Introduction

1

Abstract

Trichoderma species are filamentous fungi found colonizing various habitats including plant materials such as stems, leaves, fruits and roots. *Trichoderma* species are typically found in soil associated with plant roots. The genus is of considerable interest for their its potential in the management of plant diseases. For about 70 years, *Trichoderma* spp. have been known to attack other fungi, to produce antibiotics that affect other microbes and to act as bio-control agents. Biological control using *Trichoderma* is part of an integrated approach to manage 'Basal Stem Rot' (BSR) disease of oil palm. BSR, caused by *Ganoderma* species, is considered to be the greatest threat to oil palm production in Southeast Asia (the largest area of production of palm oil). The use of *Trichoderma* is part of integrated disease management systems that have been developed to reduce the impact of BSR disease. This management approach consists of a combination of husbandry and agronomic techniques, chemical, and biological control (especially *Trichoderma* application to the soil prior to field planting) and to develop resistant material through breeding and biotechnological approaches. Currently there is little resistance to *Ganoderma* in breeding materials and it will take time to develop such resistant materials, in which probably the resistance will never be complete but rather will be partial, so the use of *Trichoderma* as a biological control is therefore critical in the management of the disease both in the shorter-term period as well as being a longer-term part of integrated disease management systems. This introductory chapter describes the biology of *Trichoderma*, its ability to attack other fungi and how this can be harnessed as a biological control measure for BSR.

1.1 *Trichoderma* – Biology, Life-cycle and Antagonism

Species of the genus *Trichoderma* belong to one of the most useful groups of microbes and have many applications (Mukherjee *et al.*, 2013). They have been widely used as bio-fungicides and plant growth modifiers as well as being sources of enzymes for industrial use. In soil, *Trichoderma* species are used in the bioremediation of organic and inorganic wastes including heavy metals (Schuster and Schmoll, 2010; Harman, 2011a, 2011b). They are able to colonize plant parts including stems, leaves, fruits and roots; some species have been shown to grow endophytically (Bailey and Melnick, 2013). *Trichoderma* species prefer locations with a large supply of plant roots, which they promptly colonize. In addition, *Trichoderma* species attack, parasitize or derive nutrition from other fungi (Reetha *et al*., 2014). Although *Trichoderma* species have been considered as soil inhabitants, based on *in situ* diversity studies using a taxon-specific metagenomics approach, Friedl and Druzhinina (2012) suggest that only a relatively small number are adapted to soil as a habitat. Properly selected isolates interact with the plant by colonizing roots, establishing chemical communication and systemically altering the expression of numerous plant genes.

Trichoderma is easily cultured, although a specific pH is required for maximum growth where these biocontrol agents can be multiplied and target pathogens controlled. Studies of pH variation by different workers revealed that *Trichoderma* isolates show optimum growth and sporulation rates at different pH values ranging from 2 to 7 (Begoude, 2007). Earlier experiments in India showed that the most favourable pH ranges were between 5.5 and 7.5 in which total dry weight of mycelium varies between 1.41 and 1.35 g. Although all the species of *Trichoderma* produced sufficient biomass at different temperatures viz. 20°C, 25°C, 30°C and 35°C, they were found to grow best at a temperature range of 25°C to 30°C. Aeration by agitation was also studied, with the greatest biomass recorded at 150 rpm (Singh *et al*., 2014).

With respect to life cycle, for many years *Trichoderma* isolates were considered asexual (anamorph) clonal lines of formally sexually reproducing species (teleomorphs). However, it was suggested by Tulasne and Tulasne (1865, see Schuster and Schmoll, 2010) that there was a link to the sexual state of *Hypocrea* species, this link was proven experimentally by Seidl *et al*., (2009). The limited observations of the sexual stage and the reasons for this are discussed in more detail by Schmoll (2013). The organism grows and ramifies as typical fungal hyphae, 5 to 10 µm in diameter. Asexual sporulation occurs as single cells, usually green conidia (typically 3 to 5 µm in diameter) that are released in large numbers on a repetitively branched conidiophore structure. Co-intercalary resting chlamydospores are also formed, these are also single celled, although two or more chlamydospores may be fused together (Gams and Bissett, 1998; Singh *et al*., 2012).

Relatively recently, *Trichoderma* isolates have been identified as being able to act as endophytic plant symbionts. The isolates become endophytic in roots, but the greatest changes in gene expression occur in shoots. These changes alter plant physiology and may result in the improvement of abiotic stress resistance, nitrogen fertilizer uptake, resistance to pathogens and photosynthetic efficiency. Typically, the net result of these effects is an increase in plant growth and productivity (Hermosa *et al*., 2012). They are also able to induce disease suppression in soils, and this is currently their major use in oil palm. The complex mechanisms of mycoparasitism, which include direct growth of *Trichoderma* toward target fungi, attachment and coiling of *Trichoderma* on target fungi, and the production of a range of antifungal extracellular enzymes have been described (Chet, 1987; Chet *et al*., 1998; Zeilinger and Omann, 2007).

1.2 *Trichoderma* as a Biological Control Agent

Trichoderma spp. produce a wide range of extracellular enzymes, some of which have been implicated in the biological control of plant diseases (Elad *et al*., 1982; Zeilinger and Omann, 2007). The enzymes themselves were found to be toxic to fungi and mixtures of enzymes were synergistic in their antifungal properties. Different classes of chitinolytic or glucanolytic enzymes from *Trichoderma* are synergistic, as are enzymes from different organisms (Lorito *et al*., 1996).

Efficient bio-control isolates of the genus are being developed as promising biological fungicides, and their weaponry for this function also includes secondary metabolites with potential applications as novel antibiotics (Schuster and Schmoll, 2010). They are able to deal with such different environments as the rich and diversified habitat of a tropical rain forest as well as with the dark and sterile setting of a biotechnological fermentor or shaker flask. Under all these conditions, they respond to their environment by regulation of growth, conidiation, enzyme production, and hence adjust their lifestyle to current conditions, which can be exploited for the benefit of mankind. One of these environmental factors is the presence or absence of light (Schmoll *et al*., 2010).

Trichoderma spp. have also been characterized as opportunistic avirulent symbionts (Harman *et al*., 2004). The critical characteristic of this association is the penetration of the plant's root system by *Trichoderma* and the persistent survival of the fungus within living plant tissues. Recent research results, principally with cocoa, *Theobroma cacao*, demonstrate that *Trichoderma* species can persist not only within the plant's root system but also within above-ground tissues in endophytic associations (Evans *et al*., 2003; Bailey *et al*., 2006; Bailey *et al*., 2008).

Trichoderma species can be re-isolated from surface sterilized cocoa stem tissue, including the bark and xylem, the apical meristem, and to a lesser degree from leaves. Scanning Electron Microscopy (SEM) analysis of cocoa stems colonized by isolates of four *Trichoderma* species (*Trichoderma ovalisporum*-DIS 70a, *Trichoderma hamatum*-DIS 219b, *Trichoderma koningiopsis*-DIS 172ai, or *Trichoderma harzianum*-DIS 219f) showed a preference for surface colonization of glandular trichomes versus non-glandular trichomes. The *Trichoderma* isolates colonized the glandular trichome tips and formed swellings resembling appressoria. Hyphae were observed emerging from the glandular trichomes on surface sterilized stems from cocoa seedlings that had been inoculated with each of the four *Trichoderma* isolates. Fungal hyphae were observed under the microscope emerging from the trichomes as soon as 6 h after their isolation from surface sterilized cocoa seedling stems. Hyphae were also observed, in some cases, emerging from stalk cells opposite the trichome head. Repeated single trichome/hyphae isolations verified that the emerging hyphae were the *Trichoderma* isolates with which, the cocoa seedlings had been previously inoculated. Isolates of four *Trichoderma* species were able to enter glandular trichomes during the colonization of cocoa stems where they survived surface sterilization and could be re-isolated. The penetration of cocoa trichomes may provide the entry point for *Trichoderma* species into the cocoa stem allowing systemic colonization of this tissue (Bailey *et al*., 2009).

Many of the endophytic *Trichoderma* species isolated from cocoa environments are being studied for their potential to protect cocoa against diseases. Black Pod (*Phytophthora* species), Witches' Broom (*Moniliophthora perniciosa*) and Frosty Pod Rot (*Moniliophthora roreri*) are major cocoa diseases that colonize above-ground tissues. All three diseases occur in South and Central America although their distributions vary, as does their relative importance (Bowers *et al*., 2001; Wood and Lass, 2001).

1.3 Basal Stem Rot Disease in Oil Palm

Basal stem rot (BSR) was first reported in the Congo (now Democratic Republic of the Congo) in 1915 (Wakefield, 1920), and although the disease kills infected palms the incidence was considered too low to be a serious threat to crop production (Turner, 1981). In Southeast Asia, BSR caused by various species of *Ganoderma* is the only disease that is causing serious losses in field plantings, especially in Malaysia and Indonesia (Susanto, 2009a). Although the disease has been recorded in Africa, Central America and Papua New Guinea, its impact is less significant (Turner and Gillbanks, 2003). Up to 80% yield losses have been recorded with an estimated loss in revenue of US$ 500 million per year (Breton *et al*., 2010; Hushiarian *et al*., 2013). The disease is a problem on both mineral and peat soils.

In Indonesia, especially North Sumatra, where most of oil palm plantations are already in their second or third replants, *Ganoderma* incidence has increased dramatically. Furthermore, zero burning, as noted in RSPO (Roundtable on Sustainable Palm Oil) regulation, during the first generation of planting (from forest) limited the reduction of disease sources on the old forest trees which are infected by root disease, especially *Ganoderma*. In addition, there was only limited understanding of the disease and its destructive potential for oil palm; now we are more aware. In the first generation, some *Ganoderma* incidence was observed but it was considered to be only a minor problem and restricted to older oil palms. However, at replanting (at end of first generation) most *Ganoderma* infection is in the bottom part of the oil palm stem and in the bole, therefore the use of a windrowing system of this infected material provided inoculum for subsequent generations. Disease symptoms were seen earlier in the planting with the pathogen not only infecting older palms, but also young palms.

Chung (2011) estimated the economic loss of a single palm due to basal stem rot at ages of 10, 15 and 20 years to be RM 2100 ($508), RM 1400 ($339) and RM 700 ($169) respectively, so infection with the pathogen at an earlier stage in the planting has significant economic impact. In one oil palm estate in North Sumatra, Indonesia, a census was conducted in 2011, using Global Positioning System (GPS) in order to record the percentage *Ganoderma* infection of palms <6 years old, as 0-1.5%. Percentage infection increased dramatically in palms >16 years old to 13-87% (this included vacant points which usually would have been as a result of *Ganoderma* infection) with a stand per hectare ranging from 35 to 119 palms in the worst affected fields (Virdiana *et al.*, 2012b).

Consequently, BSR is particularly devastating in plantations where oil palm is grown continuously. Successive re-planting, a common practice, brings about early and more frequent disease incidence with a major impact on yield (Breton *et al.*, 2010; Purba *et al.*, 2012). Indonesia and Malaysia are the two main producers of palm oil, and the main countries impacted by *Ganoderma* infection, although infection is increasing now in PNG (Papua New Guinea) too (Pilotti *et al.*, 2004). There is therefore a concerted effort in seeking methods to manage this devastating disease (Hama-Ali *et al.*, 2014; Rakib *et al.*, 2015).

1.4 Causes of Basal Stem Rot Disease

Root and stem rots caused by *Ganoderma boninense* were first described in 1915 in the Congo, West Africa, as a disease of senescing palms (Wakefield, 1920). The disease was first identified in Malaysia by Thomson (1931). In many parts of the world, fifteen species of *Ganoderma* have been recorded as likely pathogens (Turner, 1981). Ho and Nawawi (1985) concluded that all *Ganoderma* isolates from diseased oil palms from various locations in Malaysia were all the same species, *G. boninense*. However, morphological characters of the basidiomata suggest other species of *Ganoderma* were involved, namely *G. boninense, G. miniatocintum* and *G. zonatum* (Khairudin, 1990; Idris and Ariffin, 2004).

In Indonesia, the oil palm industry has developed rapidly since 2011, replacing Malaysia as the world's biggest producer in the world. Areas of oil palm expansion in Indonesia include Sumatra, Kalimantan, Sulawesi, Papua and West Java. The expansion was sometimes opening forest areas, but also included large areas that were conversions from other plantation crops (Lubis, 2009).

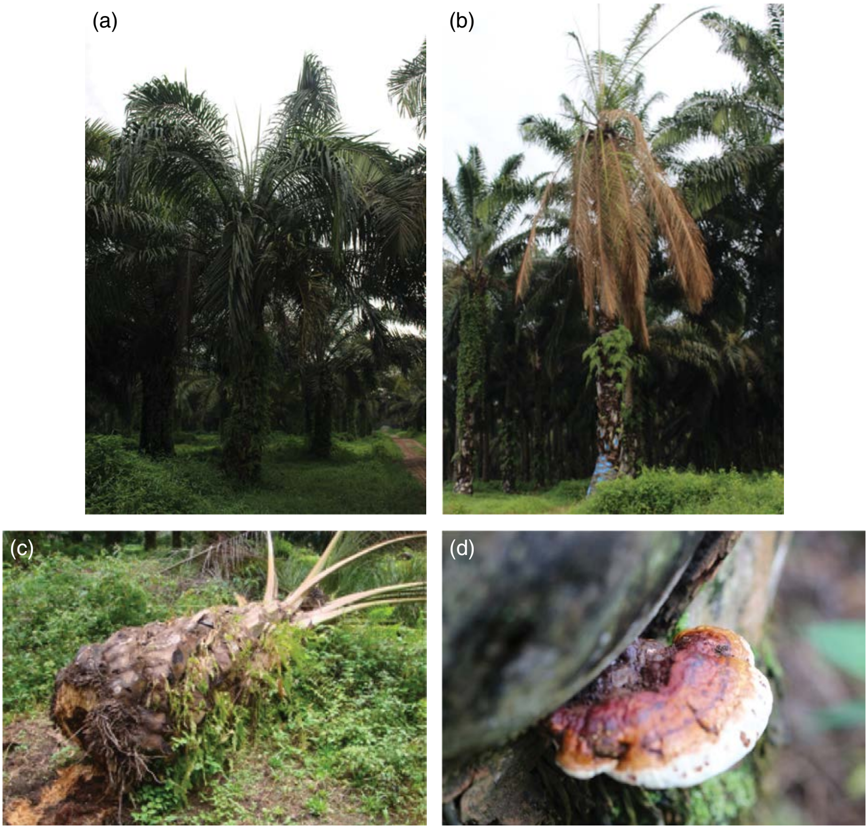

Fig. 1.1. *Ganoderma* disease symptoms: (a) Early infection (b) Advanced infection (c) Fallen palm (d) *Ganoderma* basidiocarp

1.5 Control of Basal Stem Rot Caused by *Ganoderma*

Although finding the best solution of BSR disease of oil palm is not easy, some efforts have been developed and practised to reduce infection of *Ganoderma* disease. No single method can claim to give a hundred percent success, and therefore, integrated disease management is recommended. This comprises chemical, biological and agronomic controls. Breeding efforts are also ongoing to produce resistant oil palm, but so far have only been partially successful.

(a) Cultural Control

Mounding of soil around infected palms has been utilized as a method of extending the productive life of infected palms. This stimulates renewed root development from stem tissue above the infected region and provides added stability, which compensates for reduced stem structural integrity due to decay caused by *G. boninense*. However, this treatment simply extends the life of the palm and does nothing to prevent the course of infection or transmission of the fungus (Tuck and Hashim, 1997) and could actually increase the inoculum.

Fig. 1.2. Mounding at the base of a lightly infected *Ganoderma* palm

At re-planting, shredding the oil palm stems (into slices about 10 cm thick) significantly reduced *Ganoderma* infection in the oil palm re-plant (Virdiana, 2012a). This encourages oil palm material to decay more quickly and any *Ganoderma* present in the debris becomes vulnerable to antagonism by natural microbial populations in the soil. *Ganoderma* is not a good competitor in soil (Rees, 2006).

A one-year fallow also significantly reduces *Ganoderma* infection as it reduces inoculum, again due to microbial antagonism (competition) and lack of a suitable host. However, a fallow period before re-planting will have a major impact on Internal Rates of Return (IRRs) and on an oil palm plantation's cash flow. It is therefore necessary to conduct further work to assess the optimal and minimal fallow periods to determine the best re-planting

Fig. 1.3. Shredding oil palm stems (into slices about 10 cm thick)

time. Commercially, a viable cash crop (e.g. banana, maize, legumes) could be planted as a "break crop" (Virdiana, 2012a). Soils may be sampled for DNA extraction to monitor the incidence of *Ganoderma* and other microbes in the soil, so determining soil health.

(b) Chemical control

Although many fungicides have been shown to be effective in supressing *Ganoderma* in laboratory experiments, their effectiveness in the field is poor (Susanto, 2002; Hushiarian *et al*., 2013). Rees (2006) stated there is inconclusive evidence for efficacy in reducing disease incidence or in prolonging the productive life of infected trees. However, application of the systemic fungicide triadimenol was claimed to prolong the life of infected palms (Ariffin *et al*., 2000). Consequently, chemical control of BSR has been limited and more research is required to improve this approach.

(c) Breeding

Breeding programmes for *Ganoderma* resistance have been set up in Indonesia and Malaysia. Success would be a major advance in protecting oil palm from this disease (Susanto, 2002; Breton *et al*., 2010; Purba *et al*., 2012). However, there are few potent sources for disease resistance, and little is known about the genetics of resistance. Some Indonesian seed producers have released *Ganoderma*, tolerant planting material, but this is a low level of partial tolerance and IPM (Integrated Pest Management) and other additional management methods are required.

(d) Biological Control

Trichoderma has been used as a biological control of *Ganoderma* since at least the 1920s (Harman, 2006). For oil palm, *Trichoderma* is multiplied and produced on a large scale, then used as a soil treatment. The application methods such as drenching of the new plant and manual application into the planting hole during replanting can be done as a prevention method for the root disease problem. Screening for the most effective isolates of *Trichoderma* may be conducted effectively in the nursery.

Nursery seedling trials (in pots) have shown that the highest *Ganoderma* infection (up to 100%) occurred in treatments with no *Trichoderma*. Infection was significantly higher when compared to treatments where *Trichoderma* was applied on top of rubber wood blocks (*Ganoderma* inoculum source in the pot) while the lowest infection was in treatments with *T. koningii*, *T. harzianum* and *T. virens* (Virdiana *et al*., 2012b). In addition, *Trichoderma* effectiveness has not only been demonstrated in the laboratory and in the nursery, but also significantly under field conditions. *Trichoderma* application to the soil is now implemented by a growing number of plantation companies. For example, Priwiratama and Susanto (2014) reported that the application of *T. virens* to planting holes is conducted routinely in many oil palm re-planting schemes.

The general methods involved are illustrated in Figure 1.4 and described in detail in Chapters 9 and 10.

Fig. 1.4. *Trichoderma* applications: (a) mixing soil with *Trichoderma* in the nursery (b) Manual application in the planting hole

1.6 *Trichoderma* as a Biological Control Agent for *Ganoderma*

Trichoderma isolates used in the biological control of *Ganoderma* are normally initially selected from *in vitro* studies (see Chapter 6), and from nursery testing (see Chapter 7). The ability of *Trichoderma* isolates to inhibit *Ganoderma* infection in oil palm has been observed using a glass chamber. *Trichoderma* starts to colonize *Ganoderma* isolates on rubber wood blocks

(RWB) 2 weeks after inoculation, resulting in no *Ganoderma* growth 4 weeks post inoculation with the more aggressive *Trichoderma* strains. Seedlings infected by *Ganoderma* were observed in the control treatment (no *Trichoderma*) 3 months post inoculation while those seedlings treated with *Trichoderma* showed no signs of *Ganoderma* infection 24 months post inoculation (Anjara *et al*., 2011; Anjara *et al*., 2013).

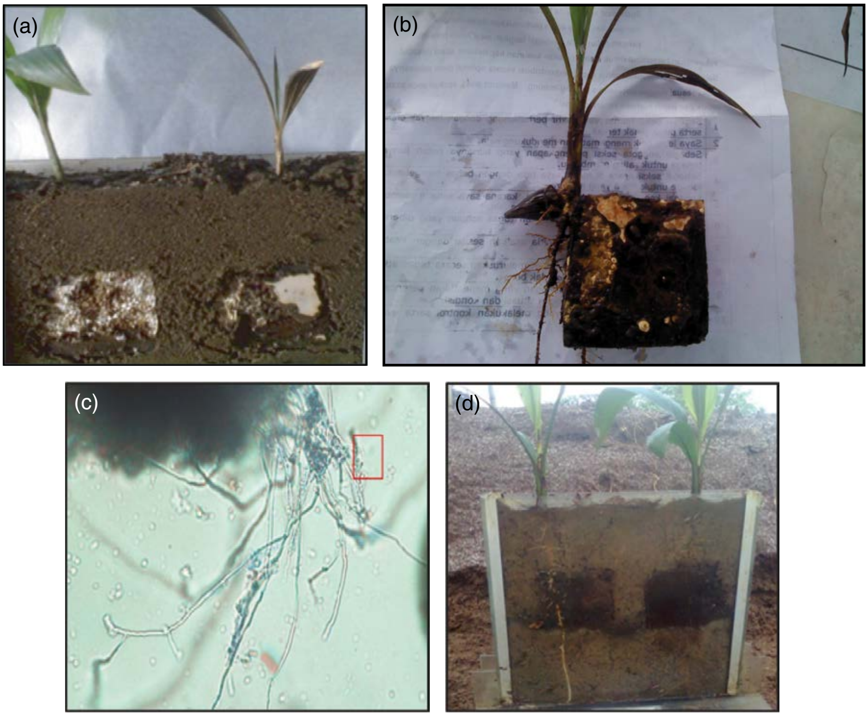

Fig. 1.5. (a) the seedling infected by *Ganoderma* in control treatment 12 weeks post inoculation; (b) seedling primary root in contact with *Ganoderma* RWB (Rubber Wood Block) as source of inoculum; (c) the photograph of *Ganoderma* mycelium taken from the root of seedling infected by *Ganoderma*; (d) seedling treated with *Trichoderma* showing no *Ganoderma* infection 24 weeks post inoculation

Microscopic studies involving *T. harzianum* showed that hyphae could penetrate *G. boninense* mycelia and so form a barrier between both mycelia. Interaction between *T. viride* and *G. boninense* was rather different with no barrier zone being observed, all *T. viride* hyphae could penetrate into *G. boninense* hyphae (Susanto *et al*., 2002).

In vitro tests have indicated that some *Trichoderma* isolates were such aggressive mycoparasites that they completely colonized *G. boninense* mycelium such that the pathogen could not be recovered - indicating the pathogen had been killed (Sundram, 2013). *Trichoderma* pre-treated seedlings (four months) were planted at four corners around *Ganoderma* infected stumps. Seedlings that were pre-treated with two kinds of *T. virens* had less disease symptoms compared to untreated positive controls where no *Trichoderma* pre-treatment had been conducted (Sundram *et al.*, 2016). In Verdant, bait seedlings are used to screen the best isolates in the field. Three or four bait seedlings are planted around a palm with a *Ganoderma* score one (light *Ganoderma*) palm combined with *Trichoderma* application into the planting hole to assess the best isolates to control *Ganoderma* in bait seedlings.

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