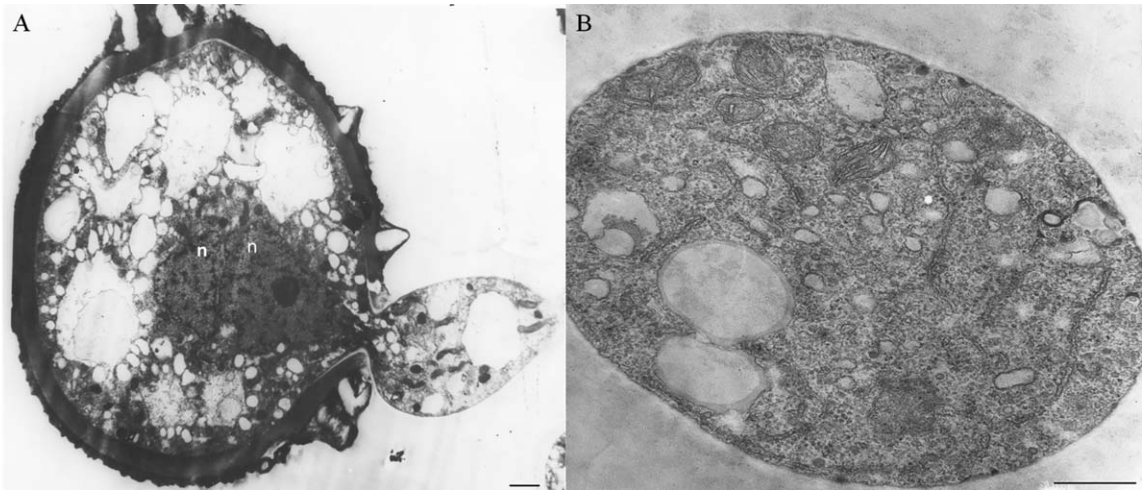

Fig. 3 *Hemileia vastatrix* urediniospore germination (Rijo and Sargent, 1983). (A) Beginning of germination, with cytoplasm passing through the germ pore with two nuclei (n) and one evident nucleolus (bar, 1 μm). (B) Transverse section of the germ tube showing different organelles (bar, 0.5 μm).

this stage, chloroses are macroscopically visible (McCain and Hennen, 1984). Hyphae invade substomatal cavities and interweave, differentiating protosori. Approximately 3 weeks after infection, urediniosporic sori protrude through the stomata in a bouquet shape. These appear as orange-coloured pustules, the typical sign of this disease (Figs 1A, 2H, I). The formation of suprastomatal sori is a trait that seems to be restricted to tropical rusts and has apparently evolved convergently in a number of rust genera (Berndt, 2012).

Ultrastructural studies have revealed that the germination of *H. vastatrix* urediniospores is accomplished through one or two germ pores (Rijo and Sargent, 1983). The germ tube wall is composed of an electron-lucent band apparently derived from the germ pore matrix, which is subjacent to the innermost urediniospore wall layer. The cytoplasm content, including the two nuclei and other organelles, passes through the germ pore (Fig. 3A). During this phase, the spore exhibits a high metabolic activity characterized by an increase in the number of mitochondria and the generation of endoplasmic reticulum, ribosomes, lomasomes and small vesicles (Fig. 3B).

β-1,3-Glucans and chitin are the major components of the cell wall of *H. vastatrix* urediniospores and infection structures (Maxemiuc-Naccache and Dietrich, 1981; Silva *et al.*, 1999). Both polymers are regularly distributed over the walls of pre-penetration fungal structures. In the intercellular hyphae and haustoria, β-1,3-glucans are also regularly distributed, whereas chitin accumulates preferentially over the internal parts of fungal cell walls, being less exposed to the eventual action of host chitinases, a general rule in rust fungi (Deising *et al.*, 1996; Silva *et al.*, 1999).

Ultrastructural studies have revealed that *H. vastatrix* intercellular hyphae and haustoria are similar to those of other rust fungi (as revised by Silva *et al.*, 1999). The cytoplasm contains abundant endoplasmic reticulum, occasional tubular complexes, mitochondria, ribosomes and lipid droplets.

Intercellular hyphae begin host cell penetration from HMCs, which have a thick multi-layered wall that attaches firmly to the host cell wall. Silva *et al.* (1999) observed that, during *H. vastatrix* haustoria formation, plant cell wall degradation was restricted to the site of host cell penetration with minimal damage to other cells. This is a common goal of biotrophic fungi, as they depend on the living host cells (Mendgen and Hahn, 2002; Schulze-Lefert and Panstruga, 2003).

The haustorium is composed of a neck, which presents a ring with two electron-opaque bands, and a body. Reaching far into the plant cell, the entire structure becomes surrounded by newly synthesized and highly modified plant plasmalemma, designated as the extrahaustorial membrane. Haustoria are thus not truly intracellular. Indeed, a zone of separation between host plasma membranes and the pathogen is established and comprises the haustorial cell wall and the extrahaustorial matrix (Harder and Chong, 1991; Mendgen and Voegele, 2005).

Similar to other biotrophs, *H. vastatrix* interacts intimately through such interfaces with the host cells, modifying the metabolic processes to serve its needs. This mode of interaction involves an effective suppression of the host immune system by effector proteins, known as effector-triggered susceptibility (Doehlemann and Hemetsberger, 2013; Hok *et al.*, 2010). In coffee plants susceptible to *H. vastatrix*, during the early stages of the infection process, a decrease in the abundance of hydrolases (sugar and peptides) and oxidases constitutively present in the apoplastic fluid of coffee leaves has been observed (Guerra-Guimarães *et al.*, 2014, 2015). However, during later stages, an increase in the abundance of defence-related proteins, such as phenylalanine ammonia lyase, peroxidase, superoxide dismutase,

chitinase, Pathogenesis-Related gene 1 (PR1), thaumatin-like, NtPRp27 protein and β-1,3-glucanase, has been detected (Guerra-Guimarães *et al.*, 2009a,b; Silva *et al.*, 2002, 2008). Furthermore, the hypersensitive cell death (hypersensitive response, HR) of guard and subsidiary cells and the encasement of some haustoria have also been observed, but in a low percentage of infection sites. Nevertheless, these plant defence responses occur too late to efficiently prevent fungal growth and sporulation (Silva *et al.*, 1999, 2002, 2006).

EPIDEMIOLOGY

CLR infections seldom kill the host plant, although severe infections affect the yield in subsequent years because they hamper vegetative development and can generate polyetic epidemics over successive seasons. Climate (including the altitude effect), shade, soil fertility and canopy architecture influence disease severity. Although a wealth of knowledge has been accumulated on epidemics and modelling, disease management strategies are still frequently ineffective. This gap may be explained by the very large number of environmental variables, their interaction at a given moment and their cumulative effect over time (Avelino *et al.*, 2004). Fluctuations in the price of coffee influence producer decisions regarding crop management, which, in turn, condition rust incidence and severity. This process seems to have become more acute in recent decades with the liberalization of prices and of cultivation quotas, and a sharp decrease in support of research and agricultural extension (McCook and Vandermeer, 2015).

A direct link between agricultural intensification and disease severity was reported during the Ceylon epidemic (Ward, 1882). Since then, epidemiology has gained several analytical tools, and multivariate studies at local, national and regional levels have contributed to a better understanding of the role of the relevant variables in the disease outcome. Usually, the peak of CLR epidemics occurs during fruit harvest; therefore, primary yield losses are frequently of low importance. Secondary losses, arising from low yield as a result of reduced vegetative growth caused by the previous epidemic, tend to be more important than primary losses (Avelino *et al.*, 1991). Modelling of rust epidemics according to agroecological variables has revealed the relevance of local agronomic factors, including shade, canopy density and soil fertility, interacting with regional environmental factors, such as rainfall (Avelino *et al.*, 2006; Boudrot *et al.*, 2016). At the global level, climate change scenarios have also been analysed in the context of CLR, with shorter incubation periods being forecasted (Ghini *et al.*, 2011) and disease-favouring scenarios mapped (Alves *et al.*, 2011). Indeed, this disease has been increasingly reported at higher altitudes in recent years (Boudrot *et al.*, 2016; Rozo *et al.*, 2012).

DISEASE CONTROL

Disease resistance breeding

The breeding of coffee plants for resistance to rust is considered to be the best disease management strategy, both environmentally and economically (Silva *et al.*, 2006). The first effective effort to select resistant germplasm was conducted in India in 1911, giving rise to the release of the cultivar 'Kent's', which replaced the susceptible cultivar 'Coorg' (revised by Rodrigues *et al.*, 1975). Several missions to Ethiopia were subsequently conducted, but no effective resistance sources were identified (Rodrigues *et al.*, 1975). In the 1950s, concern for the potential introduction of rust into the American continent (Wellman, 1953) led F. Wellman and W. Cowgill to conduct field missions in the Eastern Hemisphere, collecting more than 100 coffee types new to the Americas. In collaboration with Branquinho d'Oliveira, the work of these researchers led the USA and Portuguese governments to provide financial support for the creation of the Coffee Rusts Research Center (CIFC, Centro de Investigação das Ferrugens do Cafeeiro) in Portugal, located far from coffee-growing regions and thus centralizing research on CLR at the international level. Since 1955, CIFC has received and characterized coffee and rust germplasm and supplied breeding programmes at coffee research institutions with characterized resistance sources, together with scientific and technical information and training. One of the first practical results of the research carried out at CIFC was the demonstration that all cultivars grown at that time in America (including 'Typica', 'Caturra', 'Mundo Novo' and 'Bourbon') were susceptible to this disease (Rodrigues *et al.*, 1975). The investigation of CLR away from coffee-growing areas enabled CIFC to receive plant and fungal material from collaborating institutions around the world, which, in turn, allowed breeders in coffee-growing countries to have their genotypes characterized for resistance to races that were not present in such countries.

HDT (Híbrido de Timor) populations were derived from a plant discovered on the island of Timor in 1927 exhibiting resistance to rust among 'Typica' coffee crops (Bettencourt, 1981). In the 1950s, these populations were shown to be natural hybrids between *C. arabica* and *C. canephora*, most offering resistance to all rust races known at that time (Rodrigues *et al.*, 1975). In 1960, CIFC started a breeding programme aiming to transfer resistance from HDT to the main Arabica cultivars. Some selected F1 and F2 plants with resistance to all known races were supplied free of charge to all institutions in coffee-growing countries that requested these materials. The hybrids Caturra × HDT CIFC832/1 and Villa Sarchi × HDT CIFC832/2 synthesized at CIFC gave rise to the Catimor and Sarchimor populations, respectively. These populations, as well as others developed in Colombia (Caturra × HDT CIFC1343) and Brazil (Catuaí × HDT CIFC2570), are the sources of the majority of currently grown rust-resistant varieties.

These populations combine the resistance of HDT and the good agronomic traits of commercial varieties (Bettencourt and Rodrigues, 1988; Rodrigues *et al.*, 1975), attaining similar levels of beverage quality to those obtained from pure Arabica genotypes (Bertrand *et al.*, 2003; van der Vossen, 2003). The selection of HDT-derived genotypes adapted to local agroecological conditions and the subsequent release of cultivars have been conducted by several institutions, namely the Universidade Federal de Viçosa (UFV), the Empresa de Pesquisa Agropecuária de Minas Gerais (EPAMIG), the Instituto Agronômico de Campinas (IAC), the Instituto Agronômico do Paraná (IAPAR) and the Fundação Procafé/Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA) in Brazil; the Centro Nacional de Investigaciones de Café (CENICAFÉ) in Colombia; the Centro Agronómico Tropical de Investigación y Enseñanza (CATIE) and the Instituto del Café de Costa Rica (ICAFE) in Costa Rica; the Asociación Nacional del Café (ANACAFÉ) in Guatemala; the Instituto Salvadoreño de Investigaciones del Café (ISIC) in El Salvador; the Instituto Hondureño del Café (IHCAFE) in Honduras; the Instituto Mexicano del Café in Mexico; the Hawaii Agriculture Research Center (HARC) in Hawaii, USA; the Coffee Research Foundation (CRF) in Kenya; the Tanzania Coffee Research Institute (TaCRI) in Tanzania; the Central Coffee Research Institute (CCRI) in India; and the Dehong Tropical Agriculture Research Institute of Yunnan (DTARI) in China.

Rust resistance in HDT populations is conferred by Robusta-derived genes, such as S_H6, S_H7, S_H8, S_H9 and others not yet identified, in addition to Arabica resistance genes (S_H1, S_H2, S_H4 and S_H5). These genes, together with S_H3 (derived from *C. liberica*), condition coffee response to rust according to Flor's gene-to-gene theory (Noronha-Wagner and Bettencourt, 1967), enabling the classification of genotypes into physiological groups, ranging from resistant to all known rust races to susceptible to almost all known races (Bettencourt and Rodrigues, 1988; Várzea *et al.*, 1989; CIFC records). The usefulness of HDT populations as resistance donors led to several studies seeking to identify markers linked to resistance genes (Brito *et al.*, 2010; Diola *et al.*, 2011; Romero *et al.*, 2014), targeting downstream marker-assisted selection approaches.

The importance of HDT populations as resistance sources relies on the long durability of some of these resistance factors, which, in some cases, have been in use for more than 30 years. For instance, the genotype HDT CIFC832/2 carries additional genome introgressions compared with other genotypes (Herrera *et al.*, 2014) and presents a pre-haustorial (non-host-like) resistance (Diniz *et al.*, 2012). Indeed, the post-haustorial resistance response is typically found in most coffee–*H. vastatrix* interactions (Silva *et al.* 2002, 2006). The cytological and biochemical aspects of coffee resistance to CLR have been revised by Silva *et al.* (2006) and addressed by Diniz *et al.* (2012). In brief, both pre- and post-haustorial resistances are associated with the HR and with the activation of several genes, including receptor-like kinases, WRKY transcription factors, glycosyltransferases, lipoxygenases and PRs (Cacas *et al.*, 2011; Diniz *et al.*, 2012; Diola *et al.*, 2013; Fernandez *et al.*, 2004; Ganesh *et al.*, 2006; Guzzo *et al.*, 2009; Ramiro *et al.*, 2010; Silva *et al.*, 2006).

As the resistance in several HDT-derived genotypes is being lost (Diniz *et al.*, 2012), new sources of resistance are being investigated. Given the ample resistance found in *C. canephora*, together with the successful history of HDT, one tempting approach for the identification of new sources of resistance for Arabica coffee is to perform *C. arabica* × *C. canephora* crosses. Such studies have promised new resistance sources (Caicedo *et al.*, 2013; Herrera *et al.*, 2009; Mahé *et al.*, 2007; Romero *et al.*, 2010). To breed one of India's most popular Arabica genotypes (S.795), Prakash *et al.* (2011) developed two SCAR markers closely linked to the S_H3 gene. This is a highly effective rust resistance gene naturally introgressed into *C. arabica* from *C. liberica* (Prakash *et al.*, 2004). Partial and non-specific polygenic resistances have been evidenced in *C. canephora*, in some *C. arabica* genotypes and in some interspecific hybrids (as revised by Silva *et al.*, 2006). This corroborates previous reports suggesting that, in addition to S_H genes, other major and minor genes might condition coffee–rust interactions (Bettencourt and Rodrigues, 1988). Such studies, however, are hampered by the need for a laborious and time-consuming downstream breeding effort in order to introduce resistance factors into elite lines with adequate agronomic and quality traits.

The identification of resistance in wild *C. arabica* populations would be of interest as it avoids breeding to eliminate undesired traits from other *Coffea* species. However, the analysis of wild *C. arabica* germplasm has so far provided little support for the identification of resistance sources, as rust occurs frequently among plants in forests across the native range of *C. arabica* in Ethiopia (Samnegard *et al.*, 2014). Information regarding the susceptibility of wild germplasm to the different rust races is scarce (Rodrigues *et al.*, 1975), and the very low genetic diversity among wild populations suggests little promise of success in finding new sources of resistance (Davis *et al.*, 2012; Steiger *et al.*, 2002).

Chemical control

Chemical control of CLR is the obvious choice in the absence of a resistance genotype and of other effective disease management strategies. Chemical control represents an environmental hazard and a social concern (organic coffee is increasingly valued; Ibañez and Blackman, 2016), as well as an economic burden. For instance, in Tanzania, 50% of the coffee cultivation production costs can be attributed to the chemical control of the two main fungal diseases, CLR and coffee berry disease (Kilambo *et al.*, 2013). Preventative treatments are typically carried out with copper-based fungicides, whereas curative treatments are

conducted with systemic fungicides (e.g. epoxiconazole, pyraclostrobin). The combined or alternate use of copper-based and systemic fungicides is advised to avoid the risk of selecting fungicide-resistant rust populations (Zambolim, 2016). Research regarding the best practices for the application of such products is developed locally according to agroecological variables and epidemiology (e.g. Souza *et al.*, 2011).

Additional control strategies

The reduction in the availability of effective approved fungicides because of health and environmental concerns has made it necessary to intensify research for the development of novel, effective and sustainable disease control solutions. The effects of potassium silicate and essential oils have been tested recently, but with limited success (Lopes *et al.*, 2013 and Pereira *et al.*, 2012, respectively). However, promising results have been obtained with a resistance inducer of the benzothiadiazole (BTH) group, such as acibenzolar-*S*-methyl (Fernandes *et al.*, 2013; Guzzo *et al.*, 2001; Marchi *et al.*, 2002). BTH-treated coffee leaves overexpress genes involved in pathogenesis-related protein synthesis, the oxidative burst and cell wall strengthening, suggesting a general shift in metabolism from housekeeping to defence (Nardi *et al.*, 2006). The effects of phosphites and plant formulations based on the by-products of the coffee and citrus industries for the control of CLR have been evaluated in the glasshouse and in the field. Some of the formulations have shown an intermediate to good efficiency compared with standard fungicides, proving to be effective alternatives for the management of coffee rust and other diseases (Carvalho *et al.*, 2012; Fernandes LHM *et al.*, 2009; Monteiro *et al.*, 2013; Santos *et al.*, 2007).

Hemileia vastatrix spores are hyperparasitized by the Ascomycete fungus *Lecanicillium lecanii*. Although unable to effectively control CLR, this hyperparasite is capable of reducing spore viability and disease severity (Vandermeer *et al.*, 2010). *Lecanicillium lecanii* is primarily an entomopathogen of the green coffee scale *Coccus viridis*, which, in turn, has a mutualistic association with the arboreal nesting ant *Azteca instabilis*. The relationships between these organisms suggest that complex ecological interactions may play an important role in disease incidence and severity, potentially explaining why CLR is sometimes a severe epidemic and other times a troublesome but not devastating problem (Vandermeer *et al.*, 2014).

Bacteria and fungi present in the coffee ecosystem have been investigated for their use as potential biocontrol agents against *H. vastatrix*. Specific strains of the bacteria *Pseudomonas putida*, *Bacillus megaterium* and *B. thuringiensis*, together with two *Fusarium* sp. isolates, provide promising levels of antagonism (Haddad *et al.*, 2009, 2013, 2014; Silva *et al.*, 2012).

GENETIC AND PHYSIOLOGICAL DIVERSITY

Evidence of high pathogenic variability in CLR was recognized at an early stage and associated with the breakdown of resistance. Physiological specialization was first described by Mayne (1932, 1942), who identified four rust races. Since then, world surveys of coffee rust races have been historically pursued and amplified by CIFC from 1952 with d'Oliveira until today based on *H. vastatrix* spore samples from the most diverse coffee-producing regions of the world. Distinct races or pathotypes have been regularly identified through the differentiation of isolates on a set of coffee host materials bearing different resistance gene combinations (differentials) under prescribed testing conditions (d'Oliveira, 1954−57). Currently, more than 50 rust physiological races and 23 coffee differentials have been identified (Rodrigues *et al.*, 1975; Várzea *et al.*, 2009; and CIFC records).

Races are attributed to isolates with distinct and unique combinations of virulence genes as inferred by Flor's gene-to-gene theory and described as sequential roman numerals in order of detection (Noronha-Wagner and Bettencourt, 1967). Thus, as no further genetic confirmation has been possible so far, inferred rust race genotypes comprise virulence genes ranging from v_1 to v_9 in isolates derived from *C. arabica* and tetraploid interspecific hybrids, whereas those of the races that attack diploid coffee species are not known. Given this direct correlation with the coffee host resistance genotypes, virulence genes v_1 to v_5 can be traced back to an Arabica-type origin (v_1/S_H1 from 'Geisha'; v_2/S_H2 from 'Kent's'; v_3/S_H3 from a *C. liberica* introgression; v_4/S_H4 from 'Kaffa'; v_5/S_H5 from nearly all cultivars), whereas v_6 to v_9 reflect the additional Robusta heritage of S_H6 to S_H9 genes present in HDT and other interspecific hybrids. However, virulence profile characterization, particularly of isolates infecting HDT derivatives, can only go as far as the available collection of coffee differential genotypes allows, leaving many genotypes incompletely or entirely unidentified. As presumed from the dynamic host–pathogen co-evolutionary arms race, in which short-term selection of pathogen strains with fitness advantages is promoted, new pathotypes with increased virulence have been continuously appearing.

Within the scale of coffee differentials, race II (v_5) presents the most restricted infection spectrum and is considered to be the most common and widespread rust race in the world, acquiring a generalized occurrence, probably as a consequence of the uniform genetic background of most *C. arabica* cultivars worldwide (Bettencourt, 1981). However, the geographical distribution of rust races seems to be entirely dependent on the coffee genotypes planted locally, and the prevalence of certain races in a given area thus seems to occur accordingly (Bettencourt, 1981). Evidence of the selection pressure exerted by coffee resistance genes on the origin and distribution of pathotypes is easy to find (Várzea and Marques, 2005), but it is in India that the highest number of rust races is registered. This country holds the most ancient breeding

programme for CLR resistance in the world, involving regular and massive introductions of new or experimental resistant coffee materials into the field, to which usually follows the appearance of new pathotypes with enlarged virulence spectra (Várzea and Marques, 2005). This process recently resulted in the identification of CLR in India in genotypes resistant to all known rust races (Prakash *et al.*, 2015). Currently, the range of coffee rust virulence profiles most probably goes far beyond those of the races characterized so far.

No direct link between such high phenotypic diversity and molecular diversity has been found. Molecular makers for the characterization of variation in *H. vastatrix* populations have been elusive. Simple sequence repeat (SSR) markers, which are ideal for population genetics, failed to provide polymorphic loci with sufficient analytical resolution (Cristancho and Escobar, 2008; Rozo *et al.*, 2012). Sequencing of the rDNA-ITS region was also inadequate for sequence data analysis because of the presence of multiple copies, whereas other nuclear loci showed no variation or very few polymorphic sites (Batista *et al.*, 2010). Random amplification of polymorphic DNAs (RAPDs) and amplified fragment length polymorphisms (AFLPs) are the only informative markers documented so far, suggesting that genetic variation may be unevenly distributed along the genome.

Using RAPD markers, Gouveia *et al.* (2005) found a moderate genetic diversity and a high genetic differentiation in a global geographical range of *H. vastatrix* populations, with indications of clonal multiplication, but no evidence of population structure with regard to physiological race, host or geographical origin. Subsequent genetic diversity studies have predominantly been focused in Brazil (Cabral *et al.*, 2016; Maia *et al.*, 2013; Nunes *et al.*, 2009) and Colombia (Rozo *et al.*, 2012), addressing local populations. These populations represent the isolates with the lowest level of genetic differentiation in the study of Gouveia *et al.* (2005) compared with African and Asian populations, suggesting a possibly more recent origin. Indeed, South America was the last continent to be reached by *H. vastatrix*, and it is feasible that American isolates are derived from a few introduced since 1970 in Brazil, where it was first reported (Muller, 1971). All studies consistently report low differentiation and unstructured variability in *H. vastatrix* populations considering host and geographical origin, supported by high gene flow, probably as a consequence of effective long-distance dispersal and host germplasm exchange. Furthermore, large continuous areas of coffee plantations facilitate the stepwise movement of rust epidemics and genotype distribution (Nunes *et al.*, 2009). This pattern of evenly partitioned genetic variation found so far in CLR is consistent with the long-standing recognition of this fungus as an asexual species. However, evidence of random mating was found from an estimation of locus linkage disequilibrium (Cabral *et al.*, 2016; Maia *et al.*, 2013; Nunes *et al.*, 2009), suggesting some sort of recombination event. For instance, parasexual phenomena, including somatic hybridization, have been documented in rusts (Park and Wellings, 2012). Thus, despite the data provided by these recent studies, the mechanisms and dynamics of population genetic variation in *H. vastatrix* remain unknown. Such an endeavour is close to being accomplished by increasing the resolution of the molecular population analyses at the genome scale using next-generation sequencing of a more comprehensive rust sampling (Silva *et al.*, 2015b).

CYTOGENOMICS AND MOLECULAR PATHOGENICITY

Rusts were recently noted as the order with the largest average genome size among fungi (Talhinhas *et al.*, 2015; Tavares *et al.*, 2014). Nevertheless, the *c.* 800-Mbp genome of *H. vastatrix* (Carvalho *et al.*, 2014; Tavares *et al.*, 2014) stands out as one of the largest among rusts (Ramos *et al.*, 2015; Tavares *et al.*, 2014) and the largest genome of fungal pathogens of economic relevance. The genome of *H. vastatrix* appears to also vary in size: in a preliminary study including 11 isolates representing distinct races, the genome size ranged from 765 to 839 Mbp, with a general average ($\pm$ standard deviation) of 797 $\pm$ 27 Mbp (CIFC team, unpublished data). In addition, studies concerning the cytological characterization of the *H. vastatrix* karyotype were performed on metaphase nuclei obtained during urediniospore germination and revealed 10 chromosomes (Tavares *et al.*, 2013).

A link between biotrophic specialization and genome size expansion has been established (Spanu, 2012). Rust genomes are known to be vastly populated with non-coding regions (e.g. Duplessis *et al.*, 2011), suggesting that such non-coding regions and hypothetical polyploidy could explain the vast genome size of *H. vastatrix*. Indeed, Cristancho *et al.* (2014) suggested that 74% of the *H. vastatrix* assembled genome contains repetitive regions. A draft assembly obtained from our ongoing genome sequencing work on *H. vastatrix* accounts for less than 10% of the predicted genome size, yet a set of genes representing approximately 80% of the total number of genes in the *Melampsora larici-populina* genome was identified (CIFC team, unpublished data).

The unusually large genome size of *H. vastatrix* and the abundance of non-coding regions, including repetitive regions, could explain the difficulties in obtaining a saturated genomic sequencing. With the recent advances in genome sequencing technology, the sequencing of rusts has become feasible, and sequencing initiatives of rusts with large genomes of economic and scientific importance (e.g. *Phakopsora pachyrhizi* and *Uromyces fabae*; Loehrer *et al.*, 2014 and Link *et al.*, 2014, respectively) are ongoing, suggesting that the conditions are being met for a high-quality genome sequence of *H. vastatrix* to be obtained in the near future.

Although the genome of *H. vastatrix* has not been fully unveiled, transcriptomic approaches have already helped to gain insight into the functional genome. To this end, several technical strategies have been developed to circumvent the difficulties caused by the biotrophic nature of this pathogen, which implies that fungal samples (other than urediniospores) are physically mixed with the host in infected leaf samples. These include:

—an *in silico* approach using a bioinformatic pipeline based on homology scores and codon usage bias, applied to distinguish between fungal and plant transcripts (Fernandez *et al.*, 2012);

—a protocol developed to isolate good-quality fungal RNA from appressoria differentiated *in planta* (Loureiro *et al.*, 2015);

—a method for the selection and validation of reverse transcription-quantitative polymerase chain reaction (RT-qPCR) reference genes for fungal gene expression studies, considering the fungal biomass variation *in planta* throughout infection (Vieira *et al.*, 2011).

A transcriptomic analysis of *H. vastatrix* germinating urediniospores, appressoria and an *in planta* haustoria-rich sample (Fernandez *et al.*, 2012; Talhinhas *et al.*, 2014) led to the identification and annotation of 9234 transcripts (i.e. >50% of the genes predicted in sequenced rust genomes). Only 784 sequences were shared by the three conditions, and 75% were unique to a single library. Database comparisons further indicated that half of these *H. vastatrix* transcripts present no significant homology to genomic or transcriptomic data from other rusts, potentially representing novel or very divergent genes.

The annotation of *H. vastatrix* transcripts and a comparison of their relative abundance in each of the three sampling stages suggest a particularly active metabolism, translational activity and the production of new structures in appressoria, and intense signalling, transport and secretory activity and cellular multiplication in germinating urediniospores. In the haustoria-rich phase, results suggest intense signalling and nutrient uptake from the host to the fungus (Talhinhas *et al.*, 2014).

One hundred and forty-eight transcripts encoding putative carbohydrate-active enzymes (CAZymes) were identified, representing *c.* 45% of the CAZymes in the genomes of the poplar and the wheat stem rust fungi. These CAZyme transcripts are more frequently expressed during the early stages of infection, suggesting their involvement in the appressoria-mediated penetration of coffee leaf stomata, probably combining lytic and physical mechanisms (Talhinhas *et al.*, 2014). Among the CAZymes, chitin deacetylases (CD-As), with a potential role in avoiding recognition by plant chitin receptors and hydrolases present in the extracellular space in the leaf apoplast (Gueddari *et al.*, 2002), were analysed in *H. vastatrix*. Seven different CD-A-like genes were identified, and a phylogenetic analysis divided CD-As into two groups: one specific to *H. vastatrix* and another very similar to CD-As from different basidiomycetes, suggesting distinct biological roles (Azevedo *et al.*, 2013). Expression studies throughout the infection process showed different profiles in compatible (susceptibility) and incompatible (resistance) interactions (Azevedo *et al.*, 2013), with a peak of expression in compatible interactions coinciding with spore germination and appressoria formation (Vieira *et al.*, 2012), as noted previously in *U. fabae* (Deising and Siegrist, 1995).

Five hundred and sixteen *H. vastatrix* genes were identified as putatively encoding secreted proteins (Talhinhas *et al.*, 2014). At least 50% of these predicted secreted proteins have no homology to proteins in other rusts and may therefore be specific to *H. vastatrix*. Although less numerous, genes encoding putative secreted proteins in germinating urediniospores and appressoria RNA libraries are more abundantly expressed than those in the late infection library. The analysis of PFAM domains on the *H. vastatrix* secretome enabled the identification of 121 different PFAM domains. Transcripts with no PFAM domains and no homologies were further analysed for the presence of new conserved motifs (e.g. particular protein structure at particular positions, which suggests some common functions; Alfano, 2009; Saunders *et al.*, 2012), aiming to identify new avirulence (Avr) proteins (Gonçalves *et al.*, 2013). Three positional motifs were identified in four *H. vastatrix* transcripts, pointing to a conserved organization that resembles the structure found in the RXLR class of effector genes from oomycetes. These *H. vastatrix* genes were up-regulated in incompatible versus compatible interactions, suggesting a role in the induction of plant immunity (Gonçalves *et al.*, 2013).

CONCLUSIONS AND FUTURE PERSPECTIVES

In spite of its destructiveness, worldwide distribution and economic impact on the production of such an important cash crop as coffee, *H. vastatrix* has not been as widely studied as other rust fungi. As a pathosystem, the *H. vastatrix*–coffee interaction has some biological peculiarities and can be used as a historical, economic and epidemiological case study. The complex developmentally regulated infection process of *H. vastatrix* includes unique colonization features, such as haustorial invasion of stomata subsidiary cells before further tissue colonization and the induction of an HR as early as the appressorial stage. However, at the molecular level, much remains to be learned about the specific mechanisms underlying pathogenicity and virulence. The 516 putative *H. vastatrix* effector proteins identified may prompt the identification of coffee *R* genes encoding target proteins, providing additional tools to accelerate and improve disease resistance breeding.

The discovery and characterization of resistance in HDT populations and its deployment into commercial cultivars through breeding programmes still represent the major breakthroughs in the history of CLR disease resistance breeding. Nevertheless, the appearance of new rust races capable of overcoming such resistances raises the need to understand *H. vastatrix* virulence

evolution and to better characterize known sources of resistance and/or to discover new ones.

So far, high phenotypic diversity has not been reflected within the molecular diversity studied, and no population genetic structure has been found. Through large-scale population and evolutionary genomic studies, the efforts to unveil the *c.* 800-Mbp genome of *H. vastatrix* populations will certainly shed light not only on the reasons for such a huge genome size, but also on signatures of diversity creation. With the increasing feasibility of the generation of genomic data and sophistication of statistical methods, our understanding of *H. vastatrix* adaptive evolution will probably be boosted in the near future. This will provide deep insight into the diversity-generating mechanisms that render this a very successful and adaptable pathogen capable of overcoming resistance factors with relative ease. An improved annotated genome sequence would also greatly assist molecular research in *H. vastatrix*. Exploitation of these data, however, will still require the development or adaptation of methods for functional analysis, as well as the combination of different tools, from biochemistry to transcriptomics, to address the remaining unanswered questions about this important pathogen.

During the 150-year history of CLR, much knowledge has been gained regarding its biology, epidemiology and control, but evolving agronomic and ecological conditions, together with the evolving pathogen itself, make this a challenging pathosystem both to economy and to science. Further investment in CLR research is strongly needed, bridging biology and agronomy, in order to provide farmers with effective control strategies and durable resistance sources.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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