CHAPTER 2: LITERATURE REVIEW

The African oil palm, *Elaeis guineensis*, is a woody monocotyledon and monoecious (Corley and Tinker, 2016). Oil palm can be considered as the most productive cash crop, giving far higher yields when compared to soybean, rapeseed and sunflower (Murphy, 2007). The world palm oil production in 2014 increased to 59.3 million tonnes in comparison to the 56.3 million tonnes achieved in 2013 (MPOB, 2015). The rise of 3.0 million tonnes was due to further growth in matured oil palm areas especially in Indonesia. Current varieties of oil palm cultivated in plantations have economic life-spans between 25–30 years and produce fruits around the year (Barcelos *et al*., 2015).

Although oil palm is the basis of a lucrative commodity business, it is likely to get infected with diseases and attacked by insects. An important disease, yet still without a cure, that is affecting the present oil palm industry is basal stem rot (BSR). In 1915, the presence of the disease was first detected in the Republic of Congo, West Africa (Wakefield, 1920). In Malaysia, Thompson (1931) detected the disease that was infecting palms over 25 years of age that were due for replanting. The causal agent for BSR disease is the fungal species, *Ganoderma boninense*. It is considered to be detrimental to the oil palm industry in Indonesia and Malaysia (Ariffin *et al*., 1989; Rao, 1990). BSR progresses slowly and eventually causes the death of infected palms. The disease is capable of infecting all developmental stages of the oil palm. Palms infected early in its life usually show no symptoms until they are more than 12 years old (Paterson, 2007).

Many field studies have been carried out to monitor the disease progress and spread, followed by detection of the disease (Durand-Gasselin *et al*., 2005; Santoso *et al*., 2011;
Rees et al., 2012). Since disease progress and spread is slow, reporting of field test results have subsequently been delayed. This has resulted in limited number of publications on the outcome of field trials being reported between 2005 and 2013. Hence, most of the cited literature reviews were obtained more than a decade ago. However, there has been marked improvements with many disease detection technologies continuously being developed for field applications (Markom et al., 2009; Liaghat et al., 2014; Nurnadiah et al., 2014). Research findings on the molecular detection of the basal stem rot disease has also been forthcoming (Chong et al., 2011; Mercière et al., 2015; Ho et al., 2016).

2.1 **Ganoderma in relation to basal stem rot**

The *Ganoderma* fungus, a versatile basidiomycete, are capable of causing white rot diseases in hardwoods such as ash, maple, oak and sycamore. They mainly decompose cellulose, lignin and polysaccharides (Blanchette, 1984; Adaskaveg and Ogawa, 1990; Adaskaveg et al., 1991, 1993). *Ganoderma boninense* Pat. is known to be the causal agent of BSR in oil palm (Ho and Nawawi, 1985; Abadi, 1987; Soepena et al., 2000).

In the second and third replanting of oil palm, the symptoms can appear as early as one to two years after planting out in the field (Soepena et al., 2000). A more acute *G. boninense* problem is likely to surface over the next few years as the fungus increases its geographical range and virulence (Murphy, 2007). Singh (1991a) quantified yield losses based on fresh fruit bunch production found that it was adversely affected by incidence of the disease. Losses due to BSR could be incurred through direct reduction on the numbers of standing oil palms. This could also happen with the dwindling number in fruit bunch and weight from diseased standing palms as well as those with sub-clinical infections (Turner, 1981). The disease can result in the death of the palms
with only one-fifth still left standing in a *Ganoderma* infested field. These palms could still be producing fruit bunches either at their prime (8-15 years) or are more than 15 years old. Economic losses reaching 30% have quite frequently been reported (Corley and Tinker, 2016).

### 2.1.1 The symptoms of *Ganoderma* disease

Infected young palms usually exhibit the following symptoms: yellowing of leaves with or without the presence of spots on the lower fronds, followed by necrosis (Singh, 1991a). Other significant observations of the canopy include shorter unfolded leaves, chlorosis (pale or yellow) and necrosis (death of cells) (Arrifin *et al*., 2000). Palms may appear pale with significant retardation in growth as the disease gradually advances.

Similar symptoms, multiple unopened spears as well as pale and drooping leaf canopies, are also observed in mature palms. Once these foliar symptoms are observed in the field, 50% of the basal stem tissue could be found to be infected by the fungus. With the first appearance of foliar symptoms, infected young palms in the field may take between six months to two years before they succumb to the disease. Mature palms may take several years to die (Paterson, 2007; Cooper *et al*., 2011).

### 2.1.2 Possible sources of *Ganoderma* infection

Flood *et al*. (2000) observed the presence of BSR in oil palms planted on lands that have been converted from coconut plantations. These symptoms may develop after 10 to 14 years of planting (Flood *et al*., 2005). Utomo *et al*. (2005) also indicated that fungus starts to invade the root systems starting from the second and subsequent replanting cycles of oil palm. Spores released from the fruiting bodies (basidiomata)
were identified as the most likely sources of inocula for subsequent infection via root contact (Lim and Fong, 2005).

2.1.3 Previous studies on BSR caused by *Ganoderma sp.* in oil palm

Miller *et al.* (1999) referred to BSR as a significant constraint in oil palm plantations. In the subsequent year, Flood *et al.* (2000) categorised BSR as an increasingly important disease of oil palm for the past eight decades. Severe economic losses particularly in Malaysia and northern Sumatra has been associated with the continuous presence of the disease. According to Paterson (2007), it may take several years for the symptoms to develop in mature palms, while they are frequently undetected in immature palms. The infection rate in older palms is approximately 2% (Ariffin *et al.*, 2000). Although losses of up to 80% has been reported, these occur after many planting cycles. However, economical losses below 20% is not considered to be significant. The palms are left standing in the fields without any plans for replanting (Rao *et al.*, 2003; Conte *et al.*, 2012). These data show us that a standard measuring tool needs to be clearly defined on the seriousness of the disease.

Ariffin *et al.* (2000) described how basidiomata may initially develop from an infected root and spread towards the basal stem. The approximate locations of the diseased area inside the palm may be estimated using the positions of the basidiomata. The study by Abdullah (2000) on *Ganoderma* in coconut, favoured the spread from independent secondary inocula and neither supported root to root nor airborne spore spread.

A review by Paterson (2007) suggested otherwise, where the mode of spread was thought to be primarily from roots. This idea became noticeably accepted among
scientists doing research on ganoderma. The infection was proposed to have resulted from root to root contact between healthy tissues and diseased tissues that remained in the soil.

However, the role(s) of basidiospores still could not be ascertained (Ariffin et al., 2000). Sanderson et al. (2000) also backed the idea that for healthy roots to become diseased, they have to come in contact with infected debris. They agree that the disease is spread through this contact and without the absence of spores. Interestingly, Bridge et al. (2004) mentioned that different isolates of G. boninense varied from one another and emphasized the importance of basidiospores.

Most of the findings of these field studies were published more than a decade ago, due to the long life cycle of the oil palm and the slow mode of infection of the fungus. This also intensifies the need to increase efforts to solve the basal stem rot disease using faster advanced technologies compared to conventional methods.

2.1.4 Interaction between plants and pathogens

Plants are constantly in a battle to survive from invasions by their pathogens (Mcdowell and Simon, 2006). Pathogens often secrete virulence factors that contribute to the pathogenicity of the pathogens. These virulence factors target specific molecules within or outside plant cells (Chang et al., 2004; Tameling and Takken, 2008). Different strains of the same species of pathogen may secrete different types of virulence factors (Lukasik & Takken, 2009). Therefore, a defence system capable of recognizing distinct types of pathogen-encoded molecules has slowly developed in plants. These molecules are termed pathogen-associated molecular patterns (PAMPs). Highly conserved PAMPs in distantly related pathogens are also recognized by plants (Swiderski, et al., 2009).
PAMP detection is a type of non-host resistance in plants. It detects the presence of potential pathogens and sends early warnings to the plant. This type of defence system is also considered as one of the first line of defence in plants (Ingle et al., 2009).

Another type of defence system that has evolved in plants is known as resistance (R) proteins. Resistance proteins detect specific pathogen virulence factors as indications of potential attack. However, the virulence factor will be redefined as avirulent when detected by the host (Martin et al., 2003; Bernoux et al., 2011). To prevent further pathogen spread, this R-protein-dependent recognition system triggers a hypersensitive response. This response then activates programmed cell death at the site of infection, thus stopping further proliferation of the disease.

2.2 Mechanisms of defence against pathogens: biochemistry and physiology

Plant pathogens typically enter the hosts through cell walls. A lot of useful information has been documented on the roles demonstrated by the cuticle and various forms of cell wall strengthening (Vorwerk et al., 2004; Ho and Tan, 2015). There is no doubt that this cell wall concept has been the subject of major consideration. Paterson (2007) has proposed the lignin cell wall as the main defence mechanism against BSR disease. He also highlighted the importance of looking into developing highly tolerant or preferably resistant cell-lignin oil palm as a way to counter G. boninense infection. The possibility of differences in susceptibility to the disease within oil palm of similar genetic origin was also highlighted by Durand-Gasselin et al. (2005). He further elaborated that these variations in susceptibility can be identified within oil palms of the African (Deli, Angola and the La Mé) origins.
2.2.1 Cell wall apposition

A review paper by Garcion et al. (2007) acknowledged the role of cell wall appositions as the first line of defence in resistance to pathogens. Phenolic compounds are in abundance in the cell wall, where they can be found deposited at the site of infection (Tronchet et al., 2010; Micali et al., 2011; Underwood, 2012). Appositions are targeted within the plant cell wall at the site of attempted penetration (Collinge, 2009).

Appositions are observed in many plant species. They comprise of an outer halo surrounding the inner papilla (a fleshy projection on a plant). As demonstrated by chemical and histological analyses, the papillae consist of protein, callose, phenolic compounds, inorganic compounds and even reactive hydrogen species (Zeyen et al., 2002; Underwood, 2012). Penetration is often thought to be arrested by the presence of papillae. However, it is still unclear which particular component of the papillae is responsible for the successful defence against a specific pathogen. Results of a study on the functions of papilla in wheat infected with *Blumeria graminis* suggested that lignification of the papilla stopped further penetration by the fungus (Bhuiyan et al., 2009).

2.2.2 Callose deposition

Callose is an amorphous β-(1,3)-glucan polymer and is present in the plant cell wall (Luna et al., 2011). It is an effective barrier that prevents the entry of pathogen invasion at the site of infection. It also serves as a deposition platform for antimicrobial compounds. Callose deposition is typically triggered by conserved PAMPs and the specific delivery of chemical defences in the cellular level are provided at the site of attack (Ellinger et al., 2013; Nedukha, 2015). Some examples of potent callose-inducing PAMPs from fungal cell walls are chitin and chitosan. Other than PAMPS, callose
depositions can be activated by the presence of endogenous elicitors from herbivore- or pathogen-damaged plant tissues. Oligogalacturonides are some examples of damage-associated patterns (Ferrari et al., 2013).

2.2.3 Lignification

Lignin deposition usually occurs on the onset of pathogen invasion. It is represented as a strong barrier against potential pathogen invasion (Vanholme et al., 2010; Malinovsky et al., 2014). Lignin accumulates either only at the infection site, or over the entire wall of the infected cell or group of cells. Deposition of lignin in the plant cell wall will cause it to become more resistant to the mechanical pressure that is applied during fungal penetration. This prevents access to cell wall degrading enzymes (Bhuiyan et al., 2009; Wang et al., 2013).

Lignification is important for plant growth and structural development of plant cell walls. However, depending on the developmental process of the plant, the monomeric composition of lignin can differ greatly. Therefore, lignin in vascular tissues are significantly different from defence lignin accumulated by an elicitor treatment. This suggests that genes controlling lignin biosynthesis are differentially regulated during defence (Bhuiyan et al., 2009; Wang et al., 2013).

2.2.4 Roles of phytoalexins in the defence response

Phytoalexins are low molecular mass secondary metabolites. They possess antimicrobial activities that are often induced by stress or during plant defence. In their natural forms, phytoalexins are toxic towards nematodes, fungi, bacteria and higher animals. They are classified as weak antibacterial and antifungal agents (Ahuja et al., 2012; Thakur and
Sohal, 2013). However, phytoalexins have been shown to be detected in host plants at the sites of pathogen attacks (de León and Montesano, 2013).

Phytoalexins show biological activity towards a variety of pathogens. They are thus considered as molecular markers of disease resistance (Ahuja et al., 2012). Phytoalexins were introduced based on the findings on *Solanum tuberosum* tuber tissues that have been infected with an incompatible race of *Phytophthora infestans*. In response to the incompatible interaction, the tuber tissues produced substances (phytoalexins) that managed to inhibit the pathogen. These substances gave a form of protection to the tuber tissues against later infections by other compatible races of pathogens (Coleman et al., 2011; Pedras et al., 2011).

### 2.2.5 Hypersensitive response

In multicellular organisms, programmed cell death (PCD) often takes place during routine physiological processes (Garcion et al., 2007). This phenomenon has been observed during developmental processes. It also occurs in response to both biotic and abiotic stresses. PCD is often observed in plants. It is also known as the hypersensitive response following fungal, bacterial or viral infections. This HR is described as the accelerated failure of the plant cells to function properly, ultimately resulting in death at the site of the attempted infection. HR is often presumed to hinder the spread of a disease towards healthy neighbouring tissues by confining the pathogen. However, plants can also initiate HR without requiring induction by disease causing microbes (Zurbriggen et al., 2010; Bashir et al., 2013).

In the decade since then, the field of plant PCD has begun to mature. Although much of the work in this field has drawn heavily on comparative analyses using paradigms derived from animal systems, especially those of the apoptotic pathways, distinctive features and adaptive characteristics that correlate to the lifestyle of plants are also
beginning to be recognized. Several highly conserved or more distantly related components have also been revealed genetically to regulate PCD across eukaryotes (Hofius et al. 2009; Coll et al., 2010; Chichkova et al., 2010). In a few cases, it has become possible to join these components into pathways (Sundström et al., 2009). Single-gene mutations in most of these PCD components are viable and their effects on cell death induction and execution are usually quantitative in nature. These observations are thus consistent with the view that plant PCD pathways involve combinatorial modules to insure their proper control under a constantly changing environment that is superimposed on internal developmental cues (Bozhkov and Lam, 2011).

2.3 Disease control and management strategies

At present, there is no ultimate solution towards the spread of BSR in oil palm plantations. Efforts are therefore, more focused on short term control and developing a management system to contain the outbreak of BSR. Several mechanisms have been practiced in the oil palm industry to prevent and control BSR problems in plantations. Finding a solution to BSR problem in oil palm is possible, but it will require a thorough understanding of the biological nature of the fungus, including its life cycle and interaction with the oil palm.

2.3.1 Current agronomic practices

One of the more effective measures to control BSR is to exercise good agronomic practices. To prevent the further spread of mycelia from the invading pathogen, trenches were dug around diseased palms adjacent to healthy palms (Chung, 2011). Trenches were thought to act as natural barriers between root-to-root contacts. However, this particular practice has not been demonstrated to be successful (Turner, 1981). This is because the depth of the trenches was not deep enough to hamper the growth and
movements of the roots to neighbouring palms. Some trenches were not regularly maintained to serve the intended purpose.

Collecting basidiomata of *G. boninense* from diseased palms (Sanderson *et al.*, 2000) and painting them with carbolineum (fungicidal paste) (Turner, 1981) to prevent spore dispersal was also recommended. However, if the spores have no direct infective ability (not virulent), then this effort is rendered futile. The increased BSR incidence in oil palm has been associated with poor agronomic practices, such as poorly drained soils, occasional flooding, nutrition imbalances and deficiencies (Teh *et al.*, 2010; Chung, 2011). Currently no conclusive evidence has been used to support these factors.

### 2.3.2 Surgery and soil mounding

Turner (1968) proposed to control BSR by means of surgery and soil mounding. Initially, this was done through excision of diseased tissues as a form of treatment, but the results were variable. Later, Turner (1981) suggested that surgery be carried out, by using harvesting chisels, by excising the infected tissues away from the outer layer of the infected trunk. Diseased tissues from above and below the soil level were also removed using mechanical surgery (Singh, 1991b). With the removal of lesions, a protectant chemical (coaltar or thiram) was subsequently used to treat the exposed surfaces to prevent further fungal invasion.

When surgical methods are to be conducted, the age of the oil palm is an important factor to be considered. Turner (1981) reported that surgical treatments on palms more than 12 years old were more successful. This is because the disease lesions occur on the surface of the woody stems of older palms (Singh, 1991b). Infections almost always reoccur after this kind of surgical treatment if lesions are not effectively removed.
Another approach to control BSR is to mound the base of the infected palms with soil. A combination of *Trichoderma* biofungicide and endophytic fungus (arbuscular mycorrhizal fungus) is often incorporated into the mounded soil. Hasan and Turner (1998) showed that by incorporating soil mounding after surgery, the yield and vigour of mature disease palms could be increased. The economic life of *G. boninense* infected oil palms could potentially be prolonged by this treatment. It is believed that this combination of fungal cocktail helps to promote the growth of roots for nutrient absorption. Without the presence of arbuscular mycorrhizal fungus, soil mounding alone could increase the life expectancy of the oil palm. Weakened boles are thus prevented from being toppled by strong wind.

### 2.3.3 Fungicides

The introduction of fungicides coupled with proper application techniques could potentially be the answer to controlling BSR. Screening of fungicides against *G. boninense in vitro* showed that numerous fungicides were strongly inhibitory towards the growth of *G. boninense* (Jollands, 1983; Lim *et al.*, 1990). Loh (1976) and Khairudin (1990) conducted field trials to control BSR by using systemic fungicides. However, the results of these studies were inconclusive. These fungicides could be applied either through soil drenching or trunk injection) although the latter was the preferred method (Ariffin, 2005). The most effective fungicide cocktail for trunk infection was a mixture of carboxin and quintozene. It has been shown to effectively slow down disease progression, thereby extending the life cycle of *G. boninense* infected oil palms (Ariffin, 2005).

### 2.3.4 Replanting techniques

Besides control measures for existing stands, there are also some control measures at
replanting to control the disease in new oil palms. This can be achieved by adopting the correct technique during land preparation as the oil palm replanting process is regarded as an important part of the agronomic practice for controlling BSR (Chung, 2011). These control measures were based on the hypothesis that BSR spreads through root-to-root contact by infectious mycelia. In fields previously planted with coconuts or oil palms, the primary source of infection during replanting is the leftover tissues of former stands. By performing proper sanitation procedures, it is thought that the spread of the disease can be greatly reduced. Sanitation procedures involved the disposal of old stands (Chung, 2011).

Theoretically speaking, any approaches of disposing old stand by destructing or reducing the levels of *G. boninense* inoculum in the soil should have a beneficial effect on the subsequent replanting. Turner (1968) reported that the relationship between replanting techniques adopted and disease incidence are closely related. In the case of replanting coconut with oil palm, disease incidence has been reported to be rampant especially in coastal marine clay soils (Navaratnam, 1964). *G. boninense* is a saprophyte to coconuts. It prevails in the trunks and stumps of coconut that were left in the soil. Upon replanting, the fungus then infects the oil palm.

Smallholder farmers often practice under-planting coconut with oil palm. The under-planting of coconut with oil palm followed by poisoning or not poisoning and then felling of old coconut stands has been practiced by these smallholder farmers (Chung, 2011). Basidiomata of *G. boninense* are subsequently produced when the coconut stand starts to decompose in the field. *G. boninense* can be found in coconut plantations on dead husks and basidiomata have been observed not only on dead trunks and roots but also on living coconut seedlings. Outbreaks of BSR often occur in replanting areas
where the stumps had been retained in the ground. This is especially so in areas where oil palm has been planted after coconut. *G. boninense* infection may become apparent in oil palms as early as the first few years after planting. However, this is usually so when palms are over five years old (Singh, 1991a; Ooi and Heriansyah, 2005).

### 2.4 Current approaches to detect BSR

Scientists working on BSR disease have managed to identify *G. boninense* as the pathogenic fungus that disrupts the growth of the oil palm (Durrand-Gasselin *et al*., 2005; Paterson, 2007). The palm will ultimately die and collapse once the disease manifests itself onto the palm. Therefore, by identifying the causal agent of the disease, it serves as the starting point to the research and development into the disease. Methods to accurately detect the presence of the disease are currently still unavailable.

#### 2.4.1 Non-molecular techniques

One of the earliest methods to detect BSR was based on foliar observation of the palms in the fields. Symptoms such as mature leaves wilting, canopy drooping downwards, unopened spears are frequently observed and diagnosed as diseased palms (Ariffin *et al*., 2000). The other more obvious symptom is the presence of the basidiocarp at the base of the trunk.

Another diagnostic method to test the presence of the fungus was to use drills to sample diseased tissues in an infected palm. *Ganoderma* was then cultured on Ganoderma selective media and then maintained them on potato dextrose agar plates (Ariffin *et al*., 2000). Identification of the fungus is based on the presence of a brown halo surrounding the fungus and determination of morphological characteristics of the fungus. However, the correct and efficient techniques to detect the fungus were questioned. Many lacked
accurate taxonomy information whereby confusion arose with the identification of some species within the same genus (Hushiarian et al., 2013). A lot of time is wasted on performing these methods and they are deemed inaccurate. However, if basidiocarps are detected at the base of the palms, it could be a sure sign that the disease has already infested the trunk tissues of the palm.

More recent and advanced techniques of detecting BSR disease include the use of electronic nose (e-nose), unmanned aerial vehicles (UAV) and high resolution field spectroradiometers. The e-nose is a device capable of mimicking the human olfactory system. It has functions for detecting, recognizing and classifying volatile compounds and odours (Markom et al., 2009; Baietto et al., 2010; Abdullah et al., 2011; Abdullah et al., 2014). Many more developments of e-noses are growing due to the increasing interest in food quality control and detection of plant diseases. Most devices that are currently available are difficult to operate and expensive, except for a device specifically for odour recognition which has easy to use algorithms (Abdullah et al., 2011).

The use of UAVs of drones especially in the agriculture sectors have gradually increased. UAVs are able to gather detailed spatial information in real time. Due to its relatively low cost, the use of UAVs in conservation and ecological research has also increased (Getzin et al., 2012; Peña et al., 2013). The spatial and temporal data that are collected by the drones are accurate and can be used to understand disease transmission and environmental factors (Fornace et al., 2014). The use of UAVs could avoid the constraints with using satellite data. The collection of satellite data could be marred by cloud contaminations, long repeat times and low spatial resolution. However, the
practical use of UAVs for field research is limited to their use to specific applications and settings (Fornace et al., 2014).

Another approach which is gaining popularity is by using a spectroradiometer to capture diseases in plants. The distribution of a spectral power by a source can be measured using a spectroradiometer. Radiometric, photometric and colorimetric quantities from the spectral power distribution of light can be determined using this approach. Light sources can be measured, characterized and calibrated for various applications. High resolution field spectroradiometers are increasingly being used to analyse spectral and inspect vegetative diseases (Shafri et al., 2009; Lelong et al., 2010). One of the main challenges in using a spectroradiometer for automated detection of vegetative disease is the fine-tuning of its spectral parameters. This is required to enhance and subsequently fine-tune the potential of hyperspectral data to be used for early detection of diseases (Shafri et al., 2009; Lelong et al., 2010; Tawfik et al., 2013). Using a spectroradiometer, different stages of BSR disease infection was investigated by Liaghat et al. (2014). They examined the efficiency of reflectance spectroscopy to detect BSR disease in the early stages of infection and subsequently managed to differentiate the spectra between *Ganoderma*-infected and healthy oil palm leaves.

**2.4.2 Molecular techniques**

The earliest molecular attempts to detect the presence of *Ganoderma* were based on immunoassays. To detect the presence of *G. boninense* using ELISA (enzyme-linked immunosorbent assay), polyclonal antibodies specific to the crude mycelial proteins of the fungus were used (Karthikeyan et al., 2008; Kandan et al., 2010). Other than the crude mycelial protein, the polyclonal antisera against basidiocarp protein and monospecific protein (62 kDa) were also used in the detection using ELISA.
Another molecular technique to detect and identify Ganoderma was the use of ribosomal DNA (rDNA) sequences that codes for ribosomal RNA (rRNA). The non-coding rDNA regions contain internal transcribed spacer regions (ITS). This ITS regions have also been extensively used in biodiversity screening, evolutionary studies and phylogenetic analyses of fungi. The ITS regions are generally more variable than the rRNA genes and they have been used to explore the genetic variations of the fungus (Zheng et al., 2009; Chong et al., 2011; Yuskiangi et al, 2014).

In other studies, high-performance liquid chromatograph was used to study ergosterols. Ergosterol is a membrane sterol which is present in almost all fungi. It has been detected in other Ganoderma species (G. lucidum, G. applanatum, G. lipsiense) (Mohd As’wad et al., 2011). The concentration of ergosterol varies between the same fungal species depending on the physiological state of the fungus. Quantification of this component has also been done using nuclear magnetic resonance and gas chromatography coupled with mass spectrometry. These analyses could be used as a detailed diagnostic method for the early detection of BSR disease (Mohd As’wad et al., 2011; Toh Choon et al., 2012; Muniroh et al., 2014). Ergosterol has also been used to detect fungal contamination in sugar beet and wheat (Rossard et al., 2010; Wiwart et al., 2011).

2.5 Development of resistant varieties

To complement the effort taken in the detection of BSR, scientists are now looking into an alternation solution to control the disease. Oil palms that are resistant to G. boninense are being screened and developed as a long term solution to BSR disease (Durrand-Gasselin et al., 2005; Breton et al., 2006). However, we need to clearly understand the complexity of the disease to be able to contain it effectively. Screenings of resistant materials from existing fields have proven to produce results but it is very time
consuming (Durand-Gasselin et al., 2005). Crossings of palms from different backgrounds, followed by progeny testings have to be established prior to determining the resistance or susceptibility of each material. The differences in susceptibility of the Deli material within the *E. guineensis* germplasm were detected by de Franqueville et al. (2001). These Deli materials were planted in Indonesia in areas with high BSR incidence. The ability to artificially inoculate oil palm seedlings with *G. boninense* opens up the possibility of screening for resistance to BSR in the nursery. Using a root inoculation technique, significant differences in susceptibility among 43 palms with different genetic backgrounds including *Dura × Dura* (*D × D*), *Dura × Pisifera* (*D × P*), *Tenera × Pisifera* (*T × P*), and *Tenera × Tenera* (*T × T*) have been identified. Of the 43 palms, the most susceptible was (*D x D*; Elmina × Elmina) while the most resistant was (*D × P*; Zaire × Cameroon) (Idris et al., 2004). This tolerant palm was determined based on low severity of foliar symptoms and slow disease progression in the root and stem tissues.

Although extensive field trials have been conducted with the hope of identifying truly “resistant” planting material against BSR, little success has been found. Durand-Gasselin et al. (2005) stated that there is no *Ganoderma*-free variety, and it is advisable to remove all highly susceptible materials in the subsequent planting cycles once they have been tested.

In the 1980s, the prospect of improving crops for enhanced resistance to plant diseases through genetic manipulation was gaining tract with the onset of plant transformation. Different varieties of crops with agronomically important traits have moved towards genetically modifying the plants to be resistant to pathogens. In maize and cottonseed, a preharvest host plant resistance strategy against *Aspergillus flavus* has been developed
to control aflatoxin contamination (Cary et al., 2011). This strategy utilizes genetic engineering to enhance resistance against potential pathogens. The production of crops with agronomically useful levels of resistance has been achieved with the increased knowledge of plant-fungal interactions and the advancement of cloning disease resistance genes. Production of transgenic oil palm carrying fungal resistance genes with the potential for protecting oil palms from *G. boninense* infection is being investigated (Ariffin, 2005).

### 2.6 Proteomics – Technologies and applications

Proteomics is an area of study that examines the protein complement of the genome. It is a combination of techniques for protein separation (e.g. two-dimensional electrophoresis) with analysis and characterization of separated proteins (mass spectrometry). One of the direct approaches of building knowledge that will address many fundamental biological questions is by studying the machinery responsible for carrying out most biological functions (proteins). Furthermore, a direct approach to analyze proteins may reveal more accurate information than that inferred from mRNA measurements (Maier et al., 2009; Laurent et al., 2010). This is because more evidence is suggesting the disparity between mRNA levels and protein abundance. The disparities include regulatory processes that occur after mRNA is transcribed (Vogel and Marcotte, 2012). These include post-translational modifications, translational degradation and protein degradation.

Technological advancement and continuous improvements in the proteomics workflow have brought about many scientific researches to be published. Many branches of proteomic studies have emerged and these include protein separation, identification, quantification and sequence analysis. Other studies include structural, interaction,
modifications and cellular proteomics. However, the key techniques used are two-dimensional electrophoresis (2-DE) coupled with mass spectrometry (MS).

Proteomics has been extensively used to investigate and study infectious diseases, clinical applications and cancer research in humans (Pitteri et al., 2009; Legrain et al., 2011; Geyer et al., 2016). A review by Hamrita et al. (2010) stated that proteomics profiling had been performed by many research groups on the human breast tumours, tumour cells and tumour fluids to look for biomarkers and to understand the molecular mechanisms associated with breast carcinoma. The same objectives have been applied to plant research. Proteomics analyses have been applied to study the growth course in rice (Nozu et al., 2006), disease infections in rice (Chi et al., 2010), host-pathogen interactions (Schmidt and Völker, 2011) and development of elite soybean cultivars (Qin et al., 2013).

2.6.1 Two-dimensional electrophoresis (2-DE)

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) is capable of separating and resolving thousands of proteins in a single sample. The identities of the resolved proteins are determined by mass spectrometry. Furthermore, 2D-PAGE is also used to compare abundance of proteins between control and treated samples. It allows the classes of proteins to be determined due to these responses. Proteins that participate in similar or related process may show correlated differences in expression. This information can then be used to define the function(s) of proteins (Senkler and Braun, 2012).

2D-PAGE involves the extraction of proteins, followed by the isoelectric focusing of the extracted proteins based on their pIs (isoelectric points) (Oliveira et al., 2014).
These proteins are then separated on polyacrylamide gels based on their molecular weight. The profiles of the separated proteins can then be visualized after the gels were stained.

For comprehensive proteome analysis, every protein would ideally be resolved as one detectable spot. However, low abundant proteins may not be detectable by 2D-PAGE and they may be omitted from subsequent analyses (Baracat-Pereira et al., 2012; Fröhlich et al., 2012).

This could be overcome by targeting these proteins (e.g., membrane proteins, low molecular weight proteins etc) using mass spectrometry. Furthermore, approximately 2000-3000 spots can be observed using standard visualization methods on a typical gel. In this case, several proteins will co-migrate to the same spot position, hence masking their accurate quantitation and identification using mass spectrometry (Baracat-Pereira et al., 2012; Fröhlich et al., 2012). Therefore, preparations of protein samples are crucial. Proteins could also be run through a pre-fractionation process, or focused on a narrow ranged immobilized pH gradient (IPG strips). The largest IPG strips (24 cm) that offers greater resolution could also be used to explore the proteome of lower-abundance proteins.

2D-PAGE is not without technical difficulties. Scientists have encountered problems with image resolution and reproducibility, proteins with extreme pIs, visualization methods and image analysis. Difficulties with the compatibility of mass spectrometric techniques are generally faced in the identification of proteins. However, many publications have managed to solve most of these problems and it has become a
relatively easy task to run 2D-PAGE (Karp et al., 2007; Vincent et al., 2009; Koroleva and Bindschedler, 2011).

2.6.2 Mass spectrometry (MS)

Analysis of complex protein samples have been made easy by mass spectrometry with the gene and genome sequence databases made accessible to the public. MS is the current choice for proteomic studies with the innovative breakthrough in protein ionization methods (Han et al., 2008). Quantitative analysis of cellular and organellar proteomes have been made successful with high resolution MS methods (Walther and Mann, 2010). Traditionally, MS has been widely used for the quantitative measurement of specific small molecules, drug metabolites and hormones, with excellent precision and high specificity and high throughput (Nakamura and Oda, 2007). Nowadays, it has been widely used in differential analyses in disease research, particularly human cancers and crop diseases (Sadiq and Agranoff, 2008; Prieto et al., 2014).

The mass-to-charge ratio (m/z) of gas-phase ions is measured in an MS analysis. A basic mass spectrometer consists of an ion source, a mass analyzer and a detector. Analytes that enter through the ion source are converted into gas-phase ions. These ionized analytes are then separated by the mass analyser based on their m/z ratio. The number of ions at each m/z value is finally recorded by the detector. The mass spectrometers rely importantly on the separation of ions, hence the ionization source should be tailored depending on the application (Han et al., 2008; Zhang et al., 2013).

The matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI) are two most frequently used ionization methods. They are categorized as soft ionization techniques. They are termed ‘soft ionization’ techniques since the ions
undergo little fragmentation as they were created with low internal energy (Whitelegge, 2013).

In MALDI, protein samples are mixed with an organic matrix and co-crystallized on a metal target. The matrix is then excited using a pulsed laser. Thermal energy rapidly heats up the molecules and subsequently desorbs the ions into the gas phase. On the other hand, ESI ionizes molecules directly from solution and then sprayed into the mass spectrometer at atmospheric pressure so it can be easily interfaced with a liquid chromatography equipment (Han et al., 2008; Walther and Mann, 2010).

There are three types of commonly used mass analyzers for proteomics research: ion trap, quadrupole (Q) and time-of-flight (TOF). For ion traps, the mass analyzer first traps the ions for a certain time interval prior to scanning in the detector (Yates, 2004). Ion traps are able to determine the mass of a given peptide as well as its sequence. As the name suggests, quadrupoles consist of four parallel cylindrical rods. Ions are separated based on the stability of their trajectories in the oscillating electric fields when electric fields are applied to the rods (Whitelegge, 2013). In the TOF analyzer, an electrical field accelerates ions to the same kinetic energy as the velocity of the ions depending on the m/z ratio (Nakamura and Oda, 2007). Therefore, TOF measures velocity of the ions, from which the m/z ratio is determined.

A hybrid mass spectrometer is a device for tandem mass spectrometry (MS/MS). It consists of a combination of two or more different mass analyzers (Han et al., 2008). MS/MS quantitates peptides more precisely and is widely used for analysis of complex mixtures (Dzieciatkowska et al., 2014). Some of the hybrid instruments include triple quadrupole, quadrupole TOF, TOF/TOF.
2.6.3 Bottom-up and top-down proteomics

Proteomic experiments often involve concurrent analyses of many proteins from complex biological samples. The crucial feature in proteomic analysis is therefore the separation of peptides and proteins. Many MS-based proteomic strategies have been developed and improved to fulfill the various analytical and biological requirements (Fig 2.1). For these reasons, two principle proteomics approaches are employed to deliver simplified molecules from complex mixtures to the ionization source.

In top-down proteomics, intact or whole-proteins are analyzed typically using ESI. This method identifies the protein by comparing the precursor and product ion masses with those from protein databases. The main aim of top-down proteomics is to increase throughput for whole proteome analysis while preserving the inherent value of an intact protein mass measurement (Whitelegge, 2013). In bottom-up proteomics, proteins are digested into smaller peptide mixtures using trypsin. Prior to tandem mass spectrometry, these peptide mixtures are fractionated by multidimensional chromatography. When complex protein mixtures are used in the bottom-up approach, it is called shot-gun proteomics (Han et al., 2008). In short, the bottom-up method measures proteolytic peptides while the top-down approach analyzes intact proteins (Zhang et al., 2013).
Adapted from Han et al. (2008). *Current Opinion in Chemical Biology, 12*: 483-490

**Figure 2.1**: Strategies for protein identification and characterization using mass spectrometry. Proteins extracted from biological samples can be analysed by bottom-up or top-down approaches. In the bottom-up approach, proteins in complex mixtures can be separated before enzymatic or chemical digestion. This is followed by direct peptide mass fingerprinting-based acquisition, or further peptide separation coupled to tandem mass spectrometry. Alternatively, the protein mixture can be directly digested into peptides using the shotgun approach. In the top-down approach, proteins in complex mixtures are fractionated and separated into less complex mixtures. This is followed by intact protein mass measurement and intact protein fragmentation.