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Banana weevil borer (*Cosmopolites sordidus*): Plant defense responses and Control options

Dissertation

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ABBREVIATIONS

CSIR	Council for Scientific and Industrial Research
CRI	Crops Research Institute
CABI	Centre for Agriculture and Bioscience International
FAO	Food and Agriculture Organization of the United Nations
et al.	all, and others
glimmix	Generalized mixed model
glm	General linear model
IPM	Integrated pest management
LD50	Median lethal dose
ID50	Median inhibitory dose
LSD	Least significant difference
R^2	Coefficient of determination
pH	Negative logarithm of the hydrogen ion concentration
TEAC	Trolox equivalent antioxidant capacity
TPC	Total phenolic content
PCI	Percent coefficient of infestation
MJ	Methyl jasmonate
PFA	Paraformaldehyde
P-HCl	phloroglucinol-HCl
HCl	Hydrochloric acid
GAE	Gallic acid equivalent

AAE	Ascorbic acid equivalents
UV	ultra-violate
ABTS	2,2’-azinobis-3-ethylbenzothiazoline-6-sulfonic acid
SPAD	Soil plant analysis development chlorophyll meter
CV.	Cultivar

Chapter 1.

1.0 General introduction

1.1 Banana weevil

Banana weevil *Cosmopolites sordidus* Germar (Coleoptera: Curculionidae), is a narrow oligophagous pest to highland banana (*Musa* species), plantain (*Musa x paradisiaca*) and Abyssinian banana (*Ensete ventricosum*) cultivars (Gold *et al.*, 2003b; CABI, 2014). Banana weevil was first identified by Germar in 1824 who named it *Calandra sordida* before its current name, *C. sordidus* (Germar) with synonyms such as *Sphenophorus striatus* (Fahraeus) and *S. cribricollis* (Walker), *S. sordidus* (Germar) among others (Gold *et al.*, 2001; CABI, 2014). The genus Cosmopolites, belongs to the family Curculionidae (weevils and snout beetles), order coleopteran and class Insecta. It has many English common names such as banana borer, banana rhizome weevil, banana weevil bore, plantain weevil, among others (CABI, 2014). Calling it banana weevil throughout this thesis will mean the same to plantain and Ensete unless otherwise stated.

The banana weevils trace their origin from the Indo-Malayan region (South East Asia), since then, weevils have spread to almost all tropical and subtropical banana/ plantain growing regions of the world (Figure 1.2), possibly through infested planting materials (Gold *et al.*, 2001; Gold *et al.*, 2003b; Blomme *et al.*, 2013; CABI, 2014). For example, by 1920, weevils were reported in sub-Saharan Africa, Central America, Pacific and the Caribbean among others, and continued to spread where control measures and quarantine were not effective (Blomme *et al.*, 2013). The banana weevils have limited mobility which limits gene flow and likely evolution of local biotypes. However, biotypes of banana weevils have considerable genetic diversity within a population of the same region than that from geographically disparate areas (Gold *et al.*, 2003b). According to Gold *et al.* (2001) review, weevils take 10 years from their inception in a new area to gain pest status, and this is influenced by ecological conditions, cultivar susceptibility and management practices. Banana weevils, optimally thrive at high humidity with temperature ranges of 23-26°C and altitude between 1000 and 1400 meters above sea level (Gold *et al.,* 2001), for they are susceptible to desiccation an indication that high or low temperatures affect their survival.

Adult banana weevils are black, measuring 10 to 15 mm in length, nocturnally active and characterized with negative phototropism and thigmotactism, strong hygrotrophism,

gregariousness and death mimicry (Gold *et al.*, 2001; Shukla, 2010). The life span of adult weevil is 1 to 4 years, and they keep a close distance at the banana mat while feeding on crop residues like newly cut or rotting pseudostems, decaying rhizome and borrow as far as 50 cm under the soil (Shukla, 2010). On average each female weevil lays 1 to 4 eggs per week singly placed in crevices made at the collar of the plant rhizome (1 to 2cm) above the ground level with their rostrum. Eggs (0.5 x 2mm^2) hatch into larvae at 6 to 8 days and goes through 5 to 6 instar larval stages for 2-6 weeks to pupation which lasts 1 week to become adult. Larvae, ecologically occupy a different microenvironment from the adult, possibly due to their modest mobility and savage feeding that requires them to be at the breeding site, unlike the wandering adults. This makes larvae to be the most destructive life stage (Figure 1.1), yet difficult to eliminate with non-systemic pesticide applications (Gold *et al*.,2001; Njau *et al.*, 2011).

Figure 1.1 Hidden feeding mode of weevil larvae Source: Bakaze, 2017)

It is argued that banana weevil is one of the most serious pests of the Musaceae family with annual yield losses between 25 to 75% in endemic banana/plantain growing regions (Speijer *et al.*, 2001; Gold *et al.*, 2003b; Njau *et al.*, 2011; CABI, 2014). Damages start when weevil laid eggs

hatch into larvae that feed and develop while causing direct damage to the rhizome. During feeding, larvae create a network of galleries in the rhizome that weakens the plant. In younger plants, larvae feeding causes chlorosis, drying of cigar leaf, and eventual death of the plant. In already established plantation with susceptible cultivars, larvae feeding interferes with established and emerging roots which affect nutrient and water uptake, reduce plant vigor, delay flowering, and expose the plant to opportunist pathogens which altogether affect plant stability during windy weather. A 5 to 44% bunch weight reduction occurs in the 1st to 4th ratoon, banana mats die out and plantation life is shortened due to their damage (Rukazambuga *et al.*, 1998; Speijer *et al.*, 2001).

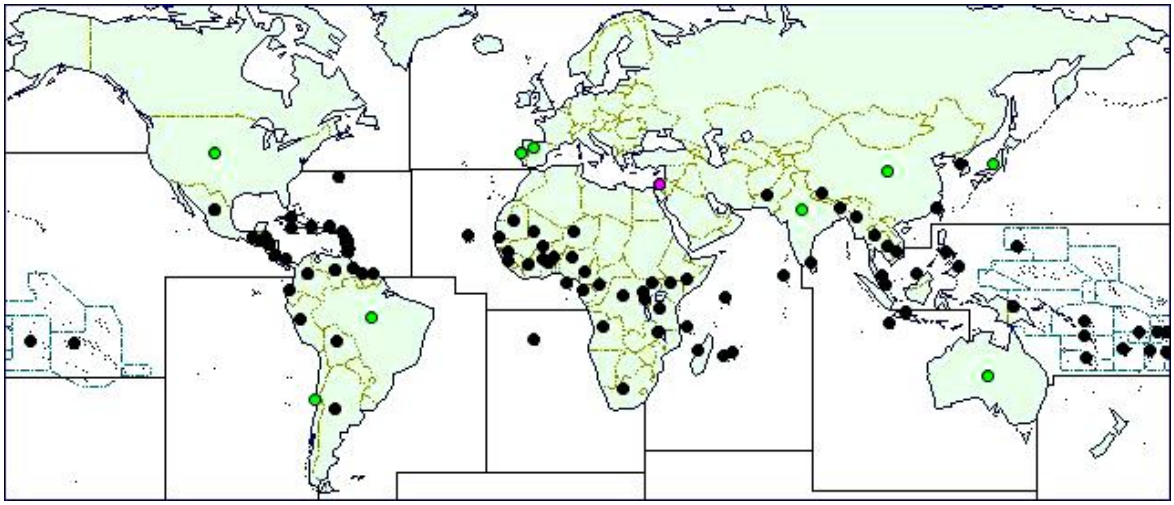

Figure 2 1.2: Map of the world showing banana weevil distribution black dots (present with no further detail) green dots (confined and subjected to quarantine); adopted from (CABI, 2014)

Bananas and plantains are monocots belonging to the order Zingiberales, family *Musaceae,* with two genera Musa and Ensete. All cultivated bananas are processed from two diploid wild banana species; *Musa acuminate* and *Musa balbisiana* with AA and BB genome respectively. The processed *Musa* species are grouped into three genomes based on their ploidy levels of eleven chromosomes; diploid (AA, BB, AB), triploid (AAA, AAB, ABB) and tetraploid (AAAB, AABB, ABBB). Most plantain or processed bananas have AAB and ABB genome e.g. “French and Horn” plantain, while most dessert bananas are pure acuminate (AA or AAA) e.g. “Gros Michel”. Most tetraploids are a result of breeding programs e.g. dessert FHIA-02 (AAAA) and FHIA-01 (AAAB),

cooking/dessert FHIA-03 (AABB). As with all monocotyledons, *Musaceae* have fibrous shallow adventitious roots that originate from the rhizome (underground corm).

Bananas/ plantain plants are herbaceous and there are vegetatively propagated using suckers. The banana mat consists of an underground corm (rhizome) that bears the lateral buds from which suckers emerge, Figure 1.3. These suckers may be left *in situ* to form the crop cycle (ratoon) or removed to serve as planting materials elsewhere. The apparent pseudostem is composed of leaf sheaths while the true stem bears a single terminal inflorescence. It is the rhizome and the emerging suckers that are primarily targeted for by banana weevils.

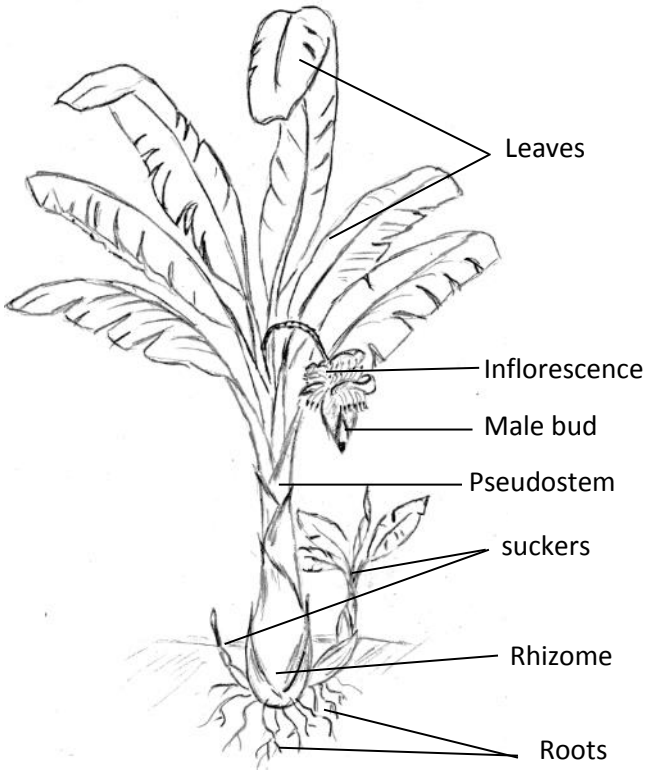

Figure 1.3 Herbaceous banana/plantain mat with suckers on the rhizome (Source: Bakaze, 2017)

It is estimated that 90% of banana/plantation production is done by small subsistence farmers in their backyard (Gold *et al.*, 2001). This contributes to banana being the eighth most important food crop in the world and fourth in the developing countries after rice, wheat and maize in terms of the gross value of production and food consumption (Dusunceli, 2014). It is cultivated by over 130 tropical and subtropical countries, for it is nutritious with high energy sources of no

cholesterol, high potassium, vitamin C and B6, health antioxidants like dopamine and catechin (USDA, 2016). Bananas originated from South East Asia and were introduced to Africa by Arab traders at around 500AD. Asia is the leading producer of banana in the world with 43% (of which 50% is produced by India), followed by Africa with 29% (of which a quarter of it is produced by Uganda) and America with 27%. In sub-Saharan Africa, bananas and plantains are mainly grown by small scale farmers as a staple food. Uganda is the leading producer with 28%, followed by Ghana with 10% Tanzania with 9%, Nigeria 8% Cameroon, and Rwanda. Most of the cultivated bananas and plantains are consumed locally with approximately 200-240kg per capita in East Africa and 134.6kg in West Africa (FAO 2014). Therefore, any sustainable effective weevil control strategy to consider, should be of low cost, easy to adapt, safe for homestead growers and readily available in order to benefit the small holder farmers while sustaining its production.

1.2 Management of banana weevil

Predominantly, banana weevil management relies on chemical pesticides, despite recommendations of culture methods (mulching, crop sanitation and pseudostem traps), use of resistant bred lines ("M2", "M9", "M20", and "M27" cooking type) (Kubiriba *et al.*, 2016), biological methods (predators, fungi, bacteria, protozoa, parasitoids) and mass trapping using pheromone lures (Gold *et al.,* 2003a). Currently, a single control strategy cannot give the IPM preferred long term efficacy, unless, other strategies are considered. Integrated pest management (IPM) has been advocated since the 1970s (Knipling, 1972) to ensure that pesticides and other interventions are economically justified to reduce human health and environmental risks. FAO argues that IPM should emphasize the growth of healthy crops with the least possible disruption to agro-ecosystem, and to encourage sustainable natural pest control mechanisms (Veres, 2013). IPM has also been suggested by Gold *et al.* (2001) as an approach that may successfully target the different life stages of *C. sordidus*.

Chemical insecticides, target wandering adult weevils excluding eggs and larvae that are deposited inside the plant tissues. To control the subsequent adults that emerge from larvae, repeated application of insecticides is done, a practice that contributes to insecticide resistance among the weevil population (Edge *et al.*, 1975; Collins *et al.*, 1991; Gold *et al.*, 1999; Mongyeh *et al.,* 2015). Owing to increased awareness on the indiscriminate use of synthetic pesticides,

persistent hazardous effects, banned pesticides (WHO and IPCS, 2009) and weevil developed insecticide resistance (Edge *et al.*, 1975; Gold *et al.*, 1999; WHO, 2012), much interest has been shown in searching and integrating the effective non-synthetic control methods. Among the interventions evaluated in this study are; exploitation of host resistance (Dixon and Paiva, 1995), the use of botanical plant derivatives (Scott *et al.*, 2008), and entomogenous fungi (Lazreg *et al.*, 2007; Roy and Cottrell, 2008).

1.3 Host resistance

This is the collective heritable characteristics by which a plant species, race, clone, or individual may reduce the possibility of successful utilization of that plant as a host by an insect species. To develop a successful IPM strategy for any crop, knowledge about plant resistance guides the rational use of other management interventions that are not harmful to the non-target organism, environment, and consumers. Insect plant relationships evolved million years ago, for they are the sole source of nutrients to almost all insects and other organisms like bacteria, fungi, protists and vertebrates (Freeman and Beattie, 2008). Plants to survive all these organisms had to develop different structures, chemicals and protein-based defenses to detect and stop the invading micro and macro-organisms (Freeman and Beattie, 2008). Banana weevil to successfully attack the banana/plantain, involves plant location, host plant acceptance to oviposit and suitability of larval survival. Therefore, host plants may affect any of those processes which result in resistant, tolerant and susceptible plants to the insect pest attack (Mithöfer and Boland, 2012).

Painter (1958), divided host resistance into three categories; antixenosis, antibiosis and tolerance. Antixenosis is the non-preference reaction of insect pests toward the host to oviposit, feed, or shelter. Banana clone with “A” genome (Yangambi-KM5 (AAA), Culcuta-4 (AA)), possess an antixenosis resistance, for they lack sufficient stimulant compounds like 1, 8 Cineole and phenolic glucoside salicin (Ndiege *et al.*, 1996). instead, they have compounds that repel weevils from ovipositing or feeding (Pavis and Lemaire, 1996). Antibiosis on the other hand refers to plant properties that adversely affect the physiology of attacking insect pests e.g. absence of essential nutrient which inhibit insect development (Ortiz *et al*. 1995). Banana cultivars with a “B” genome such as Pisang Awak (ABB), predominantly have antibiosis mechanism of resistance (Ndiege *et al.* 1996). Tolerance in banana cultivars is attributed to growth vigor and relatively large rhizomes, for example, gross michel, Cavendish (AAA) and Pisang Awak (ABB) cultivars (Kiggundu *et al.*, 2007).

Plants utilize preformed physical and chemical barriers (constitutive defense) to obstruct pest attack and associated damages. Loon *et al.* (2006) argue that plants have a wide range of induced defense which includes but are not limited to; molecules, biochemicals, and morphological changes. Plants have evolved signalling defense mechanism that is invoked when they are faced by biotic or abiotic stresses (Rolland *et al.*, 2002; Howe and Schaller, 2008). And many pathways particularly ethylene, jasmonic acid and salicylic acid are involved in signalling between stress perception and response. Howe and Schaller (2008) stated that such defense response occurs both locally at the site of the attack and systemically in undamaged tissues as it is reported in banana treated with dead *Fusarium oxyporum* f. sp. *Cubense* (Thakker *et al.*,2013). The induced defense may entail oxidative burst, production of antimicrobial metabolites, programmed cell death or cell wall lignification to block further entrance and damages.

Although most highland banana (AAA) and plantain (AAB) cultivars are susceptible to *C. sordidus*, other *Musa* clones, such as Culcuta-4 (AA), Kisubi (AB), Yangambi-Km5 (AAA), Kayinja/ Pisang awak (ABB) and FHIA-03 (AABB) are reported to be resistant (Gold *et al*., 2003a; Kiggundu *et al.*, 2007). Studies have indicated host resistance through suberization and lignification as part of the general plant response to insect and mechanical damage (Dixon and Paiva, 1995). However, the extent it occurs in the banana rhizome tissue and its distribution patterns when challenged with banana weevils is unknown. Therefore, among the triploid *Musa acuminata* cooking type, resistant Yangambe KM5 and susceptible Mbwazirume (AAA genome) banana clones were used to study host-based resistance against banana weevils.

1.3.1 Activation of host resistance

To turn on specific defense, plants use elicitors in the saliva of chewing insects to distinguish between mechanical and insect wounding (Freeman and Beattie, 2008). Insect wounding elicits both local and systemic responses via jasmonate pathways in form of physical (cell reinforcement and lignification) or chemical defense (calcium accumulation) (Beckman, 1982; Heil *et al.*, 2002; Agrawal and Konno, 2009; Heil and Bostock, 2002). Herbivorous insects, however not only cause physiological and biochemical changes but give an insight into the nature of anatomical and morphological changes due to damages (Rittinger *et al.*, 1987; De Ascensao and Dubery, 2000). According to Howe & Schaller (2008), physical containment differs among plants and organs attacked but follows a similar pattern of accumulation of waterproof substances like suberin to form a seal. It is further demonstrated that plants differentiate between signals of chewing and

piecing sucking insects, generalist and specialist feeders, and even herbivore species of the same feeding guild (Schoonhoven *et al.*, 2005; Howe and Schaller, 2008; Lucas-barbosa *et al.*, 2017).

1.3.2 Host plant phenolics

Plant phenolics are a group of multifunctional carbon-based secondary metabolites with an aromatic ring and at least one hydroxyl, produced by the phenylalanine pathway. There are over 10,000 plant phenols, grouped into simple phenols and polyphenols based on the number of phenol units. The most significant plant phenols include Phenolic acid (benzoic derivatives and cinnamic acid) and their ester, coumarins, flavonoids (C_6-C_3-C_6), lignin (C_6-C_3)n, tannins (C_6-C_3-C_6)n, stilbenes among others (Taiz and Zeiger, 2010). They play various vital roles; many serve as defenses against herbivores and pathogens, others function in mechanical support, in attracting pollinators and fruit dispersers, others reduce the growth of nearby competing plants (Taiz and Zeiger, 2010). For instance, the protective function of lignin is linked to its physical toughness which deters herbivory, and its chemical bonding to the cellulose and proteins which makes them relatively indigestible. Freeman & Beattie (2008) demonstrated this with scleroid cells of pear fruits (*Pyrus sp)* whose cell walls were impregnated with lignin that rendered it difficult to be chewed by insect pests. In banana, phenolic compounds have also been proposed to participate in the defense mechanism against biotic stresses (Wuyts *et al.*, 2007; Ewané *et al*., 2012).

Plant phenols are either preformed (constitutive) or synthesized in response to wounding (induced). Constitutive phenolic compounds are produced in small quantities until herbivore feeding is detected due to the high metabolic costs involved in their production and storage (Freeman and Beattie, 2008). Induced phenolic compounds (phytoalexins) accumulate at the site of the attack and modulate programmed cell death to prevent further damages through localized acquired resistance (Agrawal and Konno, 2009; Ewané *et al*., 2012).

1.3.3 Lignin and suberin

Lignin is a highly branched phenolic polymer of three phenylpropanoid alcohol groups (coniferyl acid, *P*-coumaryl and sinapyl). Suberin is a lipid-phenolic biopolyester with a linear polyaliphatic chain (fatty acid derivatives and glycerol) and polyaromatic domains (ferulic acid) (Vishwanath *et al.*, 2015). The biosynthesis of monomers (monolignols) of lignin and suberin, share a phenylalanine pathway and they are joined into polymers by the action of different enzymes and deposited in the apoplast (Vogt, 2009; Fleck *et al.*, 2011).

Lignification is a common response to infection and wounding (Rittinger, *et al.*, 1987; Taiz and Zeiger, 2010). It is reported to be part of the formed antifeedant barrier of phenolic polymers, whose nature and spatial distribution in the tissue depend on the extent a plant is exposed to insect wounding (Hawkins & Boudet, 1996). Various physiological and anatomical changes occur in cells surrounding the damaged tissues, one of them is the formation of impermeable layers of cutin, suberin and lignin to protect the damaged tissue from desiccation and pathogen infections (Kolattukudy, 1980; Dean and Kué, 1987; Rittinger, *et al.*, 1987; Dixon and Paiva, 1995). It is postulated, therefore, that the level of plant lignification and suberization could be useful in the selection processes of resistant plants to insect pests.

Plant response to wounding may include, but not limited to cell proliferation, modification of the first layer adjacent to the wound by the accumulation of various antimicrobial and water-impermeable substances such as lignin and suberin (Rittinger *et al*. 1987; Lagrimini 1991; Franke & Schreiber 2007; Vishwanath *et al*. 2015). And through histochemical staining of affected plant tissue, anatomical modification due to damages can be revealed (Mitra and Loqué, 2014).

Modern pest control strategies consider host resistant breeding and transgenic approaches as key methods to reduce pest associated crop losses (War *et al.*, 2012). Host resistance against banana weevil at physiochemical levels has received less attention possibly because chemical pesticides offer a quick solution. But the current concern, to look out for eco-friendly measures, make host resistance an ideal measure to contribute to IPM strategy. Therefore, an insight into banana rhizome defense mechanism through histochemical staining, its phenolic content and antioxidant capacity both in resistant and susceptible cultivar were evaluated aimed to contribute to the IPM approach.

1.4 Botanical plant extract

Crop protection based on botanical plant derivatives against injurious crop pests is a recognized valuable tool in pest management that is different from conventional pesticides (Scott *et al.*, 2008). This was a result of banning effective insecticides due to their hazardous effects and increased public perception that crops protected with natural compounds are safer than synthetic ones (Scott *et al.*, 2003; WHO and IPCS, 2009). And the development of resistance to pesticides for example dieldrin by banana weevils (Edge *et al.*, 1975) and Anopheles mosquito to pyrethroid (WHO, 2012). For instance, Southern Chile farmers place *Cestrum parqui* branches in potato fields

to repel *Epicauta pilme* beetles, and Tanzanian farmers crush *Tephrosia* species leaves in water to control maize field pests (Altieri, 1993). Manipulation of insect pest environment through botanical extract application cause larvae mortality after ingestion or contact and repel adult insects which disrupt their oviposition and feeding, consequently reducing crop damage (Scott *et al.*, 2003). In this study, neem tree *Azadirachta indica* A. Juss, black pepper *Piper guineense* Scum and Thonn, and clove tree *Syzygium aromaticum* L were investigated, for they show promising results for control of various crop pest.

1.4.1 Neem

Neem is universally known for its diverse utility as a therapeutic and naturally occurring insecticide. It shares the family of Meliaceae with other five species; *Azadirachta excels* Jack, *Azadirachta siamens* Valeton, *Melia azedarach* L., *Melia toosendan* Sieb and *Melia volkensii* Gürke that are being studied for pesticide properties on different agricultural pests (Schmutterer, 1995; Mulla and Su, 1999). *Azadirachta indica* A. Juss is commonly known as Indian lilac (neem tree), for it is a native of the Indian subcontinent. It is now widely distributed in the drier tropical and subtropical areas of Asia, Africa, America, Australia and the South Pacific islands (Schmutterer, 1995). It is an evergreen tree with heights of 35-40 m, and under favorable rains (400-800 mm) it produces olive-like yellow fruits Fig. 1.4 that contain 1-2 seeds (Schmutterer, 1995). The most insecticidal components of neem are in its fruit seeds, the seed kernels. Neem seeds with white hard endocarp kernels contain approximately 3-4 mg azadirachtin per 1 g kernel (Schmutterer, 1995). Azadirachtin is a tetranortriterpenoid with a deterrent, anti-ovipositional, antifeedant, growth-disrupting, anti-fecundity, and fitness-reducing properties on insects (Schmutterer, 1990, 19 1995).

Figure 1.4 Fruiting neem tree in the Greater Accra region, Ghana, West Africa (Source: Opata, 2014).

In this study, dried seed kernels of *A. indica* seeds collected at the backyard of farmers were assessed for their insecticidal properties against *Cosmopolites sordidus.* Different neem products, such as leaf powder, neem seed cake, neem seed aqueous extract and seed kernel oil have varying efficacy to different insect pests. For example, neem oil at different concentrations inhibited the hatchability of *Earias vittela* eggs (Thara *et al.,* 2009), caused larvae mortality and repellency to *Aedes albopictus* (Benelli *et al.*, 2015). Aqueous seed extracts, the simple alternative to oil, when properly prepared is as effective as commercial neem extracts. For instance, with 5% aqueous neem seed extracts, Musabyimana *et al.* (2001) reported fewer weevils on treated rhizome than control. He further observed reduced feeding damage by larvae on pseudostems treated with neem seed cake compared to control. Incorporation of neem seed cake into the soils around the plant base decreased the infestation in field trials even stronger than commonly used carbofuran (Musabyimana *et al.*, 2000). Inyang and Emosairue (2005) found 35-60% repellence on a filter bioassay against the banana weevil and 65-73% repellence of 10% aqueous extracts on feeding material, respectively. Neem properties are proven versatile as it targets a diversity of organisms, such as nematodes, molluscs, mites, locusts, crickets, termites, thrips, lice, moths, flies, bugs and beetles (Schmutterer *et al.*, 1995).

1.4.2 Black pepper

Piper guineense Scum and Thonn, commonly known as West Africa black pepper is a creeping vain that share Piperaceae family with other species such as *Piper nigrum* L., and *P. tuberculatum* Jacq (Ameh *et al.*, 2011). This perennial climbing vine, *P. guineense,* is native to the tropical rain forest of Africa and can reach up to 12 m in forest clearings or on remaining trees in secondary forests (Iwu, 1993; Ameh *et al.*, 2011). It is commonly grown in West Africa, as a spice crop, accustomed medicine and as a source of pesticide due to its phytochemical compositions (Iwu, 1993; Ameh *et al.*, 2011). The pepper berries grow in racemes as shown in Figure 1.5 and vary in color from red to red-brown and turn black when dry.

Figure 1.5 Ripe and dried pepper fruits with distinctively curved stalks (Source: rarepalmseeds.com; Kofler, 2014)

Of the various secondary compounds of *Piper* species, amides (N-Isobutylamine) are of primary interest in insect control (Scott *et al.*, 2007), as they have shown a promising knockdown effect on *Lymantria dispar* larvae. While *P. nigrum* extracts had high larvae mortality of *Diprion similis* and *Malacosoma disstria* and also deterred feeding and oviposition (Scott *et al*. 2004). *Piper guineense* dried fruit extracts are effective repellent against termites in maize fields (Umeh and Ivbijaro, 1999). Even foliar spray application of *P. guineense* extracts had a strong reducing effect on numbers of *Megalurthrips sjostedti* adults, nymphs and *Maruca testulalis* larvae in cowpea (Ivbijaro and Bolaji, 1990). Inyang and Emosairue (2005) found 68-85% repellence on a filter bioassay against the banana weevil and 77-94% repellence of 10% aqueous extracts on feeding material, respectively

1.4.3 Clove Syzygium aromaticum

This evergreen clove tree *Syzygium aromaticum* L belongs to the family Myrtaceae (Weiss, 2002). It is highly adapted to tropical maritime climate where it naturally grows to the height of 15m and as a secondary-story tree in lower montane forests (Weiss, 2002). It is indigenous to the Moluccas and successfully grows in areas with an annual rainfall of 2000-3000 mm such as Indonesia, Tanzania, Zanzibar, Sri Lanka and Madagascar (Weiss, 2002). A short dry spell to initiate the floral differentiation into buds is needed shortly before flowering, and it's the buds that are harvested

and dried to dark-brown (Weiss, 2002) Figure 1.6. The dried clove buds are commonly sold as food spices in East and West African markets.

Figure 1.6 Terminal inflorescence buds (right) and dried clove buds (left) (http://upload.wikipedia.org/wikipedia/commons/c/c1/Syzygium_aromaticum_ontree

Botanical products notably from the family of Myrtaceae and Lamiaceae have long been traditionally used as insecticide against many plant pest and pathogens (Murray, 2000). The family members of Lamiaceae like *Ocimum gratissimum* and Myrtaceae such as *Syzygium aromaticum* L, contain compounds like Eugenol and β-Caryophyllene (Obeng-Ofori and Reichmuth, 1997; Vieira and Simon, 2000). The major constituent of flower buds is Eugenol and β-caryophyllene (Srivastava *et al.*, 2005; Chaieb *et al.*, 2007). Besides its medicinal value as a skin-deep application to relieve pain and to promote healing, clove essential oil is also reported to have insecticidal properties (Chaieb *et al.*, 2007). For instance, Eugenol effectively inhibited egg hatching, caused high larvae mortality and repelled *Sitophilus zeamais, Sitophilus granaries* and *Tribolium castaneum* pests (Obeng-Ofori and Reichmuth, 1997). Clove extracts gave promising results in controlling *Culex pipiens* larvae by arresting the development to the adult stage completely (El Hag *et al*., 1999) and also showed detrimental effects on the root-knot nematode *Meloidogyne incognita* (Meyer *et al.*, 2008). Therefore, extracts from *A. indica*, *P. guineense*, and *S. aromaticum,* and their chemical derivatives; Eugenol, Eugenylacetate, β-Caryophyllene, and N-Isobutylamine were assessed on *C. sordidus* egg inhibition, larviciding and adult weevil repellence.

1.5 Biological control

Biological/ biocontrol according to Eilenberg *et al.* (2001), is the use of living organisms to suppress the population density or the impact of specific pest organisms, making it less abundant or less damaging than it would otherwise be. This control option is desirable over synthetic chemical pest control, due to its minimal hazardous effects on the environment while preventing the resistance outbreak among the insect pest population. Moreover, it modifies the environment or existing practices to protect and enhance specific natural enemies that reduce the insect pest damages. Practices like additional organic matter increase the population size of natural enemies which in turn lower the insect pest population. This conservation biological insect pest control, therefore, endorses the main principle of organic farming that protects the existing natural enemies.

As an alternative to chemical pesticides, natural enemies such as entomopathogens, predators and parasitoids, are considered appropriate and plausible measures to control *C. sordidus.* Entomopathogens for instance have been suggested as insect pest control agents over a century with microbes such as fungi, viruses, bacteria and protozoa (Lazreg *et al.*, 2007; Roy and Cottrell, 2008). Naturally, pest suppressive soils contain such entomopathogens which prevent insect pests from reaching their economic threshold despite favorable environmental conditions (Vegaa *et al.*, 2009). Traditionally, entomopathogens are considered important pest mortality factors that need boosting with organic carbon source or an effective biological agent (Sahayaraj and Namasivayam, 2008).

Soil isolated biocontrol agents with a promising result against banana weevils include entomopathogenic fungi such as *Beauveria bassiana, B. brongniartii* and *Metarhizium anisopliae*; entomopathogenic nematodes like *Steinernema* and *Heterorhabditis* species; and nonpathogenic endophytic fungi like *Fusarium* and *Acromonium* species (Gold *et al.*, 2001; Shukla, 2010). Myrmicinae ants *Tetramorium guineense* Mayr and *Pheidole megacephala* Fabricius though promising, they are limited by the effect of intercropping (Dassou *et al.*, 2015). On the other, the increasing interest to use fungal pathogens to control insect pests has been due to the understanding of fungal and insect ecology, and that biological insecticide can effectively compete with traditional chemical pesticides. Besides that, entomogenous soil fungi are possibly the most adaptable infectious biocontrol agents to consider, as they are self-propagating, grow on wide

host range, infect different insect developmental stages and their density often causes the natural epizootics (Ali-shtayeh *et al.*, 2003).

In this study, three fungal isolates from *Cosmopolites sordidus* cadavers were identified and evaluated along with a renowned *Beauveria bassiana* isolate IMI372439. These were; *Curvularia senegalensis, Fusarium verticillioides,* and *Fusarium oxysporum* species complex (FOSC) that belonged to class Deuteromycete, but of different subclasses. *C. senegalensis* was to Hyphomycetes of order Moniliales, while *F. verticillioides* and FOSC to Coelomycetes, order Sphaeropsidales, and both subclasses have entomopathogenic properties (Kaushal and Singh, 2010; El-Ghany, 2015). However, terms such as deuteromyceta taxon, have been abandoned because of recent phylogenetic-based studies that classify mitosporic (asexual) fungi to their close relatives. For instance *Fusarium verticillioide* (Sacc.) Nirenberg with a facultative or heterotypic synonym *Fusarium moniliforme* J. Sheld, now belongs to class Sordariomycetes, order Hypocreales, family Nectriaceae and genera Fusarium. Its Teleomorph synonyms is Gibberella fujikuroi var. moniliformis (Wineland) Kuhlman or Gibberella moniliformis Wineland (Kvas *et al.*, 2009; Watanabe *et al.*, 2011).

1.6 Objectives and hypotheses

The current study endeavors to contribute substantially to the current IPM strategies against banana weevil. Chemical pesticide use as a single solution may have brought success but this solution is considered unsustainable and expensive in some cases, thus an advocate for a more holistic pest management approach (Lucas, 2011). This is because, no single strategy is effective enough to eradicate the pest, yet chemical pesticides that seem effective have a lot of setbacks with human health and the environment. Therefore, this study aimed to examine eco-friendly strategies that included host resistance, botanical insecticides and the use of entomopathogenic fungi in the reduction of banana weevil rhizome damages.

The specific objectives

1. To examine the role of physiochemical barriers of phenolic origin (lignin and suberin) in the rhizome of "KM5" and "Mbwazirume" banana cultivar against *Cosmopolites sordidus*.

2. To assess the bio-efficacy in the water extracts of clove buds, black pepper fruits, neem seeds and their synthetic analogs against *Cosmopolites sordidus* different developmental stages both in the laboratory and infested plantain field.

3. To test the susceptibility of *C. sordidus* developmental stages to *Curvularia senegalensis, Fusarium verticillioides,* and *Fusarium oxysporum* species complex and their efficacy in reduction of plantain rhizome damage of potted plants.

1.6.1 The working hypotheses

1. The study hypothesizes that the building blocks of the physical barriers, lignin and suberin, are similar in tolerant "KM5" (AAA genome) and susceptible "Mbwazirume" (AAA genome) banana cultivars, however, the reinforcement and modification of the barriers in response to weevil attack differ greatly.

2. Water extracted botanical plant parts of *A. indica* seeds, *P. guineense* fruit, and *S. aromaticum* flower buds*,* and their synthetic analogs; Eugenol, Eugenylacetate, β-Caryophyllene, and N-Isobutylamine are equally effective against *C. sordidus* developmental stages.

3. Fungal isolates *C. senegalensis, F. verticillioides,* and *F. oxysporum* species complex are potential candidates for biocontrol agents towards *C. sordidus* developmental stages with efficacy that compares to the virulence of *Beauveria bassiana.*

1.7 Publication

This doctoral thesis consists of three manuscripts that have been peer-reviewed in academic journals. Article (I) is published in the Annals of Applied Biology and can be accessed at https://doi.org/10.1111/aab.12638, whereas article (II and III) are published in the American Journal of Science and Engineering Research (AJSER) https://iarjournals.com/upload/341122 and https://iarjournals.com/upload/342332.pdf respectively. Each article is presented in a separate chapter.

Article 1

Bakaze, E., Dzomeku, B.M., Wünsche, J.-N., 2020. Banana defence responses to Cosmopolites sordidus feeding and methyl jasmonate application. Ann. Appl. Biol. 1–11. https://doi.org/10.1111/aab.12638

Article 2

Bakaze, E., Kofler, J., Dzomeku, B.M., Wünsche, J., 2020. Natural Compounds with Potential Insecticidal Properties against Banana Weevil *Cosmopolites sordidus*. Am. J. Sci. Eng. Res. 3, 11–22. https://iarjournals.com/upload/341122

Article 3

Bakaze, E., Dzomeku, B.M., Appiah-kubi, Z., Larbi, S., Wünsche, J., 2020a. Fungal Isolates from Banana Weevils (*Cosmopolites sordidus*) Cadaver as a Pest Control Option. Am. J. Sci. Eng. Res. 3, 23–32. https://iarjournals.com/upload/342332.pdf

Chapter 2.

2.0 Banana defense responses to *Cosmopolites sordidus* feeding and methyl jasmonate application

Abstract

Each year 25-75% of banana and plantain yields are lost due to rhizome damages caused by banana weevil (*Cosmopolites sordidus*) in growing regions of Sub-Saharan Africa. However, the specific plant defense response of the rhizome tissue concerning the *C. sordidus* attack is unknown. Consequently, in this study, we evaluated whether plant defense substances in the rhizome are correlated with the degree of larval damage and whether applications of methyl jasmonate (MJ) elicit a greater induction of the plant defense potential against *C. sordidus*. Moreover, we attempted to reveal cellular modifications in response to the root feeding herbivore through histochemical staining. The banana cultivars 'Km5' and 'Mbwazirume' with tolerance and susceptibility to *C. sordidus*, respectively, were used in a pot experiment to evaluate percent rhizome damage, leaf chlorophyll content, total phenolic content (TPC), antioxidant capacity and cell morphology in response to *C. sordidus* attack and/or MJ applications compared to untreated control plants. We found that *C. sordidus* induced rhizome damage was 30% in the susceptible cultivar but less than 5% in the tolerant cultivar. The percent rhizome damage was not related to leaf chlorophyll content but showed a significant negative linear relationship to both TPC and antioxidant capacity. Larvae feeding induced a considerably greater increase of polyphenolic defense compounds in 'Km5' than in 'Mbwazirume'; however, this response was opposite in the MJ treatment, suggesting that the phytohormone induced the susceptible plant to invest more into the synthesis of defense chemicals that in turn lead to reduced *C. sordidus* damage. Tissue staining demonstrated a greater deposition of lignin and suberin in *C. sordidus* challenged rhizome, presumably to seal off healthy tissue with a physical barrier from continued pest attack. It is concluded that MJ induces polyphenolics in susceptible 'Mbwazirume' banana that reduced *C. sordidus* damage.

2.1 Introduction

Banana and plantain, both belonging to the *Musa* genus, are an important staple food crop for the population of tropical and subtropical countries since they are produced all year round. Each year; however, substantial yield losses occur due to *Cosmopolites sordidus* infestation with the

consequences of sturdiness, loss of chlorophyll, reduced bunch size, and toppling of plants (Rukazambuga, et al. 1998; Gold et al., 2005)., While the main commercial cultivars are all susceptible to *C. sordidus* attack, only a few tolerant genotypes, for example, 'M9' and 'M2', have been developed (Kubiriba et al., 2016) and are currently promoted in Sub-Saharan Africa.

The primary site of an infestation is an orifice just below the collar, the junction between pseudostem and rhizome that are created by adult females with the rostrum to lay eggs. After hatching, the larvae, while feeding, bore a network of tunnels inside the rhizome (Gold, et al. 2003; Night et al., 2010; 2011). Wounding of plant tissue, whether by biotic or abiotic means result in a massive re-arrangement of metabolism with the primary objectives of isolating the affected tissue and limiting the extent of further damage. Several studies have evaluated secondary plant metabolism pathways in response to pest or disease damage and mechanical wounding (Bernards and Bastrup-Spohr, 2008; Dixon and Paiva, 1995; Franceschi et al., 2002; Melillo et al., 1992; War et al., 2012). Within this context, newly synthesized or pre-formed phenylpropanoid derived compounds (phenolics) are well-documented in plant defense against herbivory (Johnson and Felton, 2001; Bernards and Bastrup-Spohr, 2008).

Preformed phenolics are widely distributed in plants, even before insect or mammalian herbivory-induced damage. Their roles as a pre-formed defense against herbivory include physical barriers such as wall-bound phenolics, lignins, suberin, and cuticle associated phenolics as well as stored compounds that have a deterring (antifeedant) or direct toxic (insecticidal) effects (Johnson and Felton, 2001; Bernards and Bastrup-Spohr, 2008). Many plant species increase their local chemical or morphological defense levels but also induce systemic resistance in unchallenged plant parts after herbivore attacks (van Dam and Oomen, 2008). Induced defense, therefore, may mean the synthesis of new compounds or structural barrier after herbivore damage has occurred and include proteins and secondary metabolites that can work both locally (near the point of attack) or systemically (throughout the plant).

Of the hallmark features of preformed or induced phenylpropanoids are their anti-oxidative properties (i.e. they are readily oxidized to relatively stable quinone intermediates). But in the context of resistance to herbivores, this translates into a class of compounds that, once oxidized can interfere with proteins in the digestive tract of chewing insects and mammals. This can result in an overall decline in the nutritional quality of plant material and/ or cause oxidative stress that

affects herbivore settling, feeding, oviposition, growth, fecundity and or fertility (Zhang et al., 2004; Barbehenn et al. 2005, 2006).

Plants produce reactive by-products like semiquinone radicals and other reactive oxygen species such as superoxide anion and hydroxyl radicals against feeding herbivores. These radicals become toxic once oxidized not only to herbivores but also to plants if produced in excess (Laxa et al., 2019). Therefore, antioxidants with free radical scavenging properties counteract the likely effect of radicles to plants while allowing sufficient amount to offset insect damage (Verde et al., 2003). Meanwhile, continued herbivory feeding on the plant results in increased production of radicals and reactive species that sequentially increase antioxidants that counteract excess reactive species to prevent plant cell damage (Laxa et al., 2019).

Moreover, literature describing the role of preformed or newly synthesized lignin and suberin in banana plant/herbivore interaction is inadequate. Nevertheless, it is clear that the biosynthesis of phenylalanine, a necessary precursor, is induced by herbivore damage and there is coordinated induction of genes associated with phenylpropanoid metabolism (Bernards and Bastrup-Spohr, 2008). These plant cell-strengthening biopolymers (lignin and suberin) are reportedly deposited in the secondary wall of cells adjacent to wounded sites to induce pest tolerance and hence to prevent further damage (Biggs, 1984; Franke and Schreiber, 2007; Melillo et al., 1992; War, et al. 2012). The plants' wound periderm response isolates the non-wounded healthy tissue and such physical containment depends on the intensity, extent, and duration of the injury (Beckman, 2000). Wound response, according to Howe and Schaller (2008), differs among plant species and organs but follows a similar pattern of accumulation of waterproof substances such as suberin to form a seal. For example, Freeman and Beattie (2008) demonstrated that sclerotic cells of pear fruit (*Pyrus sp)* are impregnated with lignins rendering it difficult to be chewed by insect pests. Moreover, the incorporation of phenylpropanoid metabolites such as hydroxycinnamic acid amides in the cell wall constituted a very early response to wounding in potato tuber against beetles (Howe and Schaller, 2008). In banana, cell wall reinforcement through deposition by lignin and suberin was shown in response to infections with *Fusarium* (De Ascensao and Dubery, 2000) and *Radopholus similis* (Mateille, 1994).

Consequently, the density and spatial distribution of lignin and suberin in the plant tissue may be influenced by the degree of wounding through mechanical injury or pest attack (Hawkins and Boudet, 1996; Yeung, 1999). However, both polymers are forming a physical barrier to feeding

herbivores, while other polyphenolic compounds may affect insect growth and tissue digestibility that prevents further injury (Bernards and Bastrup-Spohr, 2008; War et al., 2012). So, in defining banana/weevil interaction, specific variations in phenylpropanoid reveal the underlying plant defense strategies and an insight into the defense role of phenylpropanoids.

Many different biotic and abiotic factors have been found to induce physical barrier formation, antifeedant and toxic compounds, including insect and pathogen attack, and wounding among others. In the context of plant defense, it is important to determine other signalling agents involved in generating defense response, as this knowledge would provide tools for further understanding of defensive processes. Jasmonate, which is an endogenous plant phytohormone, is a potent elicitor/signalling agent involved in the host defense response (Franceschi et al., 2002). Studies demonstrated that methyl jasmonate treatment can induce defense response not only to pathogens but also insect pests (Franceschi et al., 2002; Melillo et al., 1992; War et al., 2012). And its applications elicit responses similar to those induced by chewing insect herbivores (Engwa, 2018; van Dam and Oomen, 2008). However, whether these response mechanisms occur in banana and plantain rhizomes concerning methyl jasmonate treatment and or *C. sordidus* attacks are unknown. The purpose of this study was to determine if methyl jasmonate could induce the previously characterized cellular defense compounds derived from the phenylpropanoid biosynthesis and physical barriers just as herbivore wounding would do. These phenylpropanoid compounds may include but are not limited to phenolic, and macromolecules lignin and suberin, each composed of various monomeric building blocks (Fleck et al., 2011; Vogt, 2010). Therefore, we examined the effect of exogenous application of methyl jasmonate on the induced defense response of phenolic origin against weevils.

In this study, we hypothesized that: i) Plant resistance against *C. sordidus* is due to a constitutively high level of polyphenolic defense compounds; ii) applications of methyl jasmonate (MJ) would elicit defense phytochemicals in susceptible cultivars similar to that produced by resistant cultivars to reduce *C. sordidus* damages; and iii) elicited cellular modifications in response to *C. sordidus* feeding would be revealed through histochemical staining.

2.2 Materials and methods

2.2.1 Plant material

Tissue culture banana (AAA) plantlets of the sweet dessert type cultivar 'Yangambi' and the starchy cooking type cultivar 'Mbwazirume', an East-African Highland banana, were used for the experiments at the Crops Research Institute (CRI) in Kumasi, Ghana, in 2015. 'Yangambi', also known as 'Km5', is tolerant to *C. sordidus*, while 'Mbwazirume' shows susceptibility (Kiggundu et al., 2007, 2003a, 2003b; Night et al., 2011). Plantlets were grown in 10 L plastic pots, containing sterilized peat, and fertilized with a 5 g mixture of 23 t% N, 10 % P_2O_5, and 10 % water-soluble potash (K_2O) each week for three months before treatment applications. Plants were placed on tables inside an outdoor shade structure with net covers that permitted plant exposure to 60 % of the incident light. All plants were adequately watered at 2-day intervals throughout the experiment. (Ortiz et al., 1995)

2.2.2 Treatments, experimental design, sampling

Ninety-six plants were selected in late December 2014 for uniform growth characteristics with approximately 60 cm height and at least 6 fully expanded, green leaves. All experimental plants were assigned in a randomized complete block design to twelve blocks of two cultivars and four treatments. Within each block, one plant per cultivar was subjected to one of the following treatments: (1) application of 0.01 % methyl jasmonate (MJ), (2) introduction of *C. sordidus* larvae (W), (3) a combination of MJ and W (MJW) and (4) untreated controls (UTC). MJ with 0.1% Tween 20 (Merck, Munich, Germany) was applied to run-off to the whole plant once at the commencement of the experiment, using a low-pressure handheld sprayer (Gloria, Typ 133, Witten, Germany). All other plants in their vegetative growth stage were treated with 0.1 % Tween 20 to ensure that the surfactant had no treatment effect. *C. sordidus* adults were trapped from infested plantations near the CRI, using the method as described by Ogenga-Latigo and Bakyalire (1993). They were maintained during ovulation and hatching of larvae on weekly supplied freshly cut plantain rhizomes inside 10 L plastic buckets that were closed with perforated lids for aeration (Kiggundu, et al. 2003a). Each treatment plant was inoculated with three two-day-old larvae by introducing them into knife-carved notches just below the collar. Measurements and samples of three plants per treatment and cultivar were taken at 1, 4, 7, and 14 days after treatment application, respectively. Rhizome tissue samples were either snap-frozen in liquid nitrogen and stored at -20°C for quantification of TPC and antioxidant capacity or fixed in 4 %

paraformaldehyde, 0.1 M 60 % PO_4 buffer and 1 ml Tween 20 and stored at 4°C for histological analysis.

2.2.3 Rhizome damage

Rhizomes were excavated from the soil and subsequently carefully pared by removing approximately 1.5 mm of the cambium-like meristematic tissue to remove roots and to expose peripheral *C. sordidus* damage. Cross-sections were then cut by hand at about 1 cm below the collar line to expose internal *C. sordidus* feeding tunnels. Rhizome damage was determined by the percentage of external and internal feeding injury (Ogenga-Latigo and Bakyalire, 1993; Gold, et al. 2003). For that, the paired rhizome surface and cross-sectional slices were overlaid with mesh wire (5 mm^2 grid size) to count the total number of grids with and without larvae damage, respectively. The percent total rhizome damage caused by *C. sordidus* was estimated as the sum of external and internal damage at each sampling time in relation to the total number of grids per plant. Plants with rhizome damage below and above 5 % were considered tolerant and susceptible to *C. sordidus*, respectively.

2.2.4 Chlorophyll content

Chlorophyll content of the second fully opened leaf was measured at three points (base, middle and apex) with the chlorophyll meter (SPAD™-502, Minolta, Japan; (Rodriguez and Miller, 2000). Plants with *C. sordidus* infested rhizomes not only display withered, yellow leaves but also impede typically the emergence of new roots, thus reduces water and nutrient uptake and increases the proportion of wind-toppled plants. For each experimental plant, the average indexed chlorophyll content or “greenness” value was correlated with the respective percent rhizome damage.

2.2.5 Total phenolic content and antioxidant capacity

Rhizome samples were lyophilized, weighed, ground to a fine powder in liquid nitrogen using mortar and pestle and stored at -20 °C until analysis. A minimum of 100 mg of powder was dissolved in 20 ml 70 % acetone inside a 100 ml Erlenmeyer flask and overlaid with nitrogen gas for approximately 30 s to prevent oxidative reactions of the sample. The flask was then sealed with parafilm and kept for 2 h in an ice bath on a magnetic mixer. Thereafter, the solution was filtered (Whatman filter paper, grade 42, Merck, Munich, Germany) and concentrated by rotary vacuum evaporation at 30 °C. The liquid residue was diluted with distilled water to a final volume of 10 ml, aliquoted and stored at -80 °C until further analysis.

Folin-Ciocalteu method (Fischer et al., 2011) was used to express TPC in gallic acid equivalent (mg GAE g^{-1} fresh weight-(FW)) and antioxidant capacity in ascorbic acid equivalents (mg AAE g^{-1} fresh weight) by using microplates to carry out an absorbance assay with a UV/VIS spectrometer (iEMS Microplate Reader, Thermo Scientific, USA) at 720 nm wavelength. Stock solutions of gallic acid and ascorbic acid were used to prepare five calibration standards with a concentration range from 7.5 to 65 mg/L and 7.5 to 100 mg/L, respectively. For the assay, microplates with ninety-six wells (Greiner Bio-One, Frickenhausen, Germany) were loaded in triplicates of either 50 µl sample extracts, gallic acid, and ascorbic acid calibration standards or distilled water (blank) and thereafter, 60 µl of 20% (w/v) Folin Ciocalteu reagent was added to each well. The microplates were inserted into an incubator and orbital shaker (iEMS Incubator/Shaker, Thermo Scientific, USA) and after 3 min, 80 µl of 7.5 % (w/v) sodium carbonate was added to each well. Incubation continued for 60 min before the absorbance assay was carried out.

Trolox equivalent antioxidant capacity (TEAC) assay was also used to measure the antioxidant capacity of the sample as compared to the standard, trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid). The oxidation of 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) to radical cations $ABTS^{+}$ is delayed by antioxidatively active compounds, thus the degree of lag-phase is a measure of the antioxidant potential of the sample. The degree of decolorisation (converting ABTS from blue-green to colourless) induced by a compound in the sample is related to that induced by trolox, giving the TEAC value (mg TE g^{-1} fresh weight), and was measured spectrometrically at 734 nm (iEMS Microplate Reader, Thermo Scientific, USA). Stock solutions of trolox were used to prepare five calibration standards with a concentration range from 1.2 to 20 mg/L. The assay commenced with mixing 0.5 mL ABTS/ PO_4-buffer with 100 mL ABAP (2,2’-azobis (2-amidinopropan) dihydrochlorid))/ PO_4-buffer and heating the solution at 60 °C for 15 min under the light exclusion. The blue-green solution was kept on ice until analysis. To measure antioxidant capacity in the sample, microplate with ninety-six wells were loaded in triplicates of either 40 µl sample (diluted 1:10), trolox/ ascorbic acid calibration standard, or distilled water (blank) and then adding 200 µl ABTS/ ABAP solution to each well. After an incubation time of 6 min at 20 °C, the absorbance assay was carried out.

2.2.6 Histochemical analysis

Specimens were dehydrated in a graded ethanol series (six concentrations from 50% to 100%). The ethanol was then gradually substituted with 100 % xylene. Thereafter, the specimens were

infiltrated in 1:1, 1:4 and 0:1 parts of a 100 % xylene/ paraffin (paraplast Plus™; Merck; Munich Germany) mixture, respectively, before they were embedded in paraffin in a plastic mold according to the procedure described by Collins and Goldsmith (1981). Transverse sections (12-14 µm thickness) were cut using a Leica RM 2255 rotary microtome (Leica Biosystems, Wetzlar, Germany). At least four sections were cut from each of the three embedded specimens (replicates) per treatment, cultivar and sampling time. Three sections were used for different stains and one section was not stained.

Sections were attached to microscope glass slides in a water bath (50 °C), dried, deparaffinized with 100 % xylene and a pure xylene/ ethanol mixture at 1:1 ratio and then gradually hydrated through a decreasing alcoholic series (ethanol 95%, 70%, 35%, distilled water). One section was treated with a 3 % (w/v) solution of 3 g phloroglucinol (P, Merck, Munich, Germany) in 100 mL 100 % ethanol, mixed with 37N HCl at a ratio of 2:1, to stain lignin-rich cell structures red-violet within 5-10 min. A second section was treated with a 1 % (w/v) solution of 1 g Sudan black B (Merck, Munich, Germany) in 70 ml 70% ethanol and 30 mL distilled water for 5-10 min to stain suberin rich cell structures brown (Hawkins and Boudet 1996). The use of P-HCl and Sudan black B permitted to selectively quench autofluorescence of lignin and suberin, respectively (Biggs, 1984). Consequently, any residual bluish-white autofluorescence remaining following staining with P-HCl is indicative of suberin or cutin, whereas residual autofluorescence following Sudan black B staining is indicative of lignin. Thus, tissue evaluation in brightfield and fluorescence microscopy allowed the visualization of spatial distribution patterns of suberin and lignin in the same tissue (Hawkins and Boudet, 1996). A third section was treated with 0.1% (w/v) solution of toluidine blue O (TBO, Merck, Munich, Germany) in distilled water for 45 s and 0.05% (w/v) of ruthenium red (Merck, Munich, Germany) in distilled water for 1min to stain lignified tissue blue, while non-lignified tissue red (Retamales and Scharaschkin, 2014).

After washing off excess stain with tap water, sections were gradually dehydrated in the opposite direction of the procedure described above for tissue hydration. The sections were then mounted with a coverslip and mounting media (Eukitt, Merck, Munich, Germany) to the surface of the slide for cellular imaging. The stained sections were evaluated using an Axio Imager fluorescence microscope (Zeiss, Jena, Germany) that was either used in brightfield or in combination with a fluorescence filter set of 395-440 nm excitation and 470 nm emission to impart a light blue autofluorescence to both suberin and lignin. Unstained sections were used to reveal polyphenolic

materials with a bluish-white autofluorescence under UV light using the fluorescence filter combination as stated above. Pictures were taken using a digital camera (AxioVision V40, Zeiss, Jena, Germany).

2.2.7 Statistical analyses

Data analyses were done with mixed model procedures of SAS 9.4 (SAS Institute Inc., Cary, NC, USA) to evaluate the effect of randomized and repeated factors (cultivar, treatment) on percent rhizome damage, chlorophyll content, total phenolic content and antioxidant capacity. The covariance of these parameters was estimated using a restricted maximum likelihood (REML) estimation (Piepho et al., 2004; Hamlett *et al.*, 2012). REML correlated repeated measurements and permitted the analysis of unbalanced data. All data were graphically displayed with SigmaPlot (Systat Software Inc., San Jose, CA, USA).

2.3 Results

2.3.1 Rhizome damage

C. sordidus larvae induced close to 30% rhizome damage in the susceptible cultivar 'Mbwazirume', but tissue injury was nearly halved within the 2-week observation period by one application of MJ (Figure 2.1). In contrast, the tolerant cultivar 'Km5' had less than 5% rhizome damage, irrespective of treatment (Figure 2.1). There were no treatment differences at earlier sampling times (data not shown).

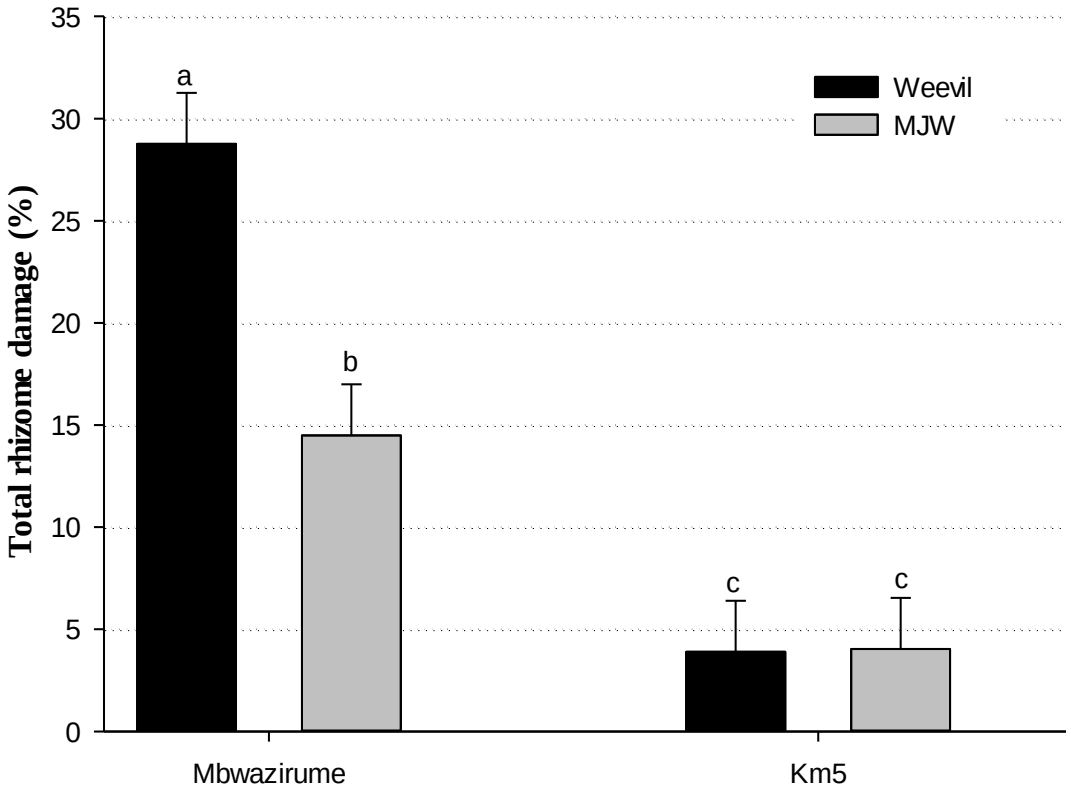

Figure 2.1: Effect of 0.01 % methyl jasmonate (MJ) and/or the introduction of 2-day-old *C. sordidus* larvae (W) on percent total rhizome damage at 14 days after commencement of treatment Vertical bars indicate standard error of the means (n=3) and treatment effects with no significant difference ($p \leq 05$) for a given cultivar are indicated by same letters

2.3.2 Chlorophyll content

The mean chlorophyll SPAD index of untreated 'Km5'was 22% t (64) = 3.68, p = 0.0005 greater than that of untreated 'Mbwazirume'. However, leaf 'greenness' of both cultivars was not

affected by treatment, except that the indexed leaf chlorophyll content of the susceptible cultivar 'Mbwazirume' dropped by 35% t (64) = 11.01, p = 0.0001) throughout the 14-day after *C. sordidus* treatment. Moreover, no relationship was found between leaf chlorophyll content and larvae induced rhizome damage in both cultivars.

2.3.3 Total phenolic and antioxidant content

The TPC, expressed as GAE equivalents, was significantly influenced by cultivar F (1, 46) = 4.81, p = 0.03 treatment F (3, 46) = 6.14, p = 0.001, unlike time of sampling. Across all sampling times, control plants of 'Km5' had 0.91 mg GAE g^{-1} FW, a 40% higher TPC than 'Mbwazirume'. Moreover, the weevil treatment, induced overall 45% more TPC in the rhizome tissue of 'Km5' than that in untreated controls, whereas there was only a 15% increase in TPC for 'Mbwazirume'. In contrast, plants of both cultivars treated with MJ and weevil (MJW) had the highest TPC, which was 2-fold greater for 'Mbwazirume' but only 5% increased for 'Km5' when compared to the weevil larvae feeding treatment. Nonetheless, MJ treatment-induced as high TPC as that of weevil treatment, but above their respective untreated control of either cultivar Figure 2.2

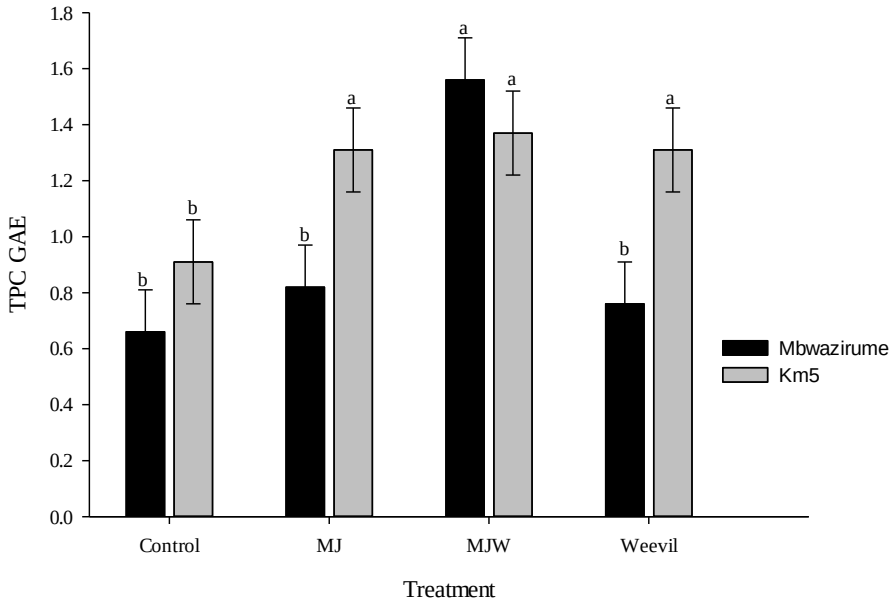

Figure 2.2 Effect of treatment on total phenolic content (TPC); untreated control treatment (UTC) 2-day-old *C sordidus* larvae (W) 0.01 % methyl jasmonate (MJ) and the combination of W and MJ (MJW) on mean the total phenolic content (TPC) expressed as gallic acid equivalent (mg GAE g^{-1} fresh weight) in the rhizome tissue of cultivars 'Mbwazirume' and 'Km5' Vertical bars indicate standard error of the means (n=12) and treatment effects with no significant difference ($p \leq 05$) for a given cultivar are indicated by the same letters.

Antioxidant capacity. In general, although both antioxidant capacity bioassays were not significantly different, AAE tended to be slightly greater than TE (Figure 3). The mean of both bioassays was significantly influenced by cultivar F (1, 44) = 10.53, p = 0.002, treatment F (3, 44) = 9.29, p = 0.0001 and time of sampling F (2, 44) = 3.27, p = p ≤ 0.04. The mean of both bioassays and all sampling times suggests that untreated 'Km5' had with 1.03 mg g^{-1} FW 26% more antioxidant capacity than 'Mbwazirume'. Besides, the 'Km5' *C. sordidus* treatment had 74% more antioxidant capacity than the control treatment, but there was only a 12% increase for 'Mbwazirume'. Plants treated with MJW had irrespective of cultivar, the highest antioxidant capacity, which was 19% greater for 'Km5' but more than 2-fold higher for 'Mbwazirume' when compared to the *C. sordidus* treatment.

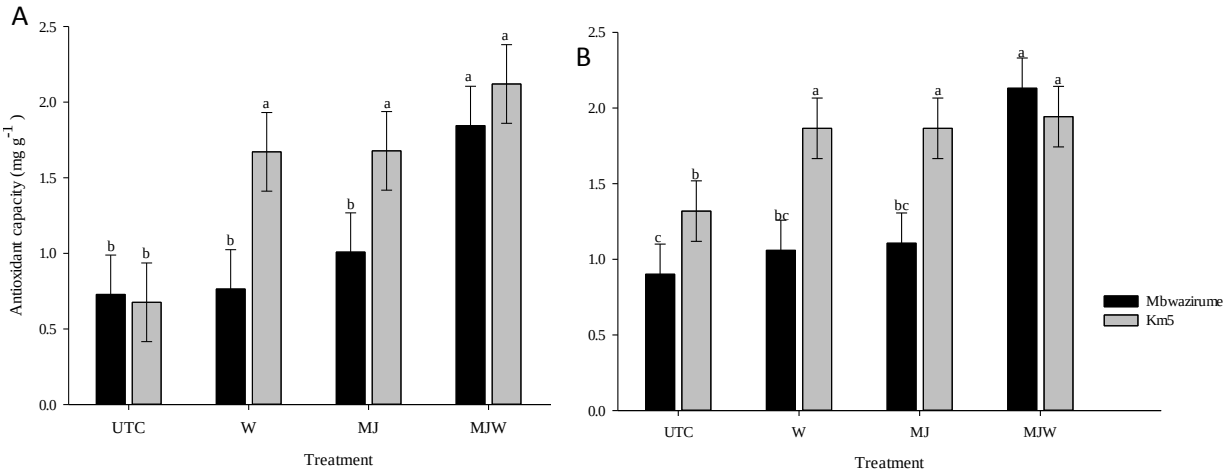

Figure 2.3 Effect of treatment on antioxidant capacity; the untreated control treatment (UTC) 2-day-old *C sordidus* larvae (W) 0.01 % methyl jasmonate (MJ) and the combination of W and MJ (MJW) on mean antioxidant capacity expressed as A) Trolox equivalents (mg TE g^{-1} fresh weight and B) ascorbic acid equivalents (mg AAE g^{-1} fresh weight) across all sampling times respectively in the rhizome tissue of cultivars 'Mbwazirume' and 'Km5' Vertical bars indicate standard error of the means (n=12) and treatment effects with no significant difference (p ≤ 05) for a given cultivar are indicated by the same letters

2.3.4 Histochemical analysis

Both stained control and *C. sordidus* challenged tissue sections, revealed the presence of lignin and suberin substances that varied with cultivar and *C. sordidus* damage levels. Comparatively, *C. sordidus* challenged tissue sections that had higher colour stain intensity than their controls. Cells proximal to *C. sordidus* damaged portions of the cultivar "Km5" and "Mbwazirume" contained more polymers that stained positive to the respective stains than cells distal to the point of

damage (Figure 2.4 to 2.6). P-HCL and Sudan black B stains revealed the presence of both polymers lignin and suberin at the point of damage more evidently than Tol Blue O + Ruthenium Red. The distribution patterns of lignin and suberin revealed under brightfield were also confirmed with residual bluish-white autofluorescence. Cells bordering the *C. sordidus* damaged tissue contained more phenolics than distant cells to the damaged site of either "KM5" or "Mbwazirume".

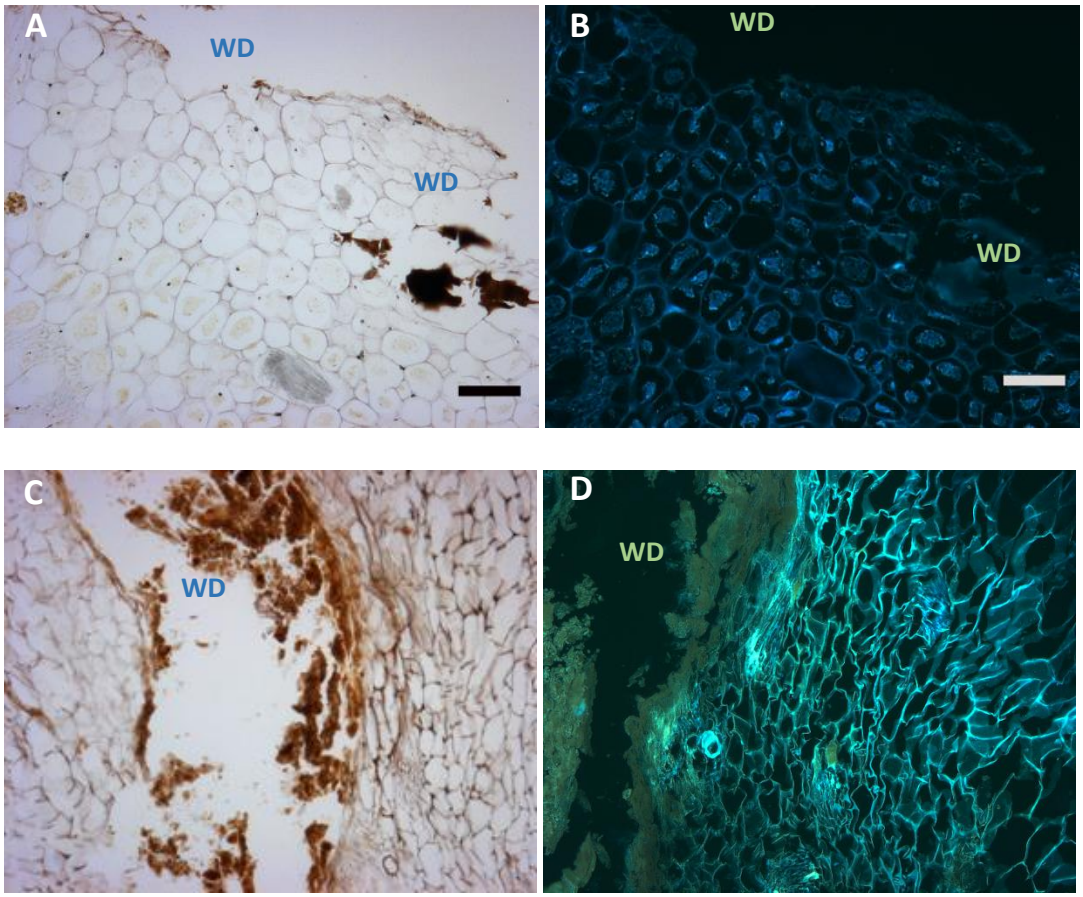

Figure 2.4 The use of Sudan black B stain; Sudan black B permitted tissue evaluation of cultivars "Km5" (A B) and "Mbwazirume" (C D) in brightfield (A C) and fluorescence (B D) microscopy visualizing spatial distribution patterns of stained suberin rich cell structures brown and bluish-white fluorescing lignin in the same tissue WD: *C. sordidus* damage Magnification 100X bar; 100µm

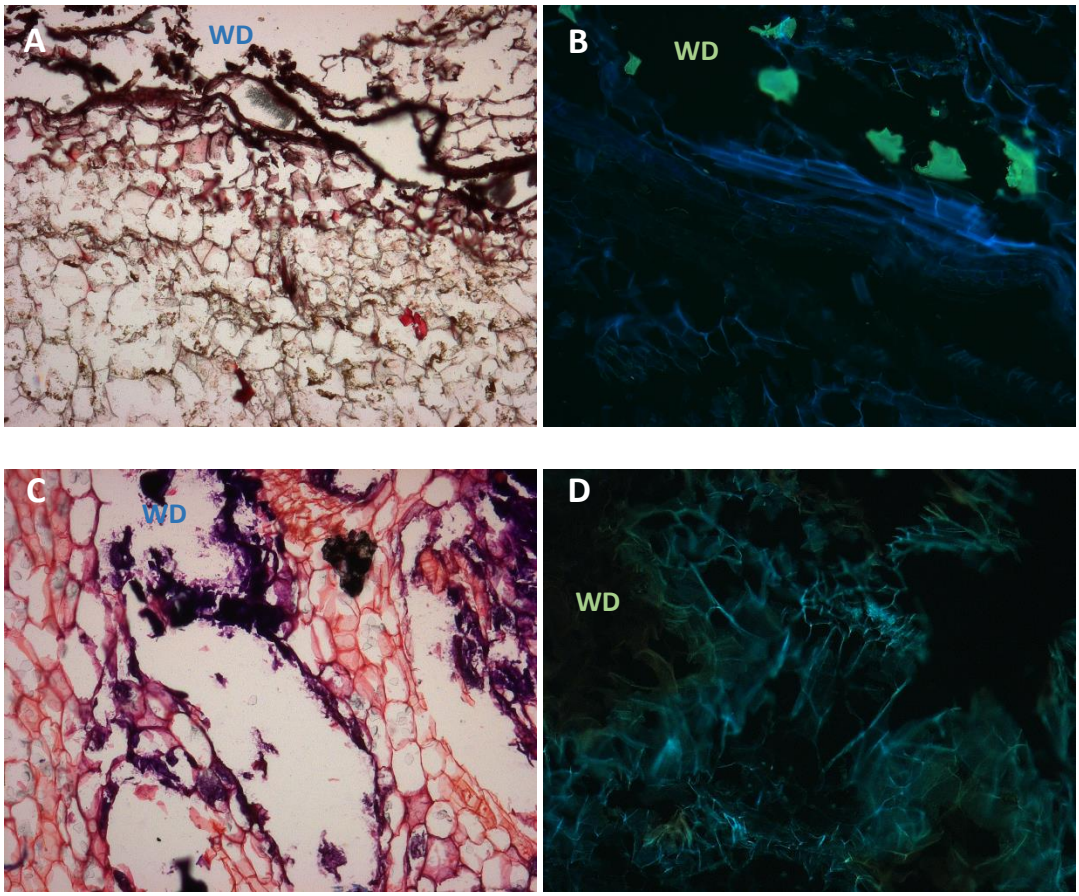

Figure 2.5 The use of P-HCl stain; P-HCl permitted tissue evaluation of cultivars “Km5” (A B) and “Mbwazirume” (C D) in brightfield (A C) and fluorescence (B D) microscopy visualizing spatial distribution patterns of stained lignin-rich cell structures red-violet and bluish-white fluorescing suberin in the same tissue WD: *C. sordidus* damage Magnification 100X

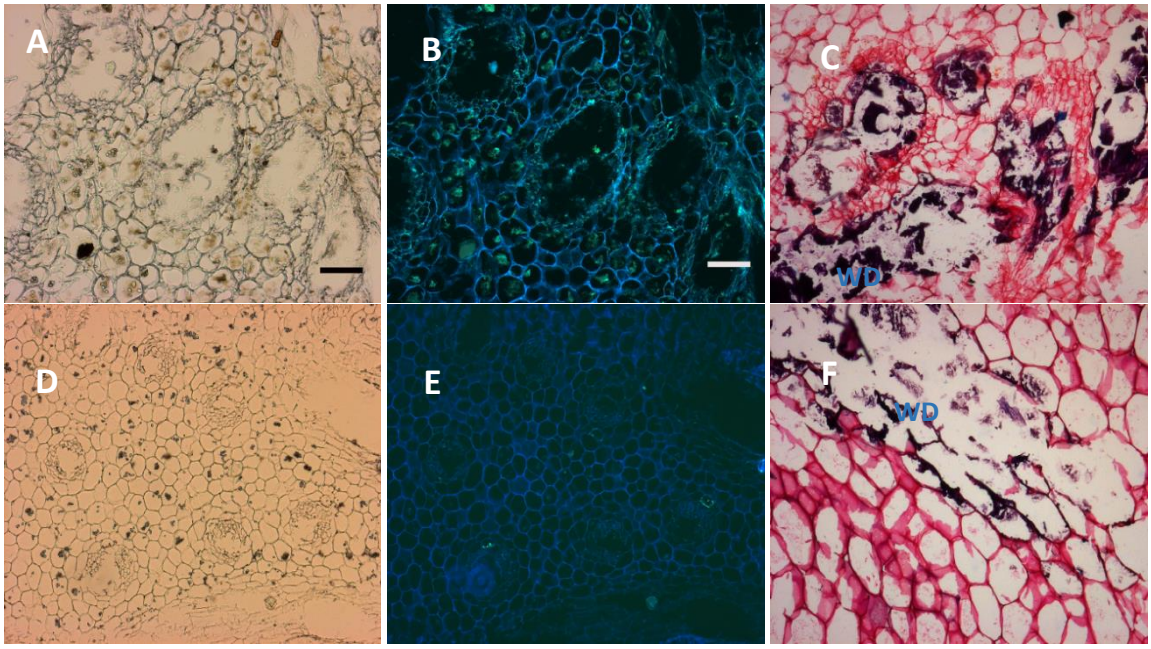

Figure 2.6 Comparison between cultivar 'Km5' (A to C) and 'Mbwazirume' (D to F). Unstained tissue sections of UTC (A and D), polyphenolics fluorescing bluish-white of UTC (B and E) under fluorescent microscopy respectively. The use of Tol Blue O + Ruthonium Red permitted tissue evaluation of the cultivars (C and F) in brightfield visualizing spatial distribution patterns of stained lignin dark blue/ violet and unlignified red in the same tissue. WD: *C. sordidus* damage, Magnification; 100X, Bar = 100µm

2.4 Discussion

2.4.1 Rhizome damage

In both cultivars, banana plants suffered considerable rhizome injury from feeding *C. sordidus* larvae at varying degrees. Such damage variations are a dependant of cultivar resistance `Km5' or susceptibility 'Mbwazirume' (Kiggundu et al., 2007; Night et al., 2011). This emphasizes the need to evaluate alternatives like MJ whose application on crops like *Picea abies* improved its resistance against *Ceratocystis polonica* (Franceschi et al., 2002). In this study, rhizome damage caused by *C. sordidus* generally decreased following the application of MJ particularly in the susceptible cultivar 'Mbwazirume' and to some extent in resistant cultivar 'Km5'. Similarly, the MJ application greatly reduced *Helicoverpa armigera* damage in *Arachis hypogae* (War et al., 2011). Although the application of MJ overall lowered the percent of *C. sordidus* rhizome damage in susceptible

cultivar 'Mbwazirume', a one-time application may be insufficient as *C. sordidus* larvae attain the 4th to 6th most destructive instar stages to pupation (Gold et al., 1999).

Applications of jasmonic acid commonly lead to increased host resistance not only in banana against *C. sordidus* but also in other plant species against numerous insect herbivores (Tran et al., 2017; War et al., 2012, 2011). Reduction in herbivore plant damage as mediated by MJ and its ally like 12-oxo-phytodienoic acid (OPDA) is either direct or indirect. In the direct, MJ enhances flavonoid biosynthesis which results in compounds like indole glucosinolate that is poisonous or deterrent to most herbivores (Tran et al., 2017). Indirectly, MJ induces proteinase inhibitors to inhibit the protease activity of the digestive enzyme in the insect guts which results in stunting and eventual death. And oxidative enzymes like lipoxygenase (LOX) which catalyze the hydroperoxidation of linolenic acid to radicals (hydroxyl radicals) that directly damage insect tissue or act as feeding repellent or indirectly through the octadecanoid pathway to attract natural enemies (War et al., 2012). Therefore, the reduction of rhizome damage in susceptible cultivar as a result of MJ application could partly complement other conventional *C. sordidus* control measures.

2.4.2 Chlorophyll content

Leaf 'greenness' largely depends on nitrogen uptake by the plant (Asai et al., 2009) and consequently, heavy *C. sordidus* damage on the rhizome not only impair root growth but also disrupt nutrient (nitrogen) translocation (Gold et al., 2005). This results in leaf chlorosis with yellowing symptoms that occur on older leaves (Zimmermann, 1968; Plantwise Technical Factsheet 2017). A no correlation between rhizome damage and chlorophyll content loss in respective cultivars may suggest, a short time exposure of banana plants to *C. sordidus* does not translate into nitrogen loss. In fact, Rukazambuga, et al. (1994) reported on the tolerance to *C. sordidus* damage among the vigorously growing banana plants compared to those that are stressed. Nonetheless, because of developed tolerance, Greenbugs feeding on sorghum leaves did not cause chlorophyll loss beyond 7 days (Deol et al., 1997). While SPAD maybe a rapid non-destructive technique in chlorophyll estimation (Deol et al., 1997; Rodriguez and Miller, 2000), attributing its loss to *C. sordidus* feeding may need ample time of exposure.

2.4.3 Total phenolic content and antioxidant capacity

The increased TPC and antioxidant capacity in the rhizome tissue upon *C. sordidus* larvae feeding and application of methyl jasmonate are in agreement with the findings of Franceschi *et al.* (2002). They demonstrated that herbivore wounding activates polyphenolic parenchyma cells through the octadecanoid pathway of jasmonate to produce phenolics. Nicholson and Hammerschmidt (1992) in their review pointed out the rapid accumulation of toxic phenols such as ferulic acid and modification of cell walls by phenolic substituent or physical barrier at the point of attack as the first plant defense strategies which function to restrain the damage and to allow synthesis of specific phytoalexins defense compounds as secondary strategies. Moreover, the phytochemicals phenolics but also flavonoids are known to deter herbivorous insects, depend on their potent antioxidant and free radical scavenging properties or reactive oxygen species generated during pest infestation (Verde et al., 2003; Tatiya et al. 2011; Brahmi et al. 2012; Brahmi et al. 2016). This, however, requires more time than is available for the rapid appearance of compounds that constitute primary defense response.

Although, there was a significant negative linear relationship between the percent rhizome damage and TPC F (1, 37) = 6.57, p = 0.01 as well as antioxidant capacity F (1, 37) = 8.93, p = 0.005, control plants had comparatively low TPC and antioxidant capacity. This observation agrees to the plant defense theory that “inducible resistance is developed to reduce the costs of constitutive defense expression” (Elle and Hare, 2000; Hare and Elle, 2002). Moreover, Valladares et al. (2007) demonstrated that ecological and evolutionary variability of induced defense-related phenolics depends on the balance between advantageous and disadvantageous consequences of such defense.

Plant phenolics and antioxidant capacity vary with plant genetics, developmental stage, growing conditions, soil factor, etc. (Saravanan and Aradhya, 2011). The tolerant cultivar ‘Km5’ had constitutively a higher TPC and antioxidant capacity in the rhizome tissue than the susceptible cultivar ‘Mbwazirume’. Larvae feeding induced a considerably greater increase of plant phytochemicals in ‘Km5’ than in ‘Mbwazirume’, a response that is likely related to the genetic make-up and specific adaptive responses of the two cultivars against *C. sordidus*. In contrast, the MJ treatment induced a higher TPC and antioxidant capacity in ‘Mbwazirume’ rhizome tissue than its UTC within two weeks of MJ application. This suggests that the phytohormone was more beneficial to the susceptible cultivar by inducing the plant to produce different types of defense

chemicals (Engwa, 2018; van Dam and Oomen, 2008), which in turn led to reduced *C. sordidus* damages. The limited effect of MJ on 'Km5' plant antioxidant capacity as compared to its UTC, is presumably due to sufficiently occurring constitutive resistance mechanisms. This view is in line with what Dixon (2001) reported, that where constitutive defense metabolites are produced in large amounts after infection, its status as phytoalexin (induced) depends on whether or not the constitutive concentrations were sufficient. Consequently, the endogenous constitutive level of plant defense compounds combined with exogenous applications of phytochemicals may play a role in the prevention and management of oxidative stress-related pest injuries such as those caused by *C. sordidus*.

2.4.4 Histological analysis

Fluorescence microscopy and or histochemistry are the common ways of identifying cellular modification in response to damage or infection. Plant cells are able to seal the immediate site of wounding with phenolic polymers to deter further herbivore feeding (Beckman 2000). In this study, there was a reduction of *C. sordidus* damage on susceptible banana cultivar attributed to the formation of the physical barrier of polymers, the lignin and suberin. The accumulation of toxic phenolics previously mentioned is not only a general rapid response to herbivore attack but also facilitates the biosynthesis of lignin, suberin, and other wound-induced polyphenolic barriers for structural reinforcement (Dixon and Paiva, 1995; Grace, 2005). The presence of such polymers according to Nicholson and Hammerschmidt (1992) occurs as a result of crosslinking phenylpropanoid esters to the cell walls of damaged plant tissues. Such hydroxycinnamic acid and their derivatives are thought to contribute to the autofluorescence of host tissues at the site of infection (Nicholson and Hammerschmidt, 1992). Similar defense mechanisms were found in the present study through histochemical staining of tissue with P-HCl and Sudan black B and fluorescence analysis which demonstrated prominent deposits of lignin and suberin at the site of damaged rhizome tissues, confirming a host plant resistant strategy against *C. sordidus*. This response could be viewed as a barrier or containment of *C. sordidus* feeding damage. Other studies (Biggs, 1984; Rittinger et al., 1987; Franke and Schreiber, 2007; Melillo et al., 1992; War, et al. 2012) report similar deposit of lignin and suberin which suggest their role in the establishment of barrier zones and impervious tissue to contain either herbivore wounding or pathogen infection among different crops. Nonetheless, the involvement of phenolic compounds

is not limited to *C. sordidus* a herbivore but was also shown for banana crown rot (Ewané et al., 2012) which suggests it as an antibiotic host plant defense strategy.

2.4.5 Conclusion

Pre-formed phenolics in both cultivars and their elevated accumulation (induction) upon *C. sordidus* feeding and or MJ application along with reduced damage in susceptible cultivar, confirms its passive and active defense role in plants against herbivores. Constitutive and timely induced host defense work together to prevent further damage caused by *C. sordidus* larvae feeding in both susceptible 'Mbwazirume' and tolerant 'Km5' cultivars. Applications of phytochemicals like MJ, therefore, will elicit the synthesis of host defense compounds in susceptible cultivar to overcome given biotic stress, a strategy that can be adopted in *C. sordidus* management after field testing.

In this study, we were able to ascertain that both constitutive and induced host defense play important roles in plant protection against *C. sordidus* in resistant and susceptible cultivar respectively. And *C. sordidus* damages do not significantly affect the chlorophyll content in the limited time of exposure. Integrated use of bright field and fluorescence microscopy in conjunction with histochemical staining technique, lignin, and suberin polymers were detected in tissue proximal to *C. sordidus* damaged points, which confirms their structural role in restricting further tissue damage.

Inducing host defense, therefore could have great potential in the management of *C. sordidus* given that their hidden feeding behaviour undermines conventional pesticides. In the nutshell, this study has provided the baseline data on the use of MJ to induce host polyphenolic defense compounds against *C. sordidus* among susceptible banana cultivar 'Mbwazirume'. However further work is needed to test MJ biological activity/ potential on-field plants, evaluate specific phenolic compounds against the pest and quantify the sufficient level needed to offer plant protection.

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Chapter 3.

3.0 Natural Compounds with Potential Insecticidal Properties against Banana Weevil *Cosmopolites sordidus*

Abstract

The banana weevil (*Cosmopolites sordidus* Germar) causes 30-70% annual yield losses to Musa species in tropical and subtropical regions. Effective control strategies against *C. sordidus* of low cost, readily available, easy to apply, and safe for homestead growers are sought. The objective was therefore to evaluate natural compounds for potential insecticidal properties against *C. sordidus*. Bio-efficacy of extracts from cloves (*Syzygium aromaticum*), black pepper (*Piper guineense*), neem (*Azadirachta indica*) and their synthetic analogs along with Carbofuran were evaluated as botanical control option against *C. sordidus*. They were assessed for egg hatch inhibitory effect, larvicidal toxicity and adult repellency in both laboratory and infested field experiments. Results showed that eggs and larvae of *C. sordidus* were most susceptible to clove extract and its synthetic analogs. Egg hatchability was inhibited to 93% by clove extract whereas to > 50% by its synthetic analogs at 0.5% concentration. Larvae mortality dependent on instar stage but at instar stage 3 it was about 80% for clove extracts and above 60% for all other treatments at 0.5% concentration, respectively. The percentage of adult weevils repelled for between 6 to 48 hours ranged from 80 - 98% with black pepper, 78 - 90% for clove and 63 - 75% for neem. In conclusion, all evaluated extracts and synthetic analogs inhibited egg hatching, caused larvae mortality and repelled adult weevil to an extent that is sufficient to reduce markedly weevil damage and to serve as an alternative to synthetic pesticides, Carbofuran.

3.1 Introduction

Integrated pest management (IPM) has been advocated for since the early 1970s (Knipling, 1972) to ensure that pest populations are suppressed below an economic relevant injury level by following a broad-based approach. It emphasizes the growth of healthy, high quality crops with the least possible disruption to agro-ecosystem and encourages sustainable natural pest control mechanisms. Despite the introduction of regular monitoring practices and mechanical and biological control measures that led to a responsible and targeted use of insecticides, fungicides and acaricides, synthetic pesticides have come under significant scrutiny in the last decade by current or impending legislation being implemented in many countries and due to breaking down

of crop resistance to pathogens and pests. Those impediments have spurred the search for crop management strategies that require lower chemical input and make use of effective non-synthetic control options in all food crops (Buss & Brown, 2014; Murray, 2007; Zeng et al., 2010). With a particular focus on small farmers in developing countries, Altieri (1993) has described key elements in the design of sustainable pest management systems.

The banana weevil (*Cosmopolites sordidus Germar*) causes extensive damage to *Musa* species, resulting in 30-70% annual yield losses (Gold et al., 2003; Njau et al., 2011; Speijer et al., 2001) in banana and plantain growing regions of East and West Africa. It is estimated that 90% of the *Musa* production is carried out on small subsistence-oriented family farms (Gold et al., 2001). Therefore, effective pest control strategies against *C. sordidus* should be of low cost, readily available, easy to apply, and safe for homestead growers. IPM strategies have been suggested by Gold et al. (2001) to target successfully different life stages of *C. sordidus*; however, weevil resistance to insecticides like dieldrin (Edge et al., 1975) is widely manifested. Consequently, crop protection strategies based on plant derivatives to control injurious pests have been recognized as a valuable tool (Scott et al., 2008).

The objective of this study was therefore to evaluate natural compounds for potential insecticidal properties against *C. sordidus*. Three plant extracts have been selected that have been previously shown to control various pests; however, they have not yet been evaluated as a biological control option against *C. sordidus*. The first pest control agent, neem (*Azadirachta indica*) and other Meliaceae species have been studied for pesticidal properties on different agricultural pests (Mulla & Su, 1999). It was, for example, shown that neem derivatives were effectively reducing the egg hatchability of Okra fruit borer (Thara et al., 2009) and had larvicidal and ovideterrent properties against *Aedes albopictus*, also known as the Asian tiger mosquito (Benelli et al., 2015). The second pest control agent, pepper (Piper) extracts and in particular various secondary compounds such as amides (N-Isobutylamine), have shown promising results for controlling various crop pests (Scott et al., 2007). Scott et al. (2008) reviewed the insecticidal activity of piperamides and concluded that piper extracts offer a unique and useful source of biopesticide in combination with other botanical insecticides such as pyrethrum. Earlier results of Scott et al. (2003) suggest that Piper extracts from two Piperaceae species, *Piper nigrum* L. and *P. tuberculatum* Jacq. could be used effectively as contact insect control agents to protect potato plants from developing *L. decemlineata* larvae at concentrations less than 0.1%. Moreover, *Piper*

nigrum extract might be useful for the control of sawflies and tent caterpillars, two common Canadian forest pest insects (Scott et al., 2007). The third pest control agent, clove (*Syzygium aromaticum*) and other species of the Myrtaceae family have been traditionally used as insecticides against many plant pests and pathogens (Murray, 2000). Main oil compounds from the clove have some potential insecticidal activity against several grain storage pests (Zeng et al., 2010). Moreover, the bioactivity of eugenol, natural oil of clove, was evaluated against four Coleoptera species (Obeng-Ofori and Reichmuth, 1997). Eugenol was highly repellent (80-100%) to all four beetle species tested and its effectiveness in terms of beetle mortality was dosage-dependent and reduced with increased length of grain storage after application. Powdered seeds of clove and *P. guineense* elicited 60-80% repellence of *C. sordidus* adults in laboratory studies (Inyang and Emosairue, 2005).

In this study, the hypothesis was tested that plant derivatives from *A. indica*, *P. guineense* and *S. aromaticum* as well as the synthetic analogs eugenol, eugenyl acetate, β-Caryophyllene, and N-Isobutylamine possess effective insecticidal properties against *C. sordidus*, thus can be used as biological control options.

3.2 Materials and Methods

3.2.1 Study site

Field experiments were conducted at the Council for Scientific and Industrial Research (CSIR), Crops Research Institute (CRI) in Kumasi (latitude 6° 41' 0" North, longitude 1° 37' 0" West), Ghana, between August and December 2015. The area is within the semi-deciduous forest region of Ghana, characterized by prevailing hot and dry air masses from the Sahara during the dry season (December to February) and a bimodal rainfall season with up to 1300 mm precipitation and tropical, south-westerlies from the southern Atlantic Ocean between March and November (Oppong-Anane 2006). The annual mean temperature is 27°C (Oppong-Anane, 2006).

Besides, laboratory experiments were carried out at the National Agricultural Research Laboratories (NARL), Kawanda, Uganda (latitude 0° 24' 30" North, longitude 32° 32' 9" East) between September and December 2016.

3.2.2 Substances with insecticidal properties

For identifying natural compounds with potential insecticidal properties against *C. sordidus*, the following plant species were selected: seeds from neem (*A.zadirachta indica*), fruit from Ashanti pepper (*Piper guineense*) and flower buds from clove (*Syzygium aromaticum*). Pepper and cloves were bought from local markets, whereas neem seeds were collected from farmers' backyards in Kumasi. These materials were sundried and milled to a fine powder using a locally made motorized mill. Extracts were prepared according to the method described by Musabyimana et al. (2001). Percentage concentration of crude extracts (w/v) at 0.2, 0.4, 0.6 and 0.8 % were prepared by soaking 20, 40, 60 and 80 g of the powder in 1 L of distilled water, respectively, for 24h and filtering the solution through a muslin cloth.

Various compounds of these plant species with reported insecticidal properties were included as synthetic analogs in the study: eugenol ($C_{10}H_{12}O_2$), eugenyl acetate ($C_{12}H_{14}O_3$) and β-caryophyllene ($C_{15}H_{24}$) from clove (Nazrul et al., 2010; Razafimamonjison et al., 2013) and N-isobutylamine ($C_4H_{11}N$) from pepper (Parmar et al., 1997; PARK et al., 2002; Scott et al., 2008). These chemicals (Merck; Munich, Germany) were used at 98% purity. Although eugenol occurs in both clove buds and pepper, it is more abundant in *S. aromaticum* (Meghwal & Goswami, 2013; Scott et al., 2008).

3.2.3 Collection and maintenance of weevils.

Adult weevils were trapped from plantations of the CRI and maintained in the laboratory in agreement with protocols described by Ogenga-Latigo and Bakyalire (1993) and Night et al. (2010). Weevils were kept on weekly supplied fresh plantain rhizomes inside 10 L plastic buckets, closed with perforated lids for aeration, and maintained at room temperature. This procedure ensured an adequate supply of eggs, larvae and adult weevils for the experiments.

3.2.4 *Egg inhibition assays*.

Two experiments, each repeated twice, were conducted to evaluate the potential efficacy of plant extracts and some of their specific synthetic analogs on the inhibitory effect of *C. sordidus* egg hatching. For the first experiment at the CRI in Ghana, extracts of clove buds, neem seed and pepper fruit at 0 (control), 0.2, 0.4, 0.6 and 0.8 % (w/v) were evaluated using 26 brown spotted eggs (4-days-old) for each extract and concentration, respectively. The second experiment at NARL in Uganda constituted of nine treatments, each applied at concentrations of 0, 0.1, 0.25,

0.5% (w/v): eugenol, β-caryophyllene, eugenyl acetate, N-isobutylamine, a combination of the clove synthetic analogs eugenol, β-caryophyllene and eugenyl acetate, a combination of the pepper synthetic analogs eugenol and N-isobutylamine as well as clove buds, pepper fruit and a mixture of clove and pepper. Each treatment at a given concentration was tested on 32 brown spotted eggs. Eggs were first soaked in a respective solution for 20 min and thereafter placed on moistened filter paper to be incubated at ambient temperature. The percentage of inhibited eggs (PR) per treatment concentration was calculated following the equation of Zeng et al. (2010) after 5 days of incubation:

where NC is the total number of hatched eggs in the control treatment and NT is the total number of hatched eggs in the treatment.

$$PR = 100 \times \left[\frac{NC - NT}{NC + NT} \right]$$

3.2.5 Larval toxicity assays

In the experiment at the CRI, extracts of clove buds, neem seeds and pepper fruit at 0.8% (w/v) were each tested twice for insecticidal effects on 20 *C. sordidus* larvae at instar stage 1 to 5, respectively. The experiment at NARL in Uganda included the same nine treatments as in the egg inhibition assays, each applied at concentrations of 0, 0.1, 0.25, 0.5% (w/v). Each treatment at each concentration was tested on 20 larvae of instar stage three (5-day-old).

Larvae in each treatment received a diet that was based on published recipes and approximated growth requirements (Shimoji and Yamagishi, 2004). The diet was a composite of different ingredients (Table 3.1), including banana rhizomes, used from weevil susceptible maiden sucker, that were sliced, solar dried and milled with a cutting mill (Fritsch, Pulverisette 15, Fritsch, Idar-Oberstein, Germany) to a fine powder. The diet was autoclaved at 121°C and 1034.21 hPa for 15 min before antibiotics (Table 3.1) was added at 55°C before dispensing. For the bioassay, 0.2 ml of each treatment concentration and sterile water for the control were randomly pipetted into 24 well plates (BD Bioscience, MA, USA). Thereafter, an approximately 1.8 ml diet at 55°C was added to each well and gently mixed before setting. Plates were allowed to cool overnight when two larvae were introduced per well. Ten wells were used for each treatment concentration and instar stage. The plates were incubated for 8 days at ambient temperature in the darkroom and

the experiments were replicated two times. Percentage mortality was calculated as per Zeng et al. (2010). The larvae were recorded dead if its body was not moving when mechanically prodded.

Table 3.1 Composition of *C. sordidus* artificial

Ingredients	**Quantity (g)**
Agar	40.0
Banana rhizome powder	80.0
Dextrose	40.0
Yeast extract	9.0
Cellulose	14.4
Casein	21.6
Vitamin B	0.045
Salt mixture	2.7
Ascorbic acid	1.8
Chlorine chloride	0.45
Methyl 4-hydroxybenzoate (Nipagine)	0.67
Inositol	0.36
Stigmasterol	0.72
Potassium sorbate	0.67
Tetracycline	0.2
Ethanol	10.0[1]
Distilled water	1000.0[1]

[1] Milliliters, adopted from (Bakaze et al., 2018)

3.2.6 Weevil repellent assay

Adult weevil repellent assays were conducted at NARL with 15 unsexed adult weevils for each extract (clove buds, neem seeds and pepper fruits), each at a concentration of 0.8% (w/v), and the substances eugenol, β-caryophyllene, eugenyl acetate and N-isobutylamine, each applied at 0.5% (w/v). A cup bioassay technique was used as described by Kumar et al. (2004). Freshly pared rhizomes from plantain sucker were soaked in 1 L of each treatment solution or water (control) for 30 min, respectively. Treated rhizome pieces were each placed in plastic buckets (37 cm width, 16 cm height), perforated with 8 holes (each of 5-6 mm diameter) at mid-position and equally distributed around the circumference.

Weevils were starved for 12 h before released into each bucket, which was subsequently closed with a lid. Each bucket was placed into a larger plastic bucket and covered with a perforated lid for aeration. A weevil was considered repelled by the treatment if found in the outer bucket after 6, 12, 24 and 48 h for extracts, and 0.5, 3, 6, 12 and 24 h for synthetic analogs after release.

Repellence was measured as the percentage number of adult weevils repelled out of the bucket at each observation time. The experiment was repeated twice at ambient room temperature.

3.2.7 Field evaluation

Field evaluations were conducted twice in a weevil-infested mature plantation in Ghana. Forty plantain plants of cv. Apantu, spaced apart at 3 m, were selected and assigned in a randomized complete block design to five replicated blocks of eight treatments. Twenty-six border plants surrounded the experimental field. Weevils were trapped for one week using a method described by Ogenga-Latigo and Bakyalire (1993) to estimate the field weevil population. Ten unsexed weevils were marked with pedicure polish (Drahokoupilová and Tuf, 2012) per replicate, using treatment specific colour labels, and released to each plantain mat a day before the first of three treatment applications.

Within each block, one plantain mat was subjected to one of the following treatments at three-week intervals: extracts (0.8% w/v) and powder (80 g) of clove, neem and pepper, respectively, Carbofuran (60 g) and water control. The selected rate of Carbofuran is commonly used by farmers to control *C. sordidus* (Gold et al., 1999; Musabyimana et al., 2000). Treatments were incorporated into soils around the respective plant mats. At the end of each treatment interval, the weevil population for each mat and treatment was monitored using the Lincoln-Peterson Index of population size (capture-mark-recapture) method (Bellemain, Swenson, Tallmon, Brunberg, & Taberlet, 2005). The number of marked and unmarked weevils were recorded for each plant mat, with unmarked weevils being marked treatment specific and marked weevils that crossed to different treatments placed back to their respective treatment mats.

3.2.8 Data analyses

Data were analysed using a generalized linear mixed model (GLIMMIX) procedure of SAS 9.4 (SAS Institute Inc., Cary, NC, USA) to evaluate for treatment effects on response variables such as percentage egg hatching inhibition, larvicidal toxicity, adult repellency and weevil population reduction. The GLIMMIX procedure assumed equal variance and was specified with a binomial distribution and logit link function for the dependent variables (Piepho W et al., 2006; Kiernan et al., 2011). Data were graphically displayed with SigmaPlot (Systat Software Inc., San Jose, CA, USA).

3.3 Results and discussion

3.3.1 Egg inhibition assay

The inhibitory effect of botanical extracts on weevil egg hatching is presented in Table 3.2. In general, the percentage of egg inhibition increased with increasing extract concentration. Among the treatments, egg hatchability was suppressed by 86-93 % for clove, 41-52 % for neem and 39-42 % for pepper. Clove and pepper extract repressed egg hatching equal or even better than their synthetic analogs (Figure 3.1). However, clove extracts and eugenol had the most potential to inhibit egg hatching, particularly at the higher concentrations, indicating that eugenol is a key ingredient for repressing *C. sordidus* egg hatchability. In contrast, neem extracts are versatile to a wide range of insect species and target different developmental stages. While, for example, neem extracts repressed 52 % weevil egg hatchability in the current study, 79 % of Okra fruit borer eggs did not hatch when exposed to neem oil (Thara et al., 2009).

Table 3.2 Treatment effects on the mean percentage of inhibited hatching of *C. sordidus***.**

Species	**Extract concentration (%)**					**Statistics**	
	0	0.2	0.4	0.6	0.8	*F-Value*	$LSD_{0.05}$
Clove	6	86	86	93	93	267.02	9.0
Neem	6	41	46	52	47	8.72	24.5
Pepper	6	39	42	39	42	60.36	7.8
F-Value		95.18	79.13	57.32	33.79	10.30	
P-Value		0.010	0.012	0.017	0.029		
$LSD_{0.05}$		16.8	16.8	22.4	29.6		11.6

Each treatment was repeated twice, each with n = 26. A least significant difference ($P \leq 0.05$).

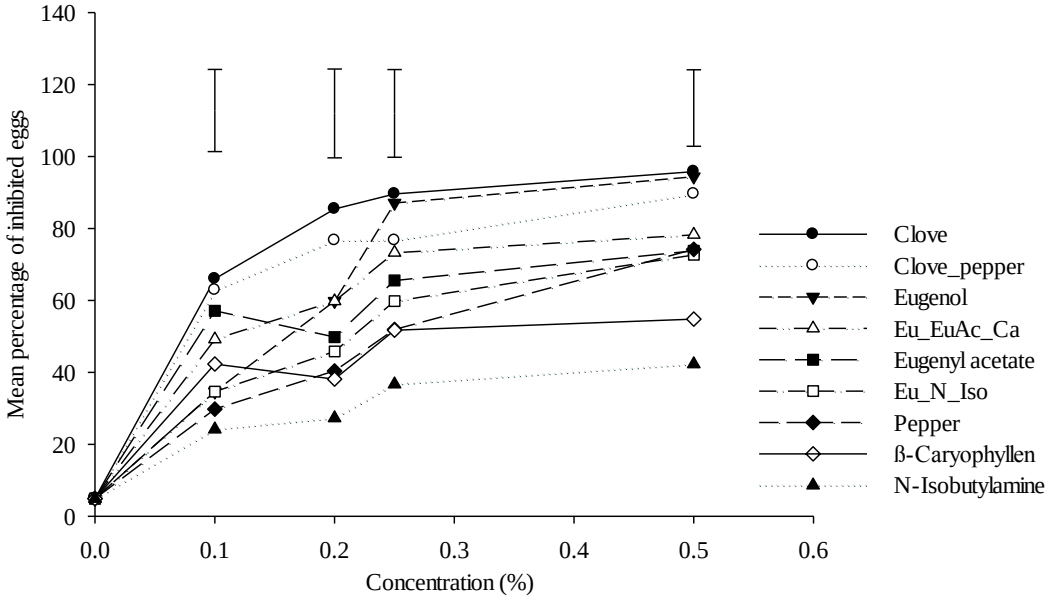

Figure 3.1 Treatment effects on the mean percentage of inhibited hatching of *C. sordidus* eggs. Each point is the mean of two experimental repeats, each with n = 32. Eu_EuAc_Ca is a mixture of clove synthetic analogs eugenol, eugenyl acetate and β-caryophyllene; Eu_N-iso is a mixture of pepper synthetic analogs eugenol and N-isobutylamine. $LSD_{0.05}$ for each concentration is represented with a vertical bar.

3.3.2 Larvae toxicity assay

All extracts at 0.8% concentration were significantly lethal to all instar larval stages (Figure 3.2) when compared to the untreated control where larvae mortality did not occur. The effectivity of the evaluated control measures against *C. sordidus* depended on the developmental stage and treatment concentration. This is in agreement with reports on age-dependent susceptibility to plant extracts (Thara et al., 2009) and dose-dependent insecticidal effects of eugenol against pests like ants, American and German cockroaches (Enan, 2001). Specifically, larvae mortality across instar stages was between 78-100 % for clove, 13-85 % for pepper and 18-74 % for neem. Musabyimana et al., (2001) reported 40 to 60 % *C. sordidus* larvae mortality due to neem extract, a range that is comparable to 19-74% instar larvae dependent mortality reported in this study. While clove extracts tended to increase larvae mortality with larvae development, the toxicity of pepper and neem extracts decreased with increasing instar larvae stages. Since neem extracts induced only less than 50% larvae mortality, except instar stage 1, its synthetic analogs were not considered for further evaluation.

The effect of clove and pepper and their synthetic analogs at varying concentrations on instar larvae stage 3 is shown in Figure 3.3. All treatments followed a similar pattern with larvae mortality steadily increasing at higher concentrations. Larvae exposure to 0.5 % induced around 80 % larvae mortality in all treatments, except for pepper extract and its constituent N-Isobutylamine with a larvicidal effect of only 60-65 %. Treatment mixtures of synthetic analogs had no synergetic effect on larvae mortality when compared to single product assays. N-Isobutylamine may be antagonistic to eugenol since the observed eugenol toxicity in a separate assay was not effective when blended with N-Isobutylamine (data not shown).

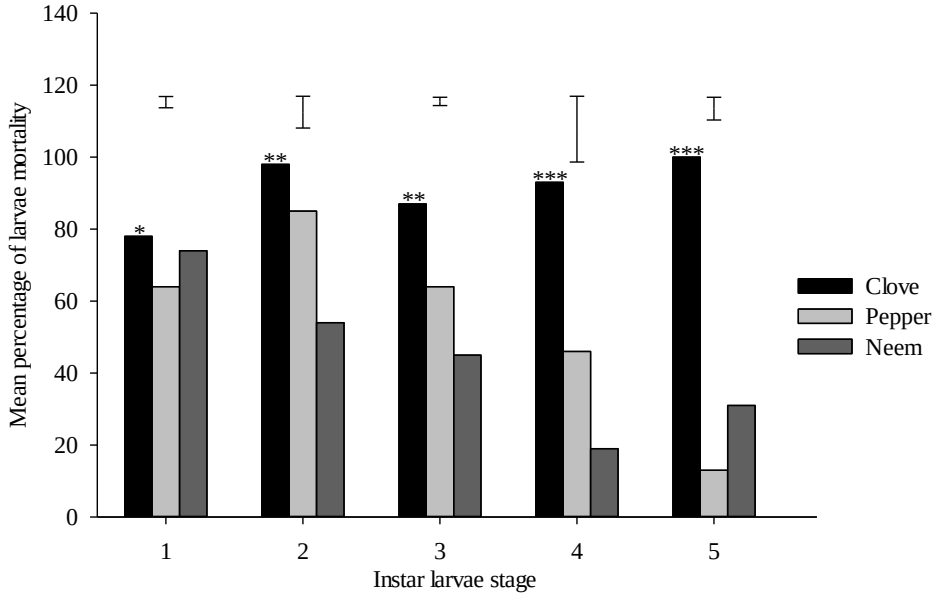

Figure 3.2 Treatment effects (0.8 % extract) on the mean percentage of *C. sordidus* larvae mortality at instar larvae stages 1 to 5. Each bar is the mean of two experimental repeats, each with n = 20. Vertical bars indicate $LSD_{0.05}$ with ***, **, * referring to significance at *P* value ≤ 0.001 and ≤ 0.01, ≤ 0.05, respectively, for each instar larvae stage.

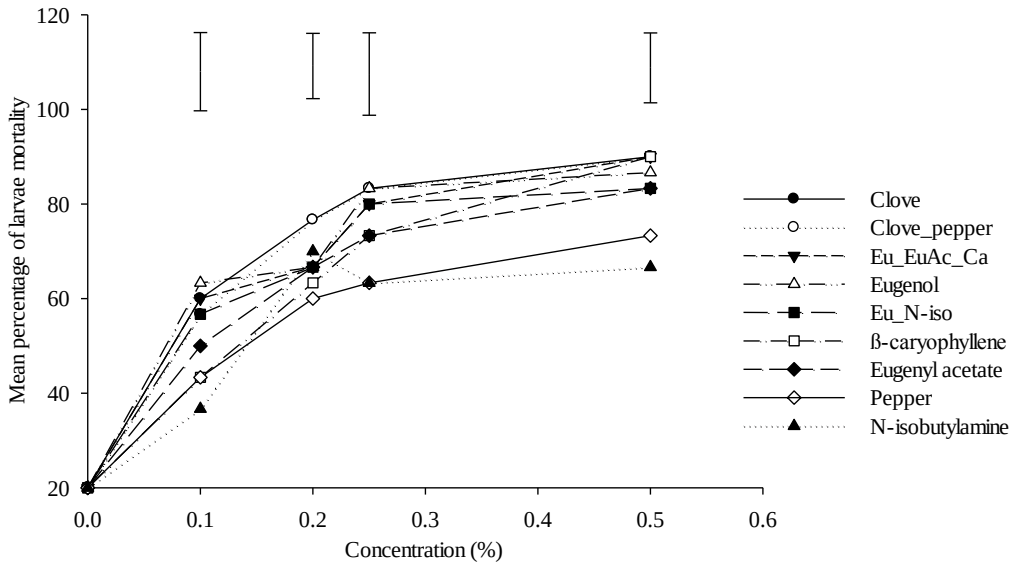

Figure 3.3 The effect of varying treatment concentrations on the mean percentage of *C. sordidu*s larvae mortality at instar larvae stages 3. Each point represents a mean of two experimental repeats, each with n = 20. Eu_EuAc_Ca is a mixture of clove synthetic analogs eugenol, eugenyl acetate and β-caryophyllene; Eu_N-iso is a mixture of pepper synthetic analogs eugenol and N-isobutylamine. $LSD_{0.05}$ for each concentration is represented with a vertical bar.

The observed 100 % mortality at instar larvae stage 5 before pupation might have been a result of increased dietary intake of clove bioactive compounds with insecticidal properties that inhibit the gut proteinases serine or cysteine in phytophagous insects (Macedo & Freire, 2011). Through such mode of action clove metabolites such as eugenol, eugenyl acetate and Caryophyllene (Razafimamonjison et al., 2013) are likely responsible for restraining *C. sordidus* performance. Similar observations were reported for grain storage pest (Sitophilus zeamais and Tribolium castaneum) (Obeng-Ofori & Reichmuth, 1997; Yan, Shuit-Hung, Hsien-Chieh, & Yen-Ling, 2002) and Culex mosquito (Culex pipiens) (Chaieb et al., 2007) when clove chemical derivatives inhibited egg hatchability and caused larvae and adult mortality. Clove extract effects on *C. sordidus* were comparable to those of their synthetic analogs which; however, exhibited a shorter efficacy. A loss of eugenol activity within 24 h of the application was also reported by Obeng-Ofori and Reichmuth (1997), results that may indicate a need for improved formulations to prolong insecticidal activity.

3.3.3 Weevil repellent assay

The repellency effect of the three plant extracts, each applied at 0.8 % concentration, to *C. sordidus* is summarized in Figure 3.4. Pepper extract effectively repelled most adult weevils, ranging between 80 to 98 %, followed by clove extract with 78 to 90 % repellency and neem extract that was least efficient with only repelling 63 to 75 % of the weevils. Moreover, neem extracts repellency of adult weevils is in good agreement with earlier studies, reporting 89 % (Musabyimana et al., 2000, 2001) and 65 to 73% repellency (Inyang and Emosairue, 2005). It is suggested, that the high efficacy of neem products in controlling *C. sordidus* is due to its key secondary metabolites azadirachtin and nimolinone. Azadirachtin works by demobilizing the ecdysteroid molting hormone (Dorn et al., 1986), preventing the larvae from developing into adults. Also, dipping plantain or banana suckers in 20 % neem extract before planting provided protection from weevil attack through repellency that discouraged egg oviposition (C. S. Gold & Messiaen, 2000).

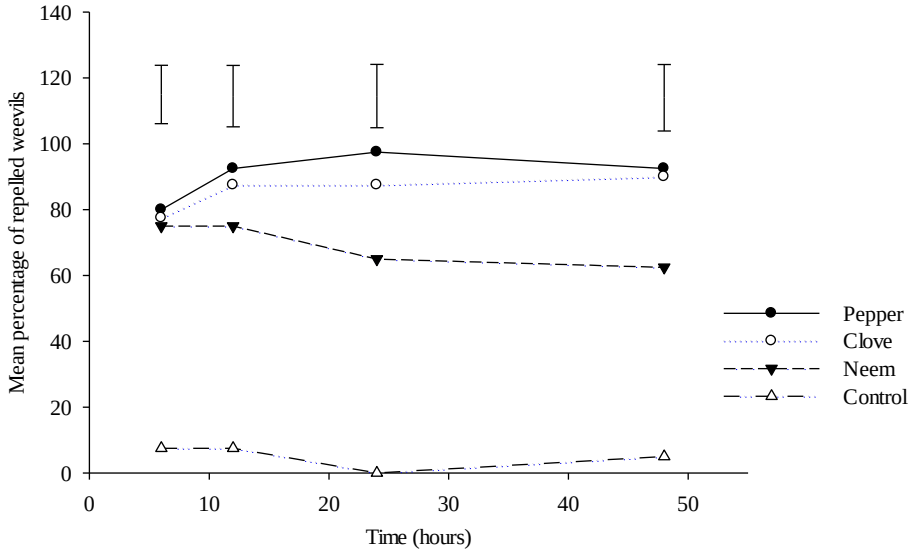

Figure 3.4 The effect of *C. sordidus* exposure to various plant extracts, each applied at 0.8 % (w/v) concentration, on the mean percentage of repelled adult weevils over 48 hours. Each point is the mean of two experimental repeats, each with n = 15. $LSD_{0.05}$ for time (hours) is represented with a bar.

The potential of various synthetic analogs of clove and pepper metabolites, each applied at 0.5 % concentration, on repellency of *C. sordidus* is shown in Figure 3.5. Repellency activity was greatest

after 3 to 6 hours of exposure to all synthetic analogs, followed by a gradual decline over the observation period of 24 hours. The repellency activity of eugenol and β-caryophyllene was still around 60 % after 24 hours; however, that of eugenyl acetate and N-isobutylamine was reduced to below 40 %. Nonetheless, the demonstrated insecticidal properties of pepper to *C. sordidus* are likely attributable to a complex plant ingredient matrix since, for example, total extracts had more repellent effect than the synthetic analog of its ingredient N-Isobutylamine. These observations are similar to reports of Samuel et al. (2016), showing that piperine, a chemical derivative of *P. guineense*, had less toxicity to Anopheles larvae than what was inducible by total plant extracts. Therefore, the limited efficacy of N-Isobutylamine on weevil egg inhibition, larviciding and repellency properties indicates that it may not be the key pepper ingredient for controlling *C. sordidus*.

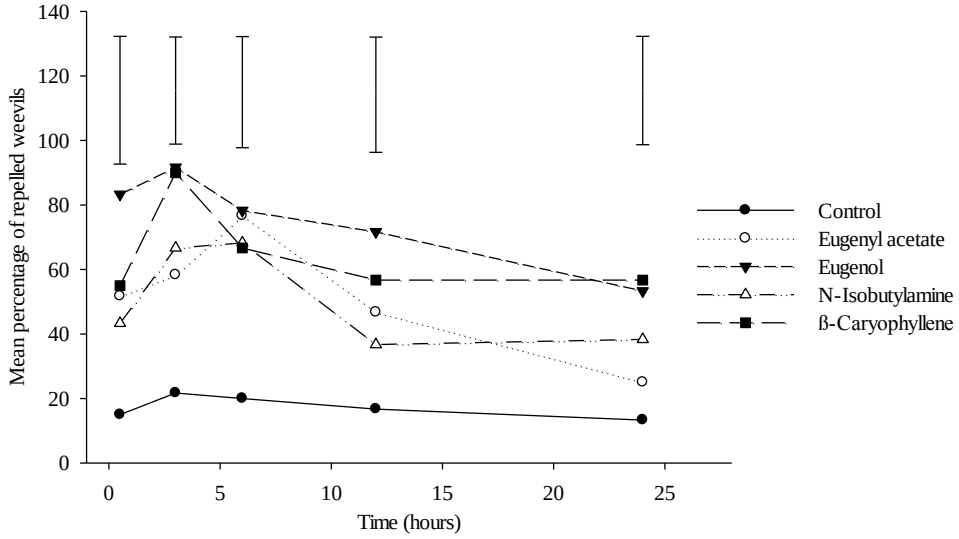

Figure 3.5 The effect of *C. sordidus* exposure to various synthetic analogs of plant metabolites, each applied at 0.5 % (w/v) concentration, on the mean percentage of repelled adult weevils over 24 hours. Each point is the mean of two experimental repeats, each with n = 15. $LSD_{0.05}$ for time (hours) is represented with a bar.

Plant extracts and synthetic analogs of their key ingredients are restrained to varying degrees the vitality of *C. sordidus* based on the evaluated parameters egg hatching, larval development and adult repellency during laboratory and field studies. Although the efficacy of plant extracts to control insect pests may vary with species, many species are susceptible to similar active

compounds (Mundi, Adamu, Ajayi, Bamayi L.J., & Egwurube, 2012; Musabyimana et al., 2001). For example, the observed larviciding effect of pepper against *C. sordidus* was also reported on *Anopheles gambia* species complex (Samuel, Oliver, Coetzee, & Brooke, 2016).

3.3.4 Field evaluation

The number of recaptured weevils was highly variable, depending on treatment and observation time (Table 3.3). Carbofuran had the most consistent toxic effect of all treatments, reducing the weevil population by 45-80% compared to the control. This effect was closely matched by the 0.8 % neem extract treatment, with 40-70% lower weevil numbers than in the control. The clove extract had 40-50% fewer weevils than in the control; however, there was less consistent efficiency throughout the experimental period. In addition, pepper extract field activity against *C. sordidus* decreased with exposure time (Table 3.3), an effect that was also reported for *P. nigrum* on Colorado potato beetles (Scott et al., 2003). This might be explained with the loss of volatility of pepper extracts and thus a time-dependent declining repellent effect on weevils. All other treatments were too variable or less efficient in controlling the weevil population.

Table 3.3 Mean number of recaptured weevils per treatment mat at three-week intervals over 9 weeks.

Treatment	**Mean number of weevils per mat at week**			
	3	6	9	Average
Control	20 [1]	10	27	19.0
Carbofuran	11	2	6	6.3
Clove extract	10	12	10	10.5
Clove powder	15	7	17	13.1
Neem extract	8	6	8	7.3
Neem powder	18	16	11	14.8
Pepper powder	9	6	29	14.7
Pepper extract	14	6	29	16.2
F-Value	1.03	1.55	1.68	1.21
P-Value	0.43	0.19	0.15	0.27
$LSD_{0.05}$	12.69	9.83	22.08	15.77

[1] Each number is the mean of two experimental repeats and five replicates per treatment, each with n = 10 per mat.

3.3.5 Conclusion

This study demonstrates the potential of using botanical pesticides such as extracts of neem, clove and pepper for controlling *C. sordidus* at varying stages throughout the lifecycle. Therefore, it can be concluded that effective alternatives to synthetic insecticides are water extractable plant metabolites that target octopaminergic neurons in invertebrates (Enan, 2001; Murray, 2000). It is recommended to use all plant extracts as a repellent, although their effectivity to repel weevils is reduced with time, possibly because of evaporation. Besides clove extract has also potential as a toxicant and egg inhibiting agent against *C. sordidus*. These natural low-cost products constitute an alternative pest management strategy for smallholder farmers and may help to reduce the occurrence of weevil resistance to synthetic insecticides. In contrast, all evaluated synthetic analogs proved to be useful repellents, but eugenol and eugenyl acetate were also effective as toxicants and egg hatch inhibitors. Proceeding studies should provide a toxicological understanding of how these botanical extracts and their synthetic analogs penetrate insect cuticle and its metabolic target to give an insight into their specific mode of action.

3.5 References

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4.0 Fungal isolates from banana weevils (*Cosmopolites sordidus*) Cadaver as a Pest Control Option

Abstract

Synthetic pesticides are routinely used to control banana weevil *Cosmopolites sordidus,* a pest that causes 40 to 70% annual yield losses of *Musa* species. Biological control agents, including entomopathogenic fungi, are appropriate alternatives to synthetic pesticides. This study, evaluated *Curvularia senegalensis, Fusarium verticillioides*, *Fusarium oxysporum* species complex and *Beauveria bassiana* on eggs, instar stage two larvae and adult *C. sordidus*. Bioassays evaluated egg hatch inhibition, larvae, and adults mortality in response to 10^6 and 10^7 ml^{-1} conidia suspension applications of each strain. Plant protection potential in terms of rhizome injury reduction was evaluated by incorporating 10^7 ml^{-1} conidia in soil potted plants at day 1 and 14 of the experiment and reciprocally releasing weevils at days 14 and 1, respectively. Both *C. senegalensis* and *F. verticillioides* at 10^7 ml^{-1} conidia resulted in > 80 % egg hatch inhibition and larvae mortality, respectively, and >55% adult mortality. In contrast, to control, fungal treated plants had >50% peripheral and internal damage reduction. Additionally, a significant relationship between the dead weevils and rhizome injury accounted for 46 % tissue damage variance. In conclusion, all fungal strains, particularly *Curvularia senegalensis*, showed potentials as entomopathogen when applied on sorghum before weevil infestation.

4.1 Introduction

The banana weevil *Cosmopolites sordidus* Germar is one of the most serious pests within the Musaceae family, affecting plantain (*Musa x paradisiaca*), Abyssinian banana *(Ensete ventricosum*) and highland banana (*Musa* species) in tropics and sub-tropics. The organism of 10 to 16 mm length whose origin being Indo-Malaysia, causes annual yield losses between 40 to 70 % in banana and plantain growing regions of sub-Saharan Africa (Gold et al., 2003; Speijer et al., 2001). *C. sordidus* are characterised by four developmental stages, *viz* egg, larva, pupa and adult. *C. sordidus* eggs laid into notches created at the collar of the plant rhizome above the ground level hatch into larvae. Damages due to feeding larvae on the rhizome affect root development, nutrient and water uptake, plant stability during windy weather and death of plants during heavy damage(Gold and Messiaen, 2000). The adults are free living (*i.e*. not confined to host plant), negatively phototropic, thigmotactic, strongly hygrotropic, gregarious and displays death mimicry, and to a small extent shown to vector *Xanthomonas campestris* pv. *musacearum* in bananas (un published). *C. sordidus* exhibit restricted feeding habit, with adults feeding on dead plant material, while larvae feed and develop mainly on corms and pseudostem (Kiggundu, 2000). *C. sordidus* life cycle is between 2 to 4 years and adults can last for 180 days without food which undermines most conventional control methods.

Frequent applications of non-systemic insecticides in the soil around the plant mat are commonly used to control adult weevils, but resistance among weevil populations is increasingly observed on organophosphates such as prothiofos, chlorpyrifos, pirimiphos-ethyl and ethoprophos) (Collins et al., 1991). Moreover, deposited eggs and developing larvae inside the plant tissue are not targeted. Integrated Pest Management (IPM) has long been advocated for because its intervention goes beyond pest control, environmentally sound and suppresses the pests through the use of a wide variety of technological and management practices. Example of IPM program against *C. sordidus* include but not limited to the following; *Beauveria bassiana* and pseudostem traps (Nankinga, 1999), *B. bassiana* and aggregated pheromone traps (Tinzaara et al., 2007), Fallow and pheromone mass trapping (Rhino et al., 2010) among others. Consequently, integrating biological control options, based on applications of natural enemies such as entomopathogens (fungi, bacteria, and protozoa), predators or parasitoids might be appropriate alternatives to the use of chemical pesticides (Lazreg et al., 2007; Roy and Cottrell, 2008).

Pest suppressive soils often contain entomopathogens, which prevent insect pests from reaching a critical threshold despite favorable environmental conditions (Vegaa et al., 2009). Soil fungi are possibly the most adaptable infectious biocontrol agents as they are self-propagating, grow on a wide range of hosts and infect different insect developmental stages (Ali-shtayeh et al., 2003). And because of a wide host range, fungi like *Curvularia* have species that may exists as endophytes epiphytes, saprophytes and as pathogen (Manamgoda et al., 2015), but yet to be reported as an entomopathogen. Nonetheless, *Fusarium* and *Beauveria* are reported as endophytes as well as entomopathogens (Ochieno, 2010). In this study, three fungal isolates from *Cosmopolites sordidus* cadavers were identified in Ghana and evaluated along with the known *Beauveria bassiana* isolate IMI372439: *Curvularia senegalensis, Fusarium verticillioides* and *Fusarium oxysporum* species complex. The objective of this study was to test in laboratory assays the susceptibility of different developmental stages of *C. sordidus* to fungal contamination and to investigate in pot experiments whether soils inoculated with various fungal conidia results in lower survival rates of eggs, larvae and adults for reducing plantain rhizome damage.

4.2 Materials and Methods

4.2.1 Fungal strain isolation and identification

Fungal isolates from the species *C. senegalensis* (Cs), *F. verticillioides* (*Fv*) and *Fusarium oxysporum* species complex (*FOSC*) were obtained from banana weevil cadaver collected from the plantain fields at the Crops Research Institute (CRI) in Kumasi, Ghana (6° 41' 0" North - 1° 37' 0" West). Fungal strains were determined following the morphological identification key described by (Mathur and Kongsdal, 2003). For confirming their identity, fungal cultures were sent to the Leibniz Institut Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany), where strains were verified by sequencing rDNA internal transcribed spacer (ITS) and rDNA large subunit (LSU) fragments (Schoch et al., 2012) using standard fungal primers (https://sites.duke.edu/vilgalyslab/rdna_primers_for_fung) for PCR; >ITS1F (forward) CTTGGTCATTTAGAGGAAGTAA and >LR5 (reverse) TCCTGAGGGAAACTTCG, and sequencing; >ITS4 TCCTCCGCTTATTGATATGC and >LR4 ACCAGAGTTTCCTCTGG (both reverse). Obtained rDNA-ITS/ LSU sequences were confirmed by databases Westerdijk Fungal Biodiversity Institute (http://www.wi.knaw.nl/), NCBI BLAST (https://www.ncbi.nlm.nih.gov/) and MycoBank (http://www.mycobank.org/). The fungal isolate *B. bassiana* (*Bb*; IMI number 372439) from the culture collection of the Centre for Agriculture and

Biosciences International (CABI) was selected as a positive standard because of its virulence to *C. sordidus* (Nankinga and Moore, 2000; Prabhavathi and Ghosh, 2014). All fungal isolates were cultured on fresh potato dextrose agar (PDA) at 27 ±2 °C in the darkroom (Kaushal and Singh, 2010).

4.2.2 Inoculum production

For inoculum production, 14-day-old sporulation cultures were flooded with sterile water, containing 0.05 % Tween 20, and conidia were gently dislodged using a sterile L-shaped glass rod. The conidia suspension was calculated by counting conidia with a Neubauer haemocytometer (BS.74B, Weber, England) under a bright-field microscope (Leica Microsystems DM500, Wetzlar, Germany). The conidia suspension was diluted as appropriate with sterile water, containing 0.05 % Tween 20, and used for inoculation experiments and as a starter kit for mass production on a sorghum (*Sorghum vulgare*) carrier as recommended by Sahayaraj and Namasivayam, 2008. Conidia produced on a carrier are more tolerant of the adverse environmental condition than conidia produced in liquid media (Mahassneh and Ali-shtayeh, 1999). The carrier was prepared by washing and soaking whole grain sorghum in water overnight. Excess water was then drained and grains were dried to attain on average 50 % moisture content. The sorghum was placed in flasks and autoclaved at 121 °C and 1034.21 hPa for 30 min. After cooling, conidia suspension at 10^9 ml^{-1} was added under a laminar flow hood. Flasks were incubated at 27 ±2 °C for 15 days and shaken daily to evenly distribute the inoculum, to avoid clumping and to guarantee uniform fungal development throughout the carrier substrate. After incubation, subsamples were suspended in sterile water with 0.05 % Tween 20 and subsequently filtered with a double-layered cheesecloth. The conidia filtrate was used to determine the conidia density per gram of colonized sorghum using the haemocytometer.

4.2.3 Banana weevil collection and rearing

Adult weevils were trapped according to Ogenga-Latigo and Bakyalire (1993) from infested plantations nearby the CRI. Weevils were maintained on weekly cut plantain rhizomes inside 10 L plastic buckets that were closed with perforated lids for aeration. Buckets were kept in the laboratory at ambient temperature. This procedure ensured an adequate supply of eggs, larvae and adult weevils for experiments.

4.2.4 Laboratory bioassays

Based on preliminary experiments, conidia suspensions of 10^6 and 10^7 ml^{-1} were selected for infection studies using different *C. sordidus* developmental stages. Twenty brown spotted weevil eggs (3-day-old), twenty larvae of instar stage two (5-day-old) and twenty unsexed adult weevils were dipped into the two inoculums of each fungus, respectively, while controls were treated with distilled water with 0.05 % Tween 20. The whole experiment was replicated three times. Treated adult weevils were put in 250 ml flasks, corked with cotton plug, while eggs and larvae were separately kept on Petri dishes with moistened filter paper. Larvae and adult weevils were supplied with freshly cut rhizome pieces and all specimens were incubated at 27 ±2 °C in the darkroom for up to 10 days. The numbers of unhatched eggs, dead larvae and dead adult weevils were counted daily from the 4^{th} day of incubation and percentage values were calculated as proposed by (Zeng et al., 2010). To confirm the infectivity of fungal isolates (Bos, 1981), about 5 specimens with visual signs of mycosis from each replicate were surface sterilized with 70% ethanol (1 min) and 1 % sodium hypochlorite (2 min) and subsequently rinsed with sterile water. After blotting off excess water, specimens were placed on moistened filter paper in Petri dishes that were sealed with parafilm. Incubation was at 27 ±2 °C in the darkroom for up to two weeks until mycosis developed and confirming microscopically (Amscope B490B-3M-digital microscope-US) that mortality was due to respective fungal infection. Microscopically re-isolated conidia from weevil eggs, larvae and adults that died as a result of respective fungal treatments, were further used to infect different weevil growth stages in an attempt to confirming Koch's postulate.

4.2.5 Plant material

Sword suckers of the plantain cultivar ‘Apantu’ were grown in 10 L plastic pots filled with sterile peat soil. Potted plants were placed 60 cm from each other under an outdoor shade structure that transmitted approximately 60 % of the incident light. Plants were weekly fertilized with 5 g nutrient mixture of 23 % N, 10 % P_2O_5 and 10 % K_2O for three months before treatment application and, in absence of rainfall, sufficiently watered. Ninety plants with uniform growth and with at least six fully expanded green leaves were selected for the experiment.

4.2.6 Experimental design of pot experiments

Experimental plants were assigned in a randomized complete block design to five blocks and eighteen treatments. Within each block, one plant was subjected to one of the following

treatments: inoculation with four fungal strains (*Cs*, *Fv*, *Bb*, *FOSC*), each with conidia applications of 20 ml of 10^7 ml^{-1} suspensions (SUS) and 20 to 25 g of sorghum carrier (SC) with conidia equivalent of 10^7 g^{-1} at the commencement (1d) of the experiment and 14 days later (14 d). Also, there were two control plants per block without fungal inoculation (UTC), one for each fungal application time. Both conidia application types were incorporated in the soil just below the plant collar. Sexed weevils (Rukazambuga et al., 1998), four female and four male adults, were introduced to each pot at either 2 weeks after fungal inoculation at 1 d or 2 weeks prior fungal inoculation at 14 d, ensuring that weevils were exposed to each fungus for 6 weeks until the end of the experiment at 8 weeks. Each pot was wrapped with insect netting to prevent the escape of the weevils.

4.2.7 Soil analysis

Soil samples at approximately 5 cm depth of each pot were taken fortnightly from 2 to 8 weeks after the commencement of the pot experiment to determine fungal density as the number of spores per gram of soil, following the method described by (Smith and Skipper, 1979) and using the haemocytometer. Additional soil samples were taken at the commencement, half-way point and termination of the experiment, respectively, and analyzed for pH, organic carbon (Rousk et al., 2009), extractable phosphorus as it affects the growth and abundance of most fungi (Kleinman et al., 2002) and soil texture classes (Kovács et al., 2004) at the CRI.

4.2.8 Chlorophyll content

Chlorophyll content at the base, center and apex of the second youngest fully opened leaf of each plant was measured fortnightly using a chlorophyll meter (SPAD™-502, Minolta, Japan). The average indexed chlorophyll content of each measured plant was used for treatment comparison and the larger the index the greater the chlorophyll loss (Deol et al., 1997).

4.2.9 Rhizome damage

At the end of the experiment, weevil mortality (Henderson and TILTON, 1955) and the percent rhizome damage as caused by feeding larvae were determined. The latter included exposing peripheral and internal feeding tunnels and counting the total number of grids with damaged and intact rhizome tissue, respectively, by using a mesh wire (grid size 1 cm^2) (Ogenga-Latigo and Bakyalire, 1993). Single grids were thereby visually evaluated by applying scores of one third, two thirds and one, according to the degree of damage level. Assessment of peripheral damage

included wrapping the mesh wire around the pared rhizome, whereas assessment of internal damage consisted of two transverse cuts at 2 and 5 cm below the collar, respectively, and overlaying the mesh wire on both tissue surfaces to count and calculate the mean cross-section damage. Percent of total rhizome damage was then determined from peripheral and internal damage caused by *C. sordidus* (Ntonifor et al., 2006).

4.2.10 Statistical analysis

The data were analyzed using a generalized linear mixed model (GLIMMIX) and General Linear Model (GLM) procedure of SAS 9.4 (SAS Institute, Inc. Cary, NC, USA) (Gbur et al., 2012). The variables were percentage egg inhibition, larvae or adult weevil mortality, soil fungal spore density, SPAD index value and percentage of rhizome damage. GLIMMIX procedure assumed equal variance and was specified with binomial distribution for dependent variables and LS-means statement for the fixed effects and unbalanced data. GLM procedure considered multiple comparisons for the p-value and confidence limits for the LS-mean differences. Results were graphically displayed using SigmaPlot 12.3 (Systat Software Inc, San Jose, CA, USA).

4.3 Results

4.3.1 Identification of fungi

The rDNA sequences of Cs (Sequence 1) GenBank accession number MT476857.1, matched *Cochliobolus geniculatus* NCBI GenBank accession JN943416.1,Westerdijk Fungal Biodiversity Institute isolate number CBS149.71, the NCBI GenBank accession number HG779001 and the MycoBank number MB296254 with a possible synonym of *C. geniculate* (Tracy and Earle) Boedijn (MycoBank 265873) (Madrid et al., 2014). The rDNA sequences of *Fv* (GenBank MT476859) (Sequence 2) also matched (100%) the CBS576.78 while FOSC rDNA sequences (GenBank MT476858) (Sequence 3) matched (99%) many forma specialist that form the complex (clades) such as *Fusarium oxysporum* f. sp. vasinfectum, f. sp. apii, f. sp. rhois, f. sp. nicotianae etc. However, FOS f. sp. cubense (Laurence et al., 2014) which causes vascular wilt disease in banana was not observed.

Sequence 1: > MT476857 [*Curvularia senegalensis*]

GGTCATTTAGAGGAAGTAAAAGTCGTAACAAGGTCTCCGTAGGTGAACCTGCGGAGGGATCATTACACA
ATAAACATATGAAGGCTGCACCGCCAACAGGCGGCAAGGCTGGAGTATTTTATTACCCTTGTCTTTTGCG
CACTTGTTGTTTCCTGGGCGGGTTCGCCCGCCTCCAGGACCACATGATAAACCTTTTTTATGCAGTTGCAA
TCAGCGTCAGTACAACAAATGTAAATCATTTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATG
AAGAACGCAGCGAAATGCGATACGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGC
ACATTGCGCCCTTTGGTATTCCAAAGGGCATGCCTGTTCGAGCGTCATTTGTACCCTCAAGCTTTGCTTGG
TGTTGGGCGTTTTTTGTCTTTGGTTTTGTCCAAAGACTCGCCTTAAAACGATTGGCAGCCGGCCTACTGGT
TTCGCAGCGCAGCACATTTTTGCGCTTGCAATCAGCAAAAGAGGACGGCACTCCATCAAGACTCTATATC
ACTTTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAAAAGAAA
CCAACAGGGATTGCCTTAGTAACGGCGAGTGAAGCGGCAACAGCTCAAATTTGAAATCTGGCTCTTTCAG
AGTCCGAGTTGTAATTTGCAGAGGGCGCTTTGGCTTTGGCAGCGGTCCAAGTTCCTTGGAACAGGACGT
CACAGAGGGTGAGAATCCCGTACGTGGTCGCTAGCTATTGCCGTGTAAAGCCCCTTCGACGAGTCGAGT
TGTTTGGGAATGCAGCTCTAAATGGGAGGTAAATTTCTTCTAAAGCTAAATATTGGCCAGAGACCGATAG
CGCACAAGTAGAGTGATCGAAAGATGAAAAGCACTTTGGAAAGAGAGTCAAACAGCACGTGAAATTGTT
GAAAGGGAAGCGCTTGCAGCCAGACTTGCTTGCAGTTGCTCATCCGGGCTTTTGCCCGGTGCACTCTTCT
GCAGGCAGGCCAGCATCAGTTTGGGCGGTGGGATAAAGGTCTCTGACACGTTCCTTCCTTCGGGTTGGC
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Sequence 2: > MT476859 [*Fusarium verticillioides*]

TCGTAACAAGGTCTCCGTTGGTGAACCAGCGGAGGGATCATTACCGAGTTTACAACTCCCAAACCCCTGT
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ACCCTCAAGCCCAGCTTGGTGTTGGGACTCGCGAGTCAAATCGCGTTCCCCAAATTGATTGGCGGTCACG
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AGTGTTATAGCCCACCGTGTAAT

Sequence 3: >MT476858 [*Fusarium oxysporum* species complex]

TCTTGGTCATTTAGAGGAAGTAAAAGTCGTAACAAGGTCTCCGTTGGTGAACCAGCGGAGGGATCATTA
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AGAGTTAAAAAGTACGTGAAATTGTTGAAAGGGAAGCGTTTATGACCAGACTTGGGCTTGGTTAATCAT
CTGGGGTTCTCCCCAGTGCACTTTTCCAGTCCAGGCCAGCATCAGTTTTCCCCGGGGGATAAAGGCGGCG
GGAATGTGGCTCTCTTCGGGGAGTGTTATAGCCCACCGTGTAAT

4.3.2 Laboratory bioassays

All fungal strains inhibited significantly egg hatching of *C. sordidus* when compared to the control treatment (Figure 4.1a). Unlike for *Bb*, the two conidia suspension used for each fungal treatment did inhibit differentially egg hatching. The percentage of egg inhibition was over 80% for both *Cs*

and *Fv* and below 50% for *Bb* and *FOSC* (Figure 4.1a). All fungal strains induced significantly greater mortality of *C. sordidus* instar stage 2 larvae compared to the untreated control (Figure 4.1b). At both conidia suspensions, high percentages of larvae mortality of above 75% were recorded for *Cs* and *Fv*. In contrast, larvae mortality was below 40% for *Bb* and *FOSC*, except for *Bb* applied at 10^7 ml^{-1} conidia suspension with a treatment effect that was similar to that of *Cs* and *Fv*, respectively (Figure 4.1b). Adult weevil mortality was between 35 to 65% across all fungal treatments and zero for the control (Figure 4.1c). For *Cs* and *Fv*, weevil mortality was significantly greater in the higher conidia suspension, an effect that was not seen for *Bb* and *FOSC* (Figure 4.1c).

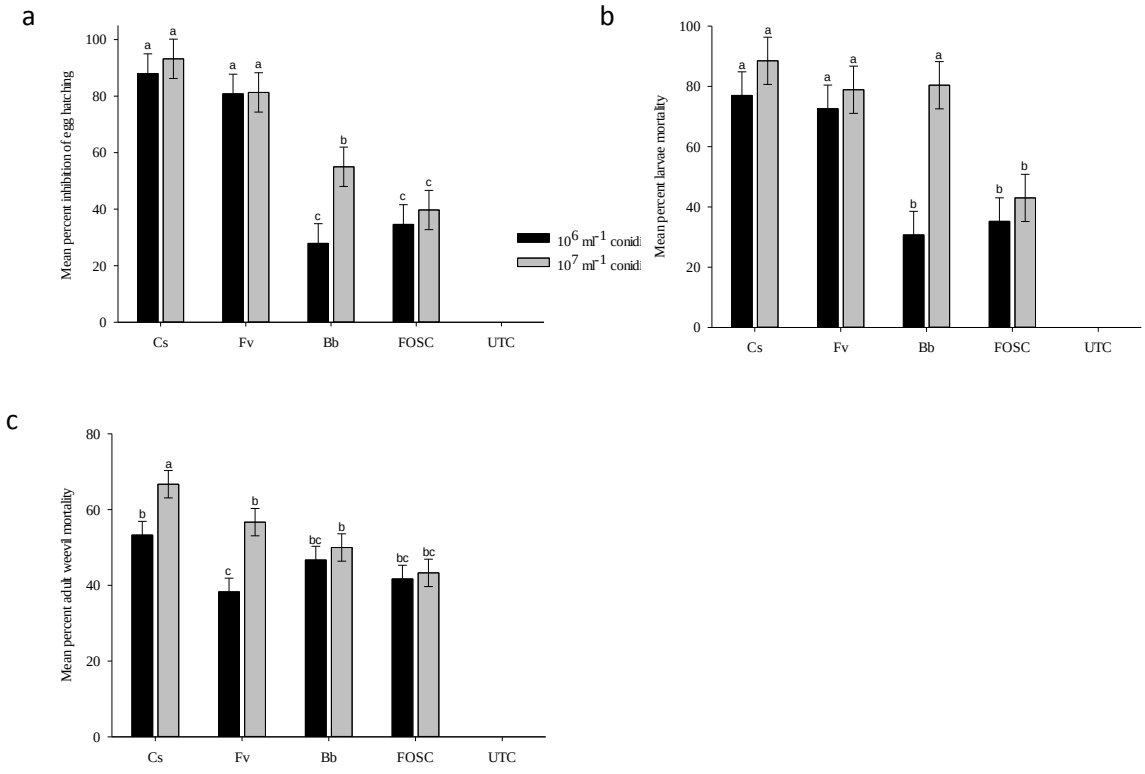
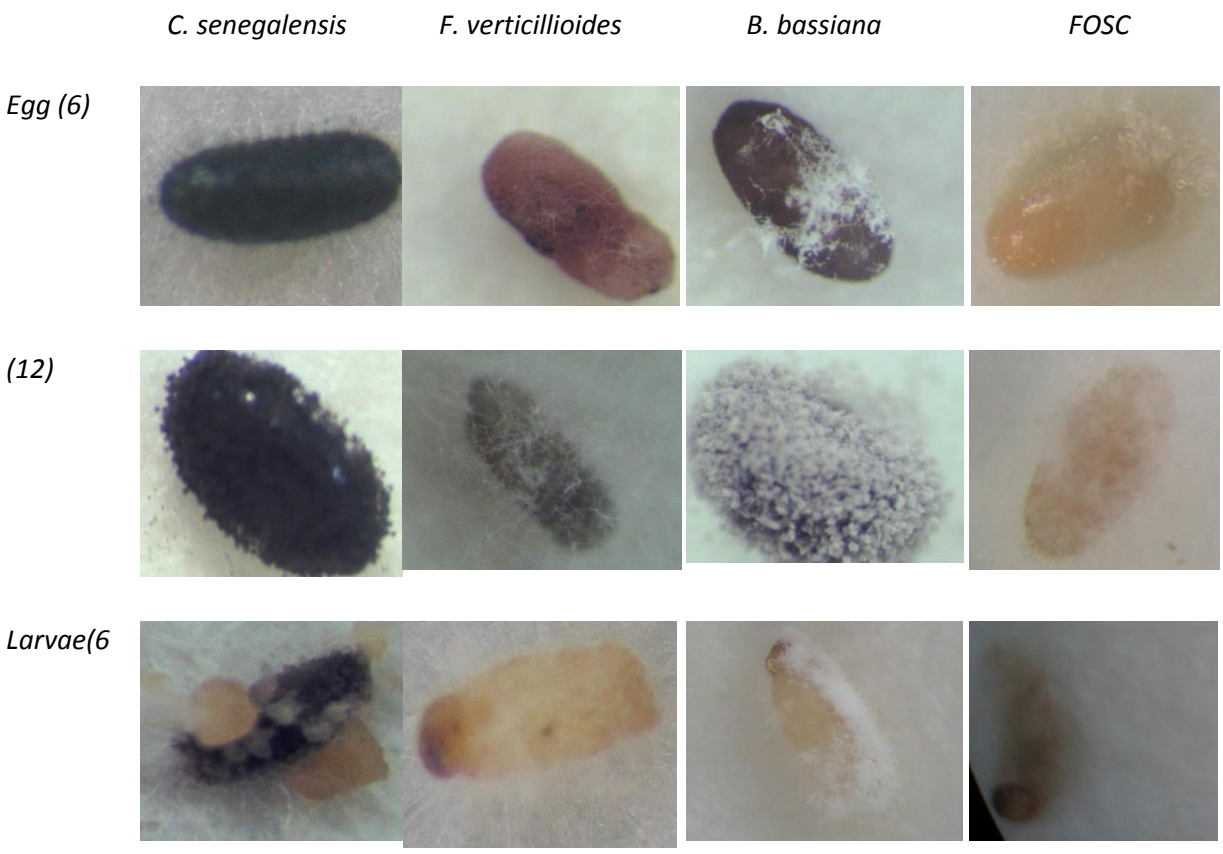


Figure 3.1 Effect of fungal treatments (*Cs* - *Curvularia senegalensis*, *Fv* - *Fusarium verticillioides*, *Bb* - *Beauveria bassiana*, *FOSC* - *Fusarium oxysporum* species complex) with 10^6 and 10^7 ml^{-1} conidia suspensions, respectively, compared to an untreated control (UTC) on mean percent inhibition of egg hatching (a), larvae (instar stage 2) mortality (b) and adult weevil mortality (c) of *C. sordidus*. Vertical bars indicate standard error of the means (3 replicates, each with n=20) and fungal strains at the two conidia suspension with no significant difference ($p \leq 0.05$) are indicated by the same letters.

4.3.3 Re-infection and re-isolation of the fungi

To confirm the infectivity of all fungal isolates, re-incubation of surface sterilized fungal inhibited eggs as well as dead larvae and adult weevils, respectively, resulted in the death of re-infected weevil growth stages and considerable mycelia growth on surface sterilized cadavers of the respective fungal strain (Figure 4.2).

Moreover, the degree of fungal induced mycoses tended to increase for all strains from 6 to 12 days of incubation. The microscopic characterization of conidia (Figure 4.3) from mycotic re-infected weevil eggs, larvae and adults, respectively, confirmed that mortality of *C. sordidus* was due to the infection of the respective fungal strain (Meyer et al., 2008; Muerrle et al., 2006) of pathogen re-isolation.



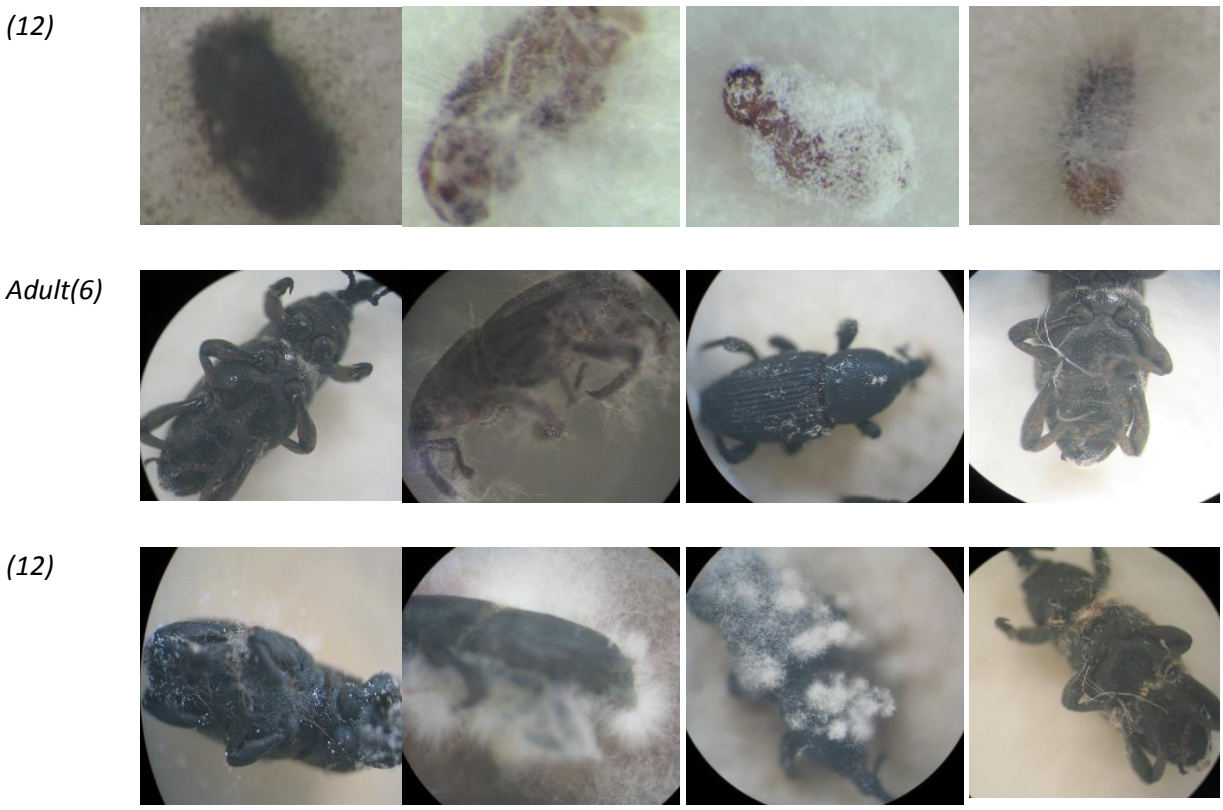
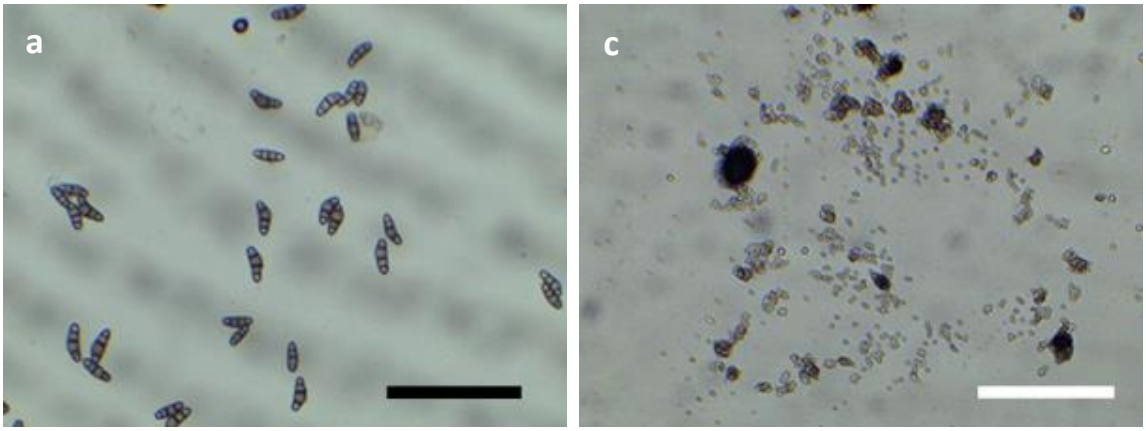


Figure 4.2 Re-growth of *C. senegalensis*, *F. verticillioides*, *B. bassiana* and *Fusarium oxysporum* species complex (*FOSC*) on surface sterilized, previously with the same fungal strains inhibited eggs as well as dead larvae and adult weevils, respectively, after 6 and 12 days of re-incubation.



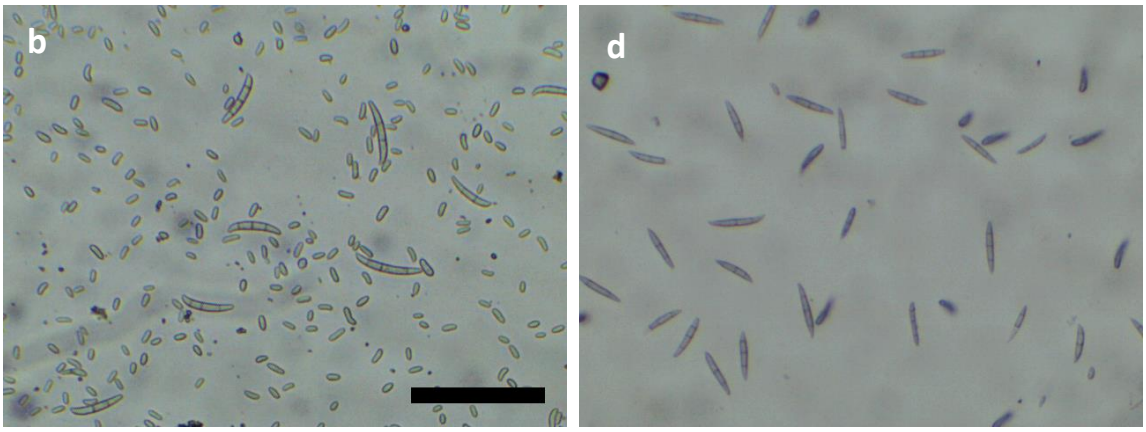
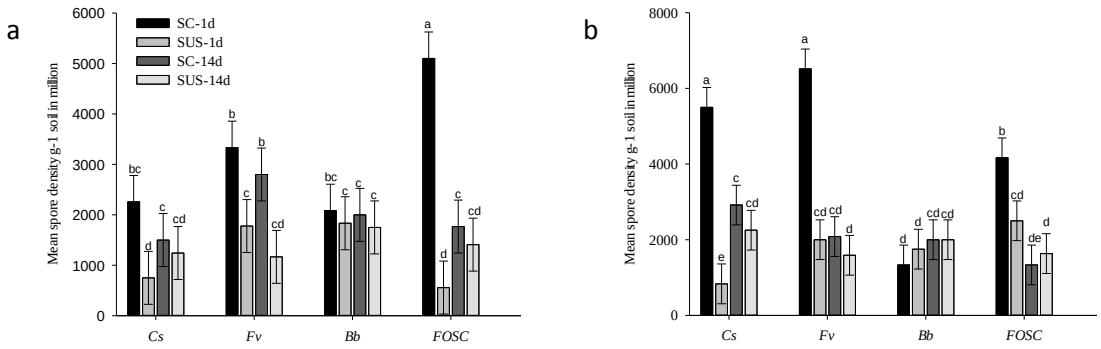


Figure 4.3 Re-isolated conidia of *C. senegalensis* (a), *F. verticillioides* (b), *B. bassiana* (c) and *Fusarium oxysporum* species complex (*FOSC*) (d) from weevil (*C. sordidus*) cadavers. Conidia magnification is 100 X.

4.3.4 Pot experiment

Soil characteristics. The soil used for the experiment was sandy loam in a moderately acidic pH range (5.5 to 6.0), a concentration of extractable P ranging between 116.4 to 489.5 ppm and organic carbon content of about 1.6%. Soil properties represent the three samples taken at the start, halfway through and at the end of the experiment.

Soil spore density. The number of fungal spores per gram of soil increased continuously for all fungal strains throughout the experimental period of 8 weeks (Figure 4.4). However, conidia of each fungus were more abundant in SC at all sampling times than what was observed in SUS. Irrespective of fungal strain, the SC-1d treatment had with 4 to 7 billion spores per gram soil the highest spore density at the end of the 8-week experimental period. All other fungal treatments reached a final spore density of below 3.5 billion spores per gram soil, except SC-14d and SUS-14d for *Cs*.



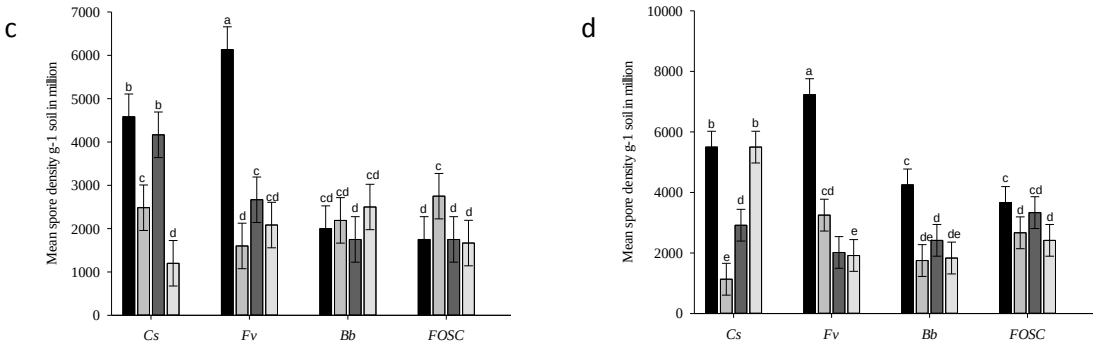


Figure 4.4 Mean number of spores per gram of soil at (a) 2-weeks, (b) 4-weeks, (c) 6-weeks and (d) 8-weeks post-inoculation with *C. senegalensis* (*Cs*), *F. verticillioides* (*Fv*), *B. bassiana* (*Bb*) and *Fusarium oxysporum* species complex (*FOSC*) applied at 20 ml of 10^7 ml^{-1} suspension (SUS) or 20 to 25 g of sorghum carrier (SC) with conidia equivalent of 10^7 g^{-1} at the beginning (1d; weevils introduced 2 weeks later) of the experiment or 14 days later (14 d; weevils introduced 2 weeks earlier). Vertical bars indicate standard error of the means (n = 5) and treatment effects at each sampling time with no significant difference ($p \leq 0.05$) are indicated by the same letters respectively.

4.3.5 Chlorophyll SPAD values

In general, neither the fungal strain nor the fungal application time to the introduction of weevils had any effect on the SPAD index of young 'Apantu' leaves. In contrast, the method of conidia application significantly affected the SPAD index for example at week 6 and 8 of sampling. 'Apantu' plants treated with SUS tended to have greener leaves than those treated with SC (Figure 4.5).

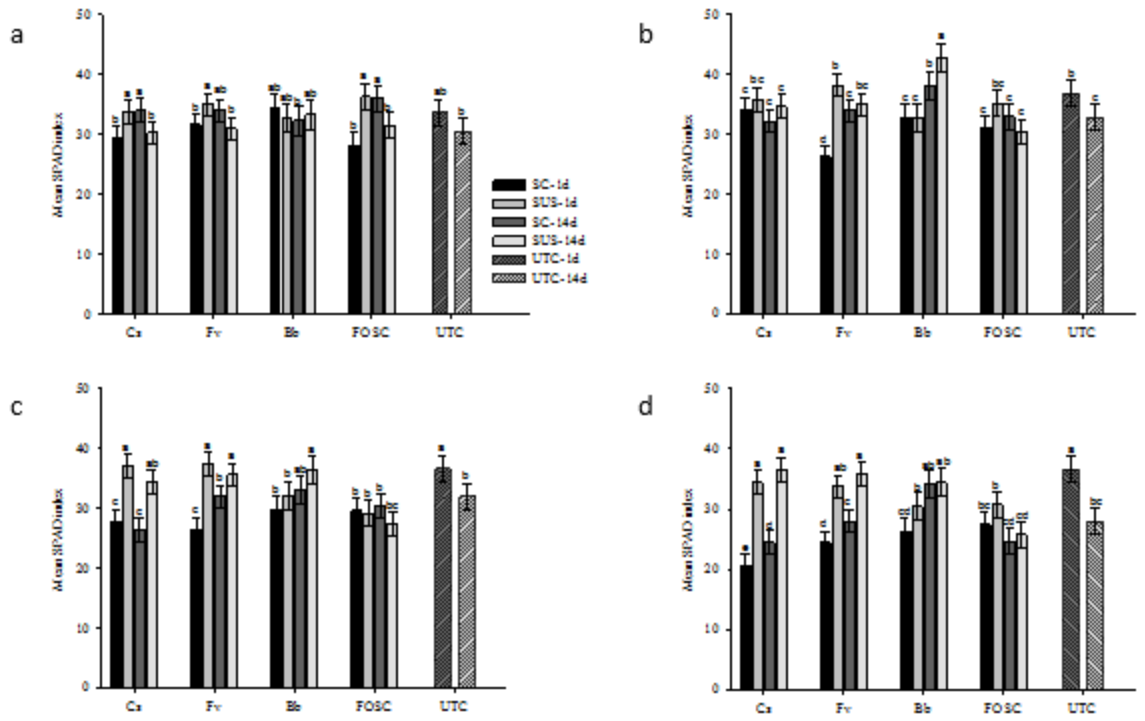


Figure 4.5 Mean SPAD index of the second youngest fully opened leaf (average of records at the base, centre and apex) of the plantain cultivar 'Apantu' at (a) 2-weeks, (b) 4-weeks, (c) 6-weeks and (d) 8-weeks of exposure to weevils (*C. sordidus*). Weevil damage was not controlled (UTC) or controlled by the fungal strains *C. senegalensis* (*Cs*), *F. verticillioides* (*Fv*), *B. bassiana* (*Bb*)and *Fusarium oxysporum* species complex (*FOSC*) applied at 20 ml of 10^7 ml^{-1} suspension (SUS) or 20 to 25 g of sorghum carrier (SC) with conidia equivalent of 10^7 g^{-1} at the beginning (1d; weevils introduced 2 weeks later) of the experiment or 14 days later (14 d; weevils introduced 2 weeks earlier), respectively. Vertical bars indicate standard error of the means (n=5) and treatment effects at each sampling time with no significant difference ($p \leq 0.05$) are indicated by the same letters.

4.3.6. Rhizome damage

Apantu' plants treated with either of the four fungal strains had only half of the rhizome injury compared to the untreated controls that had more than 40 % tissue damage (Figure 4.6a). Plants treated with *Cs* and *Bb* were about 50 % less damaged by *C. sordidus* than those treated with *Fv* and *FOSC* (Figure 4.6b). While the method of the fungal application did not significantly affect the rhizome damage caused by feeding larvae, plants treated 2 weeks before weevils were introduced had less damage than those treated 2 weeks after weevil exposure (Figure 4.6a). *C. sordidus*

caused less peripheral than internal damage (Figure 4.6b; Figure 4.7). Fungal mycelia growth, observed in the larvae feeding tunnels (Figure 4.7), likely caused larvae mortality and thus resulted in reduced rhizome damage in treated plants. There was a significant ($p \leq 0.001$) relationship between the number of dead weevils and rhizome injury, accounting for about 46 % of the variance in tissue damage (Figure 4.8).

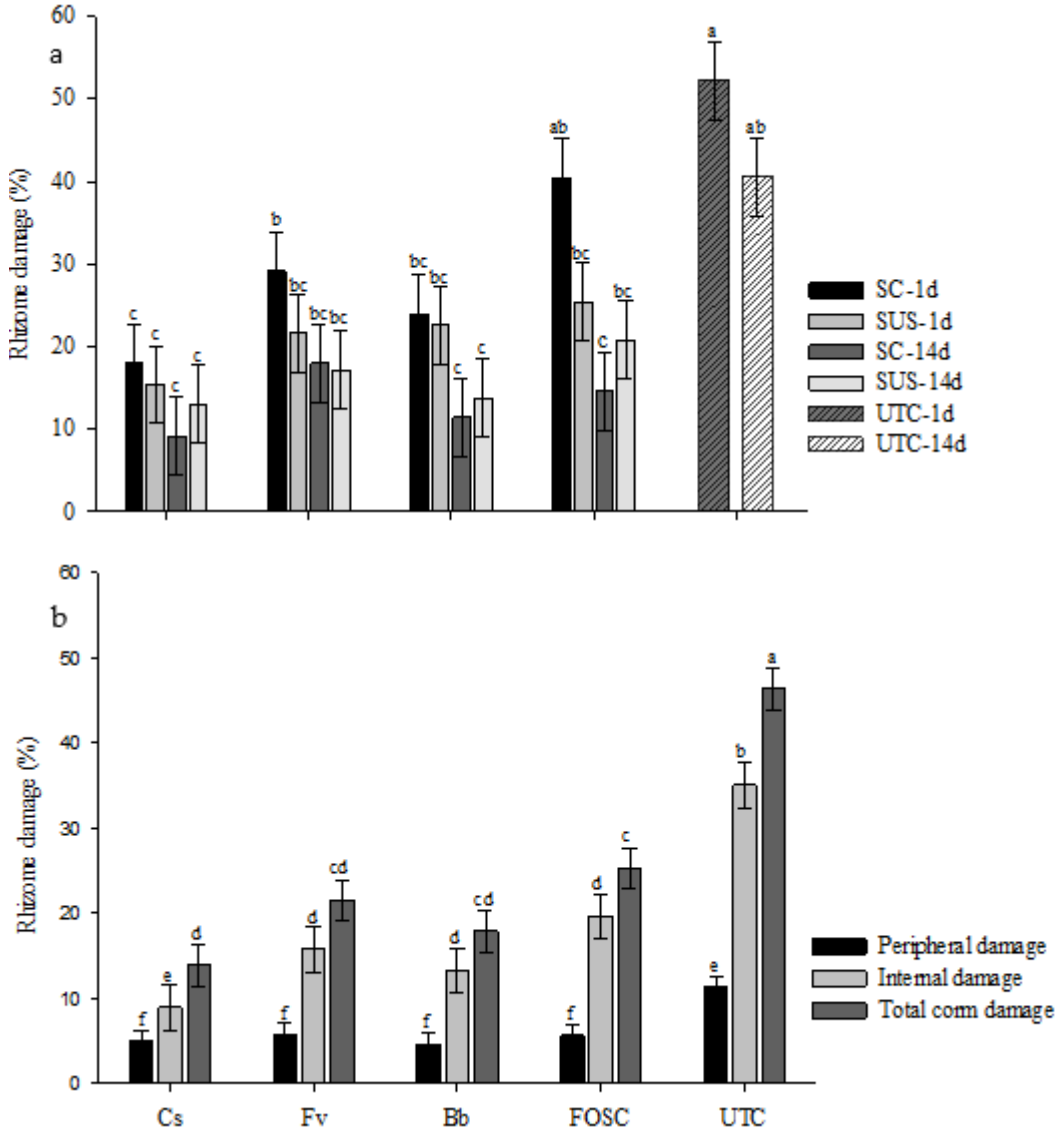


Figure 4.6 Mean percent rhizome damage of plantain, cultivar 'Apantu', after 6 weeks of exposure to weevils (*C. sordidus*): a) fungal application effects, (b) overall fungal effect on total, peripheral and internal damage. Weevil damage was not controlled (UTC) or controlled by the fungal strains *C. senegalensis* (*Cs*), *F. verticillioides* (*Fv*), *B. bassiana* (*Bb*) and *Fusarium oxysporum* species complex (*FOSC*) applied at 20 ml of 10^7 ml^{-1} suspension (SUS) or 20 to 25 g of sorghum carrier (SC) at the beginning (1d; weevils introduced 2 weeks later) of the experiment

or 14 days later (14 d; weevils introduced 2 weeks earlier), respectively. Vertical bars indicate standard error of the means (n=5) and treatments with no significant difference ($p \leq 0.05$) to untreated controls are indicated by the same letters.

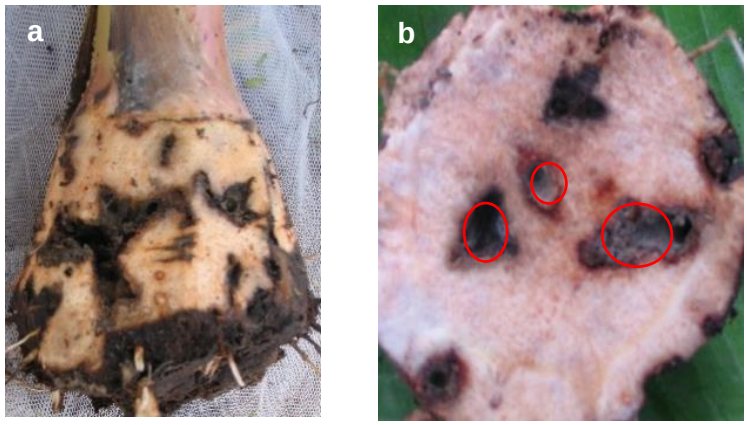


Figure 4.7 Weevil (*C. sordidus*) damage on 'Apantu' rhizomes: Pared rhizome with peripheral larvae tunneling (a); mycelia growth in larvae feeding tunnels marked with red circles (b).

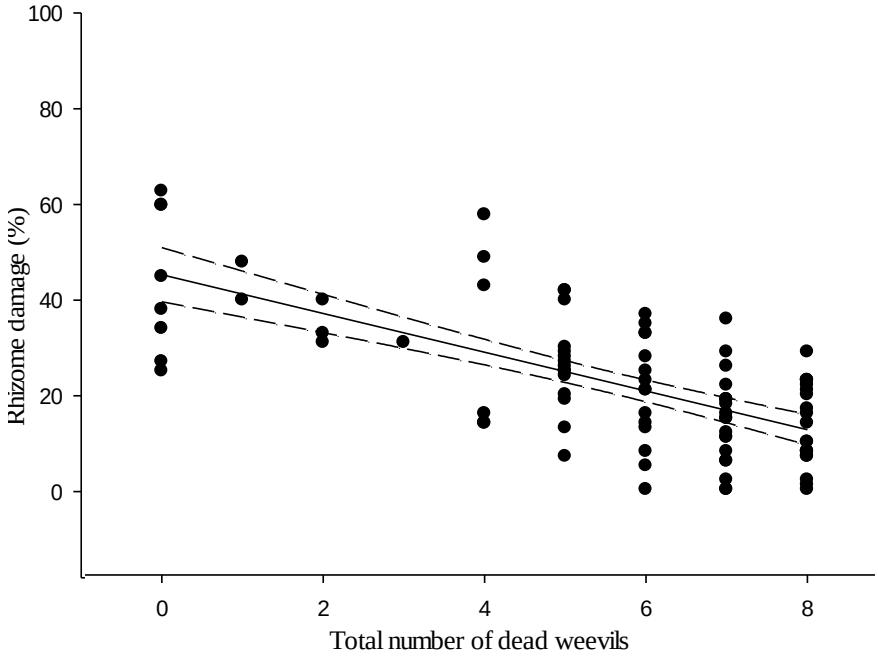


Figure 4.8 Relationship between the total number of dead weevil (n=90) and rhizome damage. Equation of the linear relationship is y= -4.1x + 45.3. R^2 = 0.46. Confidence limits are indicated by the dash lines.

4.4 Discussion

4.4.1 Identification of fungi

Fungal taxonomy is complicated due to considerable morphological and physiological variation and the limited number of morphological markers but renders important for a range of reasons for example the development of biopesticides. The isolates were initially identified up to the genus level by microscopic evaluation of colony color and conidia morphology and consequently classified to *Dothideomycetes* (*Cs*) and *Sordariomycetes (Fv* and *F. oxysporum*). Both genera have a wide host range and geographical distribution (Manamgoda et al., 2015, 2012). To identify the isolates to species level, molecular data were used such as the rDNA internal transcribed spacer (ITS) and rDNA large subunit (LSU) sequence. The rDNA ITS/LSU sequences were then compared to sequences in various databases with a 99-100 % match. The rDNA ITS is commonly used because it is regarded as a DNA barcoding marker for the identification of fungi to species level (Raja et al., 2017). Since the rDNA ITS could not separate *Fusarium oxysporum* species complex into its members (clades), the multi-locus sequence typing (MLST) approach is proposed. *Cs* and *Fv* are classified as biosecurity level (BSL) 1, while *FOSC* members to BSL 1+ (Hoog et al., 2019), both not considered dangerous organisms according to the German Committee on Biological Agents.

4.4.2 Treatment effects on eggs, larvae, and adult weevils

Entomopathogenic isolates from a host organism are typical of great virulence that leads to some level of damage (Liu et al., 2002). Of the three fungal strains isolated from weevil cadaver, *Cs* and *Fv* were more virulent against the different life stages of *C. sordidus* than *Bb* and *FOSC* (Figures 4.1, 4.2). Such variation in virulence among fungal isolates has often been reported (Alshareef and Robson, 2014; Valero-jiménez et al., 2014) and can be compensated for to some extent with increasing conidia concentration as has been demonstrated in the current study (Figure 4.1c). The pathogen's application time to the developmental stage of the pest will also affect the degree of disease severity. For example, the physiochemical properties of the egg, larvae, or adult cuticle (Grizanova et al., 2019; Inglis et al., 2001) may affect conidia adhesion and viability. The observed rapid germination and hyphal growth of *Fv* spores were also reported on *Melodogyne graminicola*, a nematode that damages rice roots (Le et al., 2016) and *Tropidacris collaris*, a grasshopper pest that consumes all plant material (Pelizza et al., 2011), a fungal characteristic that may be related to a relatively high infectivity rate. After spore attachment and germination

on the host surface, the fungus penetrates through the insect cuticle, joints or creases where the insect's protective covering is thinner, with either germ tubes or an appressorium formation to infect the weevil host (St Leger and Wang, 2010). The application of adhesive fungal suspensions may lead to an improved pathogen virulence towards insect pests with hard to penetrate cuticles. In agreement with (Njau et al., 2011), the mortality of adult weevils was generally lower than that of eggs and larvae, a variation that could be attributable to differences in the composition and thickness of the cuticle for the various stages of the weevil life cycle. After the death of the weevil, the fungus grew out through the exoskeleton and began to produce spores.

4.4.3 Soil properties and spore density

The major soil properties that affect optimum fungal germination and multiplication are pH, available phosphorus, and organic carbon, which were all within the recommended range (Miranda and Harris, 1994; Rath et al., 1992; Tawaraya et al., 1996). However, fungi with a slow growth pattern on infected cadaver such as *Cs* rely on insect carbohydrates rather than soil carbon (Vegaa et al., 2009). The observed differences in soil spore density among fungal treatments are likely more dependent on timing and method of fungal application rather than on soil properties. For example, the number of spores per gram of soil was much greater when conidia were applied on sorghum (Figure 4.4) that presumably supplied carbon and nutrient required for fungal multiplication (Zahmatkesh et al., 2017). However, the number of spores in the soil did not correlate well with fungal infectivity for example *Fv* with the greatest soil spore density (Figure 4.4) was not the most effective against weevil damage (Figure 4.6b).

4.4.4 Treatment effects on potted plants

Treatment effects on potted plants. The weevil *C. sordidus* affects Musa and Ensete species with stunted growth, leaf yellowing, loss of roots, and eventually premature death (Okolle et al., 2009); however, plants have few or no symptoms when treated with appropriate infectious biological control agents. Entomopathogenic endophytic fungi not only target the various life stages of *C. sordidus* but also grow into tunnels (galleries) within the corm (Figure 4.7) where they can infect feeding pathogens. Despite mycelia growth in larvae feeding tunnels, fusarium wilt disease and corm discoloration symptoms were not observed. The optimum temperature for most entomopathogens to grow, infect and cause disease to the insect pest host is between 15-30°C (Ansari et al., 2014; Inglis et al., 2001). The results here indicate that applications of fungal strains before weevil infestation is a preferred option for managing crop damage.

4.4.5 Conclusions

In this study, we were able to ascertain that isolates of *Cs*, *Fv* and *FOSC* are pathogenic to *C. sordidus* both in laboratory and pot experiments. All strains, but in particular *Cs*, show promising potentials as entomopathogenic fungi when applied on sorghum to plantain plants before weevil infestation. They can then self-propagate and survive in moderately acidic soils with adequate levels of organic carbon to infect and cause mortality to *C. sordidus*. However, further work is needed to test their biological potential on field plants and whether their efficacy is sufficient enough to provide crop protection against weevil damage. Entomopathogenic fungi are effective pest control options to include in IPM for *C. sordidus* while reducing the reliance of chemical pesticides and their associated risk of pesticide resistance and contribute to sustainable management practices for *Musa* spp.

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Chapter 5

5.0 General discussion

The concealed feeding of banana weevil leads to severe damage and finally death of plants if undetected. However, applying efficient measures against the weevil would require the early detection of infestation, which is practically (almost) impossible due to the difficulty to detect the small wounds from the oviposition at the bottom of a pseudostem. Yet to successfully keep weevil population and its damages below the economic threshold of 2 adult weevil per pseudostem trap after rain season (Smith, 1995), may requires integrating different control strategies that are effective, yet inexpensive, sustainable and environmentally friendly to small holder farmers. Since the use of a single approach is insufficient (Gold et al. 2003), the efficacy of host plant resistance, water extracted botanical plants and entomogenous fungi against banana weevil life stages are hereby discussed.

5.1 Host plant resistance

5.1.1 Host resistance

Although plants are sessile organisms, they have evolved a plethora of chemical (secondary metabolites) and physical defenses to protect themselves against environmental stress and defend against pathogens and herbivores. Plants defend themselves by blocking pathogenic enzymes, toxins, and biochemicals from gaining access to healthy plant cells (Kolattukudy, 1980; Biggs, 1984). They also strategically deposit defense traits at points where they are easily triggered for programmed cell death and formation of barrier upon herbivore damages. The observed structural polymers of lignin and suberin in the epidermis and cells proximal to weevil damage in “Km5” but to a much lesser extent in “Mbwazirume” is presumed a host resistance. This affirms what Beckman (2000) described about phenolic containing cells, that they are highly specialized in synthesis, storage and rapid oxidation in the case of insect wounding. For they are not only toxic to herbivores, but also to plants hence stored as glycosides in cell vacuole, xylem, guard cells, epidermal cells and subepidermal cells waiting for decompartmentalization upon insect damage (Ewané, Lepoivre *et al.,* 2012). Upon wounding, plants form lignified structures when oxidized phenolics polymerize first with each other, and thereafter to cellular proteins and or cell wall carbohydrates (Beckman, 2000). Then polymerized phenolic structures, lock up the immediate

site of wounding with substances deterrent to insect or pathogens, which make the plant resistant.

Both cultivars suffered rhizome injury from feeding *C. sordidus* larvae at varying degrees. Such damage variations are a dependant of cultivar resistance `Km5' or susceptibility 'Mbwazirume' (Kiggundu et al., 2007; Night et al., 2011). Franceschi et al. (2002) reported improved resistance against *Ceratocystis polonica* of *Picea abies* when treated with MJ. Similarly, rhizome damage caused by *C. sordidus* generally decreased following the application of MJ particularly in susceptible cultivar 'Mbwazirume' and to some extent in resistant cultivar 'Km5'. Moreover, the MJ application greatly reduced *Helicoverpa armigera* damage in *Arachis hypogea* (War et al., 2011).

Applications of jasmonic acid commonly lead to increased host resistance not only in banana against *C. sordidus* but also in other plant species against numerous insect herbivores (Tran et al., 2017; War et al., 2012, 2011). Reduction in herbivore plant damage as mediated by MJ and its ally like 12-oxo-phytodienoic acid (OPDA) is either direct or indirect. In the direct, MJ enhances flavonoid biosynthesis which results in compounds like indole glucosinolate that is poisonous or deterrent to most herbivores (Tran et al., 2017). Indirectly, MJ induces proteinase inhibitors to inhibit the protease activity of the digestive enzyme in the insect guts which results in stunting and eventual death. And oxidative enzymes like lipoxygenase (LOX) which catalyse the hydroperoxidation of linolenic acid to radicals (hydroxyl radicals) that directly damage insect tissue or act as feeding repellent or indirectly through the octadecanoid pathway to attract natural enemies (War et al., 2012). Therefore, the reduction of rhizome damage in susceptible cultivar as a result of MJ application could partly complement other conventional *C. sordidus* control measures.

5.1.2 Total phenolic content and antioxidant capacity

The increased TPC and antioxidant capacity in the rhizome tissue upon *C. sordidus* larvae feeding and application of methyl jasmonate are in agreement with the findings of Franceschi *et al.* (2002). Nicholson and Hammerschmidt (1992) in their review pointed out the rapid accumulation of toxic phenols such as ferulic acid and modification of cell walls by phenolic substituent or physical barrier at the point of attack as the first plant defense strategies which function to restrain the damage and to allow synthesis of specific phytoalexins defense compounds as secondary

strategies. Moreover, the phytochemicals phenolics but also flavonoids known to deter herbivorous insects, depend on their potent antioxidant and free radical scavenging properties or reactive oxygen species generated during pest infestation (Verde et al., 2003; Tatiya et al. 2011; Brahmi et al. 2012; Brahmi et al. 2016). This, however, requires more time than is available for the rapid appearance of compounds that constitute primary defense response.

Although, there was a significant negative linear relationship between the percent rhizome damage and TPC F (1, 37) = 6.57, p = 0.01 as well as antioxidant capacity F (1, 37) = 8.93, p = 0.005, control plants had comparatively low TPC and antioxidant capacity. This observation agrees to the plant defense theory that "inducible resistance is developed to reduce the costs of constitutive defense expression" (Elle and Hare, 2000; Hare and Elle, 2002). Moreover, Valladares et al. (2007) demonstrated that ecological and evolutionary variability of induced defense-related phenolics depends on the balance between advantageous and disadvantageous consequences of such defense.

Plant phenolics and antioxidant capacity vary with plant genetics, developmental stage, growing conditions, soil factor, etc. (Saravanan and Aradhya, 2011). The tolerant cultivar 'Km5' had constitutively a higher TPC and antioxidant capacity in the rhizome tissue than the susceptible cultivar 'Mbwazirume'. Larvae feeding induced a considerably greater increase of plant phytochemicals in 'Km5' than in 'Mbwazirume', a response that is likely related to the genetic make-up and specific adaptivity responses of the two cultivars against *C. sordidus*. In contrast, the MJ treatment induced a higher TPC and antioxidant capacity in 'Mbwazirume' rhizome tissue than its UTC within two weeks of MJ application. This suggests that the phytohormone was more beneficial to the susceptible cultivar by inducing the plant to produce different types of defense chemicals (Engwa, 2018; van Dam and Oomen, 2008), which in turn led to reduced *C. sordidus* damages. The limited effect of MJ on 'Km5' plant antioxidant capacity as compared to its UTC, is presumably due to sufficiently occurring constitutive resistance mechanisms. This view is in line with what Dixon (2001) reported, that where constitutive defense metabolites are produced in large amounts after infection, its status as phytoalexin (induced) depends on whether or not the constitutive concentrations were sufficient. Consequently, the endogenous constitutive level of plant defense compounds combined with exogenous applications of phytochemicals may play a role in the prevention and management of oxidative stress-related pest injuries such as those caused by *C. sordidus*.

5.1.3 Histological analysis

Fluorescence microscopy and or histochemistry are the common ways of identifying cellular modification in response to damage or infection. Plant cells can seal the immediate site of wounding with phenolic polymers to deter further herbivore feeding (Beckman 2000). In this study, there was a reduction of *C. sordidus* damage on susceptible banana cultivar attributed to the formation of the physical barrier of polymers, the lignin and suberin. The accumulation of toxic phenolics previously mentioned is not only a general rapid response to herbivore attack but also facilitates the biosynthesis of lignin, suberin, and other wound-induced polyphenolic barriers for structural reinforcement (Dixon and Paiva, 1995; Grace, 2005). The presence of such polymers according to Nicholson and Hammerschmidt (1992) occurs as a result of crosslinking phenylpropanoid esters to the cell walls of damaged plant tissues. Such hydroxycinnamic acid and their derivatives are thought to contribute to the autofluorescence of host tissues at the site of infection (Nicholson and Hammerschmidt, 1992). Similar defense mechanisms were found in the present study through histochemical staining of tissue with P-HCl and Sudan black B and fluorescence analysis which demonstrated prominent deposits of lignin and suberin at the site of damaged rhizome tissues, confirming a host plant resistant strategy against *C.* sordidus. This response could be viewed as a barrier or containment of *C. sordidus* feeding damage. Other studies (Biggs, 1984; Rittinger et al., 1987;Franke and Schreiber, 2007; Melillo et al., 1992; War, et al. 2012) report similar deposit of lignin and suberin which suggest their role in the establishment of barrier zones and impervious tissue to contain either herbivore wounding or pathogen infection among different crops. Nonetheless, the involvement of phenolic compounds is not limited to *C. sordidus* a herbivore but was also shown for banana crown rot (Ewané et al., 2012) which suggests it as an antibiotic host plant defense strategy.

5.2 Botanical extracts

5.2.1 Botanical extracts

The biological activity of the tested plant extracts has been reported to differentially affect bacteria, fungi, viruses, and insect pests (Jovanovi, Kosti and Popovi, 2007). The effect of these botanical extracts on insect pests may vary, but the majority of insect species are susceptible to similar active compounds in the extracts. The variations in the efficacy, however, may be a result

of material handling processes (drying time, storage conditions, and humidity). Kraus and Ermel (1995) reported on azadirachtin variations in the neem seeds due to edaphic factors, while Ermel *et al.* (1987), showed high temperature and relative humidity at storage, and compromised neem seed quality. According to Sundaram *et al.* (1997), azadirachtin evaporation from a commercial formulation in 1h was three out of five with a residual mass of 20-50%. Although in chapter 3, pepper and cloves were bought on local markets and neem seeds collected at the back yard of farmers with no clear information on drying and storage, they remained substantially effective against banana weevils as discussed below.

5.2.2 Effect of botanical extracts and synthetic analogs

The effectivity of the evaluated clove, pepper and neem extracts, and their synthetic analogs against *C. sordidus* depended on developmental stage and treatment concentration. This is in agreement with reports on age-dependent susceptibility to plant extracts (Thara et al., 2009) and dose-dependent insecticidal effects of eugenol against pests like ants, American and German cockroaches (Enan, 2001). Clove and pepper extracts had the most potential of egg hatch inhibition, larvae mortality and adult repellency as shown in the result section of chapter 3. Musabyimana et al., (2001) reported 40 to 60 % *C. sordidus* larvae mortality due to neem extract, a range that is comparable to 19-74% larvae mortality reported in this study.

Clove extracts against *C. sordidus* were as effective as that used against grain storage pests *Sitophilus zeamais,* and *Tribolium castaneum* (Obeng-Ofori and Reichmuth, 1997; Yan *et al.*, 2002), and *Culex pipiens* (Chaieb *et al.*, 2007). However, when other extracts together with clove were applied at 0.8% (w/v) concentration, they did not exhibit insecticidal activity on adult weevils, instead caused emigration (Figure 3.4). Such repellency could be the most appropriate mode of action against weevils as it does not only affect their feeding and egg deposition but also prevent the development of resistance seen with conventional pesticides.

Bioactive compounds with insecticidal properties, inhibit the gut proteinases serine or cysteine in phytophagous insects (Macedo & Freire, 2011). A mode of action that clove metabolites such as eugenol, eugenyl acetate and Caryophyllene (Razafimamonjison et al., 2013) might have followed to restrain *C. sordidus* performance. Similar observations were reported for grain storage pest (Sitophilus zeamais and Tribolium castaneum) (Obeng-Ofori & Reichmuth, 1997; Yan, Shuit-Hung, Hsien-Chieh, & Yen-Ling, 2002) and Culex mosquito (Culex pipiens) (Chaieb et al., 2007). Clove

extract effects on *C. sordidus* were comparable to those of their synthetic analogs which; however, exhibited a shorter efficacy. A loss of eugenol activity within 24 h of the application was also reported by Obeng-Ofori and Reichmuth (1997), results that may indicate a need for improved formulations to prolong insecticidal activity.

Moreover, neem extracts repellency of adult weevils is in good agreement with earlier studies, reporting 89 % (Musabyimana et al., 2000, 2001) and 65 to 73% repellency (Inyang and Emosairue, 2005). It is suggested, that the high efficacy of neem products in controlling *C. sordidus* is due to its key secondary metabolites azadirachtin and nimolinone. Azadirachtin works by demobilizing the ecdysteroid molting hormone (Dorn et al., 1986), preventing the larvae from developing into adults. Also, dipping plantain or banana suckers in 20 % neem extract before planting protected from weevil attack through repellency that discouraged egg oviposition (Gold & Messiaen, 2000). In Cameroon, neem seed extract field applications, reduced >70% weevil sucker damages and 50% plant damage (Messiaen 2000). Out of the 60-100g of recommended neem seed powder per banana mat at a 4-months interval (Musabyimana, 1999), the use of 0.8% (80g) gave satisfactory efficacy against all weevil development stages.

The demonstrated insecticidal properties of pepper to *C. sordidus* are likely attributable to a complex plant ingredient matrix since, for example, total extracts had a more repellent effect than the synthetic analog of its ingredient N-Isobutylamine. These observations are similar to reports of Samuel et al. (2016), showing that piperine, a chemical derivative of *P. guineense*, had less toxicity to Anopheles larvae than what was inducible by total plant extracts. Therefore, the limited efficacy of N-Isobutylamine on weevil egg inhibition, larviciding and repellency properties indicates that it may not be the key pepper ingredient for controlling *C. sordidus*.

The number of recaptured field weevils was highly variable, depending on treatment and observation time (Table 3.3). Carbofuran had the most consistent toxic effect of all treatments, reducing the weevil population by 45-80% compared to the control. This effect was closely matched by the 0.8 % neem extract treatment, with 40-70% lower weevil numbers than in the control. The clove extract had 40-50% fewer weevils than in the control; however, there was less consistent efficiency throughout the experimental period. Besides, pepper extract field activity against *C. sordidus* decreased with exposure time (Table 3.3), an effect that was also reported for *P. nigrum* on Colorado potato beetles (Scott et al., 2003). This might be explained with the loss of

volatility of pepper extracts and thus a time-dependent declining repellent effect on weevils. All other treatments were too variable or less efficient in controlling the weevil population.

5.3 Biological control

5.3.1 Biological control

A biological control strategy is a tool in pest management that seeks to change from the dependence on chemicals to ecological approaches. This increased interest to use biological agents to control insect pests has led to the testing of many naturally occurring microorganisms including entomopathogenic fungi. Entomopathogenic fungi are naturally important mortality factors to some insect pests. However, their limited use has been to identify strains with a rapid kill, mass production and appropriate formulation. In respect to that, presented below are the discussion of the efficacy of identified fungal Isolates, and how formulation affected their ability to reduce rhizome damage of potted plants

5.3.2 Treatment effects on weevil developmental stages

Entomopathogenic isolates from a host organism are typical of great virulence that leads to reduced level of damage (Liu et al., 2002). Using molecular data such as the rDNA internal transcribed spacer (ITS) and rDNA large subunit (LSU) sequence, isolates were identified to species level (Raja et al., 2017). Of the three fungal strains isolated from weevil cadaver, *Cs* and *Fv* were of great virulence against the different life stages of *C. sordidus* than *Bb* and *FOSC* (Figures 4.1, 4.2). They belong to genera *Dothideomycetes* (*Cs*) and *Sordariomycetes (Fv* and *FOSC*) that are known to have a wide host range and geographical distribution (Manamgoda et al., 2015, 2012).

Variation in virulence among fungal isolates has often been reported (Alshareef and Robson, 2014; Valero-jiménez et al., 2014) and can be compensated for to some extent with increasing conidia concentration as has been demonstrated in the current study (Figure 4.1c). The pathogen's application time to the developmental stage of the pest will also affect the degree of disease severity. For example, the physiochemical properties of the egg, larvae, or adult cuticle (Grizanova et al., 2019; Inglis et al., 2001) may affect conidia adhesion and viability.

The observed rapid germination and hyphal growth of *Fv* spores were also reported on *Melodogyne graminicola*, a nematode that damages rice roots (Le et al., 2016) and *Tropidacris collaris*, a grasshopper pest that consumes all plant material (Pelizza et al., 2011), a fungal

characteristic that may be related to a relatively high infectivity rate. After spore attachment and germination on the host surface, the fungus penetrates through the insect cuticle, joints or creases where the insect's protective covering is thinner, with either germ tubes or an appressorium formation to infect the weevil host (St Leger and Wang, 2010). The application of adhesive fungal suspensions may lead to an improved pathogen virulence towards insect pests with hard to penetrate cuticles. In agreement with (Njau et al., 2011), the mortality of adult weevils was generally lower than that of eggs and larvae, a variation that could be attributable to differences in the composition and thickness of the cuticle for the various stages of the weevil life cycle. After the death of the weevil, the fungus grew out through the exoskeleton and began to produce spores.

5.3.3 Treatment effects on potted plants

The weevil *C. sordidus* affects Musa and Ensete species with stunted growth, leaf yellowing, loss of roots and eventually premature death (Okolle et al., 2009); however, plants have few or no symptoms when treated with appropriate infectious biological control agents. Entomopathogenic endophytic fungi not only target the various life stages of *C. sordidus* but also grow into tunnels (galleries) within the corm (Figure 4.7) where they can infect feeding pest/pathogens. Despite mycelia growth in larvae feeding tunnels, fusarium wilt disease and corm discoloration symptoms were not observed.

The ability of fungal isolates to grow, infect and cause disease to the insect pest is dependent on optimum temperature of 15-30°C (Ansari et al., 2014; Inglis et al., 2001), soil properties (pH, available phosphorus and organic carbon) (Miranda and Harris, 1994; Rath et al., 1992; Tawaraya et al., 1996) of which were all in the recommended range for the current study. However, fungi with a slow growth pattern on infected cadaver such as *Cs* rely on insect carbohydrates rather than soil carbon (Vegaa et al., 2009). The number of spores per gram of soil was greatest when conidia were applied on sorghum (Figure 4.4) that presumably supplied carbon and nutrient required for fungal multiplication (Zahmatkesh et al., 2017). However, the number of spores in the soil did not correlate well with fungal infectivity for example *Fv* with the greatest soil spore density (Figure 4.4) was not most effective against weevil damage (Figure 4.6b). Nonetheless, application of fungal strains before weevil infestation is a preferred option, for it allowed fungal establishment which reduced *C. sordidus* rhizome damage.

In this study, we were able to ascertain that isolates of *Cs*, *Fv* and *FOSC* are pathogenic to *C. sordidus* both in laboratory and pot experiments. All strains, but in particular *Cs*, show promising potentials as entomopathogenic fungi when applied on sorghum to plantain plants before weevil infestation. They can then self-propagate and survive in moderately acidic soils with adequate levels of organic carbon to infect and cause mortality to *C. sordidus*. However, further work is needed to test their biological potential on-field plants and whether their efficacy is sufficient enough to provide crop protection against weevil damage.

5.4 General recommendation and outlook

The recommendation that can be drawn based on this current study toward banana weevil controls is based on the host resistance, use of locally available insecticidal plant material and isolated entomogenous fungi.

5.4.1 Host plant resistance

Host plant resistance is ecologically compatible with agroecosystem diversity and blends well with other direct control strategies. Pre-formed phenolics in both cultivars and their elevated accumulation (induction) upon *C. sordidus* feeding and or MJ application along with reduced damage in susceptible cultivar, confirms its passive and active defense role in plants against herbivores. Constitutive and timely induced host defense work together to prevent further damage caused by *C. sordidus* larvae feeding in both susceptible 'Mbwazirume' and tolerant 'Km5' cultivars. Plants screened with relatively high polyphenolic compounds are resistant to weevil damage, therefore when establishing a banana/plantain plantation, we recommend field design that incorporates resistant banana/plantain cultivar together with susceptible ones. This will disrupt the breeding processes of banana weevil which involves host location, acceptance to oviposit and suitability of larval survival. However, research to evaluate and quantify the damage reduction due to "resistant-susceptible planting design" should be conducted first. We, also recommend use of characterized phenolic polymers as molecular probes to investigate the expression pattern of lignification and suberization genes linked to protein, transcripts and metabolites in response to weevil damage in banana cultivars. Once these genes are identified, they could be further used as molecular markers in banana resistance breeding. Until resistant banana cultivars with similar quality/taste properties to susceptible ones are available, the protective use of Jasmonate spray on leaves could help to minimize losses through the induction

of herbivore defense polyphenols in plantations of susceptible cultivars, a strategy that can be adopted in *C. sordidus* management after field testing.

5.4.2 Botanical plant extracts

Botanical extracts of clove, neem, and pepper and their synthetic analogs (Eugenol, Eugenylacetate, β-caryophyllene and N-Isobutylamine) restrained the *C. sordidus* performance through egg hatch inhibition, larviciding and adult repellency, thus can serve as an alternative to the synthetic pesticide. We, recommend the use of any of the three extracts at 0.8 to 1% (w/v) concentration to dip banana suckers before planting. To already infested plantations, pour unfiltered extracts at the collars (primary site of weevil attack) of pseudostem of banana/plantain mats at 3 weeks interval. And since harvested pseudostem emits banana weevil attractants, we recommend the pouring of botanical extracts on every freshly harvested pseudostem stumps. The toxicology of these botanical extracts and their synthetic analogs to penetrate *C. sordidus* cuticle and their metabolic targets were beyond the scope of this study.

5.4.3 Entomogeneous fungi

Soil entomogenous fungi are biological agents that traditionally cause insect pest mortality and can serve as alternatives to synthetic pesticides once boosted up with organic carbon source and or an effective strain. Out of the three fungal isolates, *Curvularia senegalensis* and *Fusarium verticillioides,* for the first time they substantially reduced weevil damage through their direct effect on eggs, larvae and adult mortality. They are recommended, therefore, to be considered potential pathogens to all *C. sordidus* developmental stages. They were able to self-propagate, infect and grew on eggs, larvae and adults, and their density in the inoculated soil pots caused the natural epizootics to banana weevil population which reduced rhizome damage. We also recommend the application of fungal inoculum at the time of establishing a plantation by dipping the sackers in the inoculum of at least 10^6 conidia m^{-1}. Since the rDNA ITS could not separate *Fusarium oxysporum* species complex into its members (clades), the multi-locus sequence typing (MLST) approach is proposed. Protective gear should be used when handling the inoculum, for they are categorized in biosecurity level BSL-1 or risk group RG 1. In conclusion, since no single control strategy can produce the desired results as advocated by IPM programs, we recommend combining host resistance in the design of a plantation, dip the planting materials into botanical extracts and or entomogenous fungal inoculum. This will reduce the reliance on chemical

pesticides and associated risk of pesticide resistance to contribute to sustainable weevil management practices.

6.0 General References

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7. Summary

Each year 25-75% banana yields are lost to *Cosmopolites sordidus,* one of the major pests of banana/plantain plants. This loss is common with resource-limited farmers who cannot afford the frequent application of insecticides due to their cost and developed resistance by weevils. Larvae, the most destructive life stage, occupy ecologically different microenvironments from adult weevils, thus least affected by synthetic insecticides. Feeding of larvae on banana rhizomes interfere with the established and emerging roots which reduce water and nutrient uptake and consequently weaken the plant stability during windy weather. Integrated pest management (IPM) is being promoted, for a single control strategy produces limited and or unsustainable results. IPM options for banana weevils include habitat management (cultural control), biological control, host resistance, botanical control and chemical control as last resort. Of the above IPM strategies, this research evaluated host resistance, botanical plant extracts and entomogenous fungi to contribute to the overall goal of reducing synthetic insecticides use. In the evaluation of host resistance, physiochemical of phenolic origin; lignin and suberin were considered. Comparably, weevil and methyl jasmonate treatment, induced higher deposits of lignin and suberin, cellular modifications, and high total phenolic content as well as antioxidant capacity in "Km5" than "Mbwazirume" banana cultivars. Induced polyphenols reduced weevil damage to less than 5% in "Km5" compared to 11% damage in the "Mbwazirume" cultivar. However, with the onetime application of 0.01% methyl jasmonate, "Mbwazirume", had 50% reduced weevil damage compared to untreated control.

Extracts from dried clove buds (*Syzygium aromaticum*), pepper fruits (*Piper guineense*) and neem seeds (*Azadirachta indica*) and their synthetic analogs were evaluated as botanical control option to Carbofuran against *C. sordidus* in the laboratory and infested field experiments. Efficacy of plant extracts and their synthetic analogs, revealed egg hatch inhibitory effect, larvicidal toxicity and adult repellency variation. For instance, clove extracts and its synthetic analogs had the lower egg inhibitory dose (ID_{50}) of 0.08 to 0.22% than black pepper (0.24 to 0.75%), and half the ID_{50} value caused 50% larvae mortality. However, in 6 to 48 hours pepper repelled 80 - 98%, clove 78 - 90% and neem 63 - 75% adult weevils, an effect that significantly (P =0.001) reduced field weevil population.

Lastly, three Entomogenous fungi; *Curvularia senegalensis, Fusarium verticillioides,* and *Fusarium oxysporum* species complex (FOSC) were also evaluated for their ability to infect weevil eggs,

larvae and adult weevils, and to reduce weevil damage in potted plants. *C. senegalensis* and *F. verticillioides* greatly affected egg hatching and larval survival, for instance, they caused 75 to 90% eggs hatch inhibition, unlike the 25 to 55% egg hatch inhibition for *Beauveria bassiana* and FOSC. Besides that, fungal treated plants 14 days before weevils, had significantly high SPAD value (P <0.0001), less than 20% rhizome damage and predictive weevil mortality R^2 = 0.46. Rhizome damage was greatly reduced by *C. senegalensis,* followed by *B. bassiana* and *F. verticillioides,* and it is the first record to demonstrate that *C. senegalensis* and *F. verticillioides* are pathogenic to *C. sordidus.* In conclusion, an IPM that combines host resistance with locally available botanic extracts and effective entomogenous fungi may provide a sustainable intervention in the management of the weevil population and their damages to benefit both commercial and resource-limited farmers.

8. Zusammenfassung

Jedes Jahr gehen 25-75% der Bananenerträge an Cosmopolites sordidus verloren, einen der Hauptschädlinge von Bananen- / Wegerichpflanzen. Dieser Verlust tritt häufig bei Landwirten mit begrenzten Ressourcen auf, die sich die häufige Anwendung von Insektiziden aufgrund ihrer Kosten und der entwickelten Resistenz von Rüsselkäfern nicht leisten können. Larven, das zerstörerischste Lebensstadium, besetzen eine ökologisch andere Mikroumgebung als erwachsene Rüsselkäfer und sind daher am wenigsten von synthetischen Insektiziden betroffen. Die Fütterung von Larven mit Bananen-Rhizomen stört die etablierten und entstehenden Wurzeln, wodurch die Wasser- und Nährstoffaufnahme verringert wird, und schwächt folglich die Pflanzenstabilität bei windigem Wetter. Das integrierte Schädlingsmanagement (IPM) wird gefördert, da eine einzige Bekämpfungsstrategie nur begrenzte und / oder nicht nachhaltige Ergebnisse liefert. IPM-Optionen für Bananenrüsselkäfer umfassen Habitatmanagement (kulturelle Kontrolle), biologische Kontrolle, Wirtsresistenz, botanische Kontrolle und chemische Kontrolle als letztes Mittel. Von den oben genannten IPM-Strategien bewertete diese Studie die Resistenz des Wirts, botanische Pflanzenextrakte und entomogene Pilze mit dem Ziel, zum Gesamtziel der Reduzierung des Einsatzes synthetischer Insektizide beizutragen. Bei der Bewertung der Wirtsresistenz werden Physiochemikalien phenolischen Ursprungs; Lignin und Suberin wurden berücksichtigt. Vergleichsweise induzierte die Behandlung mit Rüsselkäfern und Methyljasmonat höhere Ablagerungen von Lignin und Suberin, zelluläre Modifikationen und einen hohen Gesamtphenolgehalt sowie eine höhere Antioxidationskapazität in „Km5“ als in Bananensorten mit „Mbwazirume“. Induzierte Polyphenole reduzierten den Rüsselkäferschaden in „Km5“ auf weniger als 5% im Vergleich zu 11% in der Sorte „Mbwazirume“. Bei einmaliger Anwendung von 0,01% Methyljasmonat hatte "Mbwazirume" jedoch eine um 50% verringerte Schädigung des Rüsselkäfers im Vergleich zur unbehandelten Kontrolle.

Extrakte aus getrockneten Nelkenknospen (Syzygium aromaticum), Pfefferfrüchten (Piper guineense) und Neemsamen (Azadirachta indica) und ihre synthetischen Analoga wurden im Labor und in befallenen Feldversuchen als botanische Kontrolloption für Carbofuran gegen C. sordidus bewertet. Die Wirksamkeit von Pflanzenextrakten und ihren synthetischen Analoga zeigte eine hemmende Wirkung auf das Schlüpfen von Eiern, eine larvizide Toxizität und eine Variation der Abstoßungsfähigkeit bei Erwachsenen. Zum Beispiel hatten Nelkenextrakte und ihre synthetischen Analoga die niedrigere Eihemmungsdosis (ID50) von 0,08 bis 0,22% als schwarzer

Pfeffer (0,24 bis 0,75%), und die Hälfte des ID50-Wertes verursachte eine 50% ige Larvensterblichkeit. In 6 bis 48 Stunden stieß Pfeffer jedoch 80 - 98%, Gewürznelke 78 - 90% und Neem 63 - 75% erwachsene Rüsselkäfer ab, ein Effekt, der die Feldrüsselkäferpopulation signifikant (P = 0,001) reduzierte.

Zuletzt drei entomogene Pilze; *Curvularia senegalensis, Fusarium verticillioides* und *Fusarium oxysporum* species complex (FOSC) wurden ebenfalls auf ihre Fähigkeit hin untersucht, Rüsselkäfereier, Larven und erwachsene Rüsselkäfer zu infizieren und Rüsselkäferschäden in Topfpflanzen zu verringern. *C. senegalensis* und *F. verticillioides* beeinflussten das Schlüpfen von Eiern und das Überleben der Larven stark. Beispielsweise verursachten sie eine Hemmung des Schlupfes von 75 bis 90%, im Gegensatz zu der Hemmung des Schlupfens von 25 bis 55% bei Beauveria bassiana und FOSC. Außerdem hatten mit Pilzen behandelte Pflanzen 14 Tage vor Rüsselkäfern einen signifikant hohen SPAD-Wert (P <0,0001), weniger als 20% Rhizomschaden und eine prädiktive Rüsselkäfersterblichkeit R2 = 0,46. Der Rhizomschaden wurde durch *C. senegalensis*, gefolgt von *B. bassiana* und *F. verticillioides*, stark reduziert, und es ist die erste Aufzeichnung, die demostratiert, dass *C. senegalensis* und *F. verticillioides* für *C. sordidus* pathogen sind. Zusammenfassend kann ein IPM, das Wirtsresistenz mit lokal verfügbaren Pflanzenextrakten und wirksamen entomogenen Pilzen kombiniert, ein nachhaltiges Eingreifen in das Management der Rüsselkäferpopulation und ihrer Schäden zum Nutzen sowohl kommerzieller als auch ressourcenbeschränkter Landwirte ermöglichen.

9. Author's declaration

I Elyeza Bakaze, hereby declare that this doctoral thesis is a result of my work and that no information presented that has not been officially acknowledged, this include aids and literature used. The content and the wording are entirely my work, for I am aware that the digital version of my thesis can and or will be checked for plagiarism with the help of a software program. Furthermore, I assured that the work has not been used partly or in full for achieving any academic degree.

…… …….

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・Working on a PhD thesis titled: "Evaluation of eco-friendly management strategies of banana weevil *Cosmopolites sordidus* Germar" under the supervision of Prof. Dr. Jens Wünsche.

・Evaluation of three fungal strain isolates; *Curvularia senegalensis, Fusarium moniliforme (F. verticillioides*), and *Fusarium oxysporum* species complex against *Cosmopolites sordidus* Germar (Coleoptera: Curculionidae) both in the laboratory and potted experiment.

・This involved fungal isolation, culturing and morphological identification, and sequencing their rDNA internal transcribed spacer (ITS) and rDNA large subunit (LSU) fragments which were later compared to GenBank and MycoID databases.

・Evaluation of host plant resistant mechanism against *C. sordidus*, through histochemical analyses of cellular structural modification, and quantification acetone soluble polyphenol and their antioxidant capacity.

Business or sector Institut Für Kulturpflanzenwissenschaften (340 f)

01/05/2012–31/08/2013

National Agricultural Research Laboratory (NARL) under Agricultural Banana support project II (ABSPII) funded project, Kampala (Uganda)

・Bulking nematode transgenic lines in such a way to raise enough plants to screen for nematode resistance and retain backups in tissue culture and enough copies for planting confined field trial (CFT). I was involved in medium preparation (proliferation medium), sub culturing plants and weaning/potting plants. I also participated in challenging potted plants with parasitic nematodes that were microscopically isolated and cultured on carrots in the laboratory.

・Selection of resistant lines to parasitic nematodes and the corresponding backup weaned and planted in the CFT. I participated in the planning and planting of CFT.

・Molecular characterization of CFT transgenic lines; this involved working in a team of which I was charged with DNA extraction using CTAB as the buffer and running PCR with Actin primers first and thereafter with gene specific primers to verify the presence of the gene(s). I also used the PCR positive lines to do protein analyses which included extraction, quantification with Bradford, ELISA, and SDS-PAGE before doing western blotting. With the Western, I worked with conjugated antibodies which were anti goat bred with my protein of the transgenic plants. Then compared expressed proteins to nematode damage data on roots.

EDUCATION AND TRAINING

02/09/2015–Present
PhD. Agriculture Science
Hohenheim University, Stuttgart (Germany)

05/09/2007–16/01/2010
Masters of Science degree in Molecular Biology
Makerere University, Kampala (Uganda)

2002–2005
Bachelors of Vocational Studies in Agriculture/Education
Kyambogo University, Kampala (Uganda)

01/02/2000–20/12/2001
Uganda Advanced Certificate of Education
Luwero Secondary School, Kampala (Uganda)
Subjects offered; Biology, Agriculture, Geography and General paper

ADDITIONAL INFORMATION

Publications
Bakaze, E. 2011. Evaluation of *Bacillus thuringiensis* crystal (CRY6A) and Carica papaya cystatin toxins against the banana weevil (*Cosmopolites sordidus*) using a novel diet and construction of a plant transformation vector with two stacked toxin genes. Makerere University, URL:http://hdl.handle.net/10570/3967.

Bakaze, E., Dzomeku, B.M., Appiah-kubi, Z., Larbi -Koranteng, S., Wünsche, J., 2020a. Fungal Isolates from Banana Weevils (Cosmopolites sordidus) Cadaver as a Pest Control Option. Am. J. Sci. Eng. Res. 3, 23–44.

Bakaze, E., Dzomeku, B.M., Wünsche, J.-N., 2020b. Banana defense responses to *Cosmopolites sordidus* feeding and methyl jasmonate application. Ann. Appl. Biol. 26 August, 1–30. https://doi.org/10.1111/aab.12638

Bakaze, E., Kiggundu, A., Tushemereirwe, W., 2018. Use of Artificial Diets with Plant Material to evaluate Banana Cultivars for Resistance to Cosmopolites sordidus. Uganda J. Agric. Sci. 18, 103–109. https://doi.org/http://dx.doi.org/10.4314/ujas.v18i2.4

Bakaze, E., Kofler, J., Dzomeku, B.M., Wünsche, J., 2020c. Natural Compounds with Potential Insecticidal Properties against Banana Weevil *Cosmopolites sordidus.* Am. J. Sci. Eng. Res. 3, 11–22.

Conferences

Banana weevil borer (*Cosmopolites sordidus*): infestation, damage and control options; at Science Policy Forum "Advances in research in agriculture and food security in Ghana and theirimplications" Findings of the Biomassweb project**,** 8 December 2017, FARA headquarters, Accra.

Efficacy of crude extracts and major chemical components of clove, pepper, and neem against *Cosmopolites sordidus*", 50 Jahrestagung der Deutschen Gartenbauwissenschaftlichen Gesellschaft in Freising 2015

Efficacy of crude extracts and major chemical components of clove, pepper, and neem against *Cosmopolites sordidus.* ELLS Scientific Student Conference in Warsaw, 2014

Successful in vitro rearing of banana weevil *Cosmopolites sordidus* on artificial diet and its potential forrapid screening of genotypes for resistance at the INTERNATIONAL ISHS-PROMUSA SYMPOSIUMat Salvador, Bahia- Brazil October 10th– 14th 2011

References

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