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Faculty of Biosciences
Department of Plant Sciences

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Sugarcane Production in Malawi: Pest, Pesticides and Potential for Biological Control

Sukkerrørpoduksjon i Malawi: skadedyr, plantevernmidler og potensial for biologisk kontroll

Trust Kasambala Donga

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TRUST KASAMBALA DONGA

Norwegian University of Life Sciences
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Department of Plant Sciences

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PhD supervisors:

Professor Richard Meadow

Norwegian University of Life Sciences, Department of Plant Sciences, P.O. Box 5003, N0-1432 Ås, Norway

Dr. Ingeborg Klingen

Norwegian Institute for Bioeconomy Research, Biotechnology and Plant Health Division. P.O. Box 115, NO-1431 Ås, Norway

Professor Ole Martin Eklo

Norwegian Institute for Bioeconomy Research, Biotechnology and Plant Health Division. P.O. Box 115, NO-1431 Ås, Norway

Professor Bishal Sitaula

Norwegian University of Life Sciences, Department of International Environment and Development Studies, P.O. Box 5003, N0-1432 Ås, Norway

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Trust Kasambala Donga

Ås, July 2018

Summary

Sugarcane is an importance source of energy and livelihoods worldwide. The production of sugarcane is significantly affected by several insects, weeds and pathogens commonly referred to as pests. In addition, climate scientists predict that climate change or variability will affect sugarcane production and its associated pests. Chemicals called pesticides, beneficial pathogens and insects called natural enemies or biological control agents are used to control these pests. Little is known about the diversity and richness of both pest and natural enemy species nor the properties of the pesticides used against them in Malawi. Few studies indicate that insects such as stemborers and aphids, and weeds are the most common pests; and that their control is heavily dependence on pesticides. Plant pathogens also infect sugarcane but are controlled using cultural methods. However, pesticides are harmful to the environment and improper use may lead to human poisoning. Knowing the main pests and using pesticides that are least harmful to the environment and natural enemies coupled with good crop management practices may contribute to solving this problem.

To document pest composition and how they were controlled, a review of literature, questionnaire and farm surveys were conducted in the major sugarcane growing areas of Malawi. The questionnaire survey was administered to 55 farmers and 7 representatives of 1474 farmers. We collected 221 insect samples from 48 sugarcane fields and isolated beneficial fungi from 12 soil and 60 plant samples collected from 12 sugarcane fields in southern Malawi, respectively. The best way to inoculate sugarcane was also determined in a potted experiment conducted using a commercially available formulation of beneficial fungi (*Beauveria bassiana* strain GHA). We identified the fungi and insects samples to genus and/or species level largely using morphological characteristics. Molecular characterization based on partial sequencing of Bloc gene region of 50 fungal samples and cytochrome oxidase subunit I (COI) gene region in 65 insect samples, respectively, were conducted to support morphological identifications. Separate DNA polymorphism and phylogenetic analyses were performed for the insect and fungal samples. Environmental and human health risks associated with pesticides in use were determined using the environmental impact quotient (EIQ) and World Health Organization (WHO) Classification of Pesticides by hazard. We also explored the likely impact that climate change or variability will have on the type and amount of pesticides used in sugarcane production using Malawi as a case study.

The results indicated that weeds, insect pests and plant pathogens infest sugarcane in Malawi. The main insect pests were Lepidopteran stemborers (*Chilo partellus* and *Busseola fusca*), soil-dwelling insects' pests (*Heteronychus licas* and *H. arator*, *Anomala* sp.), sugarcane thrips (*Fulmekiola serrata*), red spider mites (*Tetranychus urticae*), aphids (*Sipha flava*) and the fall army worm (*Spodoptera frugiperda* sp. 1). DNA polymorphism analysis revealed low genetic differentiation among *C. partellus* and *B. fusca* populations. A total of 16 pesticides were used to manage the pests. These are slightly to moderately hazardous to humans, 50% are highly toxic to bees and 70% can contaminate the environment. Individuals who sprayed these pesticides had minimal protective wear. At least 65% had experienced skin irritation, headache, coughing and running nose as a result of being exposed to these pesticides. Climate variability will alter the amount and type of pesticides through negative effects of high temperature on the efficacy of less toxic pesticides especially cypermethrin, increased pest severity and leaching of sorbed pesticides through high rainfall intensity and increased frequency of floods.

Beneficial fungi in three genera namely *Beauveria*, *Metarhizium* and *Isaria* were identified from soil and sugarcane samples collected from southern Malawi. More isolates (81.7%) were collected from soil than from plants (36.7%). The majority of these isolates (72%) were *Beauveria* species. Molecular identification and phylogenetic analysis identified the *Beauveria* isolates as *B. bassiana* and were closely related to *B. bassiana* AFNEO_1 clade isolated from the coffee berry borer, *Hypothenemus hampei* in coffee fields of South America and in Africa. However, the Malawian *B. bassiana* clearly clustered in a separate clade. This is the first report of *B. bassiana* occurring as an endophytes of sugarcane; and *B. bassiana*, *Metarhizium* and *Isaria* species occurrence in agricultural fields in Malawi.

Results from the sugarcane inoculation experiment showed that *B. bassiana* could be effectively inoculated in sugarcane using foliar and soil sprays, and stem injections. Stem injections were highly effective (75%) compared to foliar sprays (43%) and soil sprays (25%) plants inoculated, respectively. The inoculated *B. bassiana* was recovered in both old and new leaves and stem tissue, even though the recovery rate decreased with time. However, plants that had got stem injections were much shorter that plants that had foliar and soil inoculation, and control plants.

The results especially those on natural occurrence of beneficial fungi particularly *B. bassiana* and *Metarhizium* sp. will be useful in the control of not only of pests in sugarcane but also in several crops mainly vegetables.

Sammendrag

Sukkerrør er en viktig kilde til energi og som levebrød over hele verden. Produksjonen av sukkerrør er betydelig påvirket av insekter, ugras og plantesykdommer ofte betegnet som skadegjørere. I tillegg forutsetter klimaforskere at klimaendringer eller variasjon i klima vil påvirke sukkerrørsproduksjonen og tilhørende skadegjørere. Kjemiske plantevernmidler og biologiske kontrollmetoder brukes til å kontrollere disse skadegjørerne. I Malawi kjenner vi lite til forekomst og diversitet av skade- og nytteorganismer i sukkerrørproduksjonen eller til egenskapene til plantevernmidlene som brukes. Tidligere studier tyder på at ulike sommerfugllarver, bladlus og ulike ugrasarter er blant de vanligste skadeorganismene og at kontroll er sterkt avhengighet av plantevernmidler. Plantevernmidler kan imidlertid være skadelige for helse og miljø. Å kjenne de viktigste skadegjørerne og bruke plantevernmidler som er minst mulig skadelige for miljøet og nytteorganismer kombinert med god agronomi, kan bidra til å løse dette problemet.

For å dokumentere sammensetningen og kontroll av skadegjørerne, ble litteratur gjennomgått og det ble sendt ut spørreskjema til bøndene i hovedområdene for produksjon av sukkerrør i Malawi. Spørreundersøkelsen ble sendt ut til 55 bønder og 7 representanter for 1474 bønder. Videre samlet vi 221 insektsprøver fra 48 sukkerrørfelt, isolerte nyttesopp fra 12 jordprøver og 60 planteprøver fra 12 sukkerrørfelt i det sørlige Malawi. Videre ble det utført potteforsøk med sukkerrør får å finne den beste måten å inokulere sukkerrør med nyttesoppen (*Beauveria bassiana* stamme GHA). Vi identifiserte nyttesopp- og insektsprøver til slekts- og / eller artsnivå primært ved hjelp av morfologiske egenskaper. Molekylær karakterisering basert på delvis sekvensering av Bloc-genregionen av 50 nyttesoppprøver og cytokromoksidase-underenhet I (COI) -genregionen i henholdsvis 65 insektsprøver ble utført for å understøtte morfologiske identifikasjoner. Separate DNA-polymorfisme og fylogenetiske analyser ble utført for insekt- og nyttesopp prøvene. Miljø og helsefare knyttet til bruk av plantevernmidlene ble bestemt ved bruk av miljøindikatoren EIQ (Environmental Impact Quotient) og Verdens helseorganisasjon (WHO) sin klassifisering av plantevernmidler og helsefare. Vi undersøkte også den mulige innvirkningen av klimaendringer eller variasjon i klima på bruk av plantevernmidler i sukkerrørsproduksjon med Malawi som et casestudie.

Resultatene viste at ugras og insekt- og edderkoppdyr er skadegjørere i sukkerrør i Malawi. Blant disse hører de viktigste skadedyrene til larver av tre ulike sommerfuglarter (*Chilo*

partellus, Busseola fusca og Spodoptera frugiperda sp. 1), de jordboende scarabidene (*Heteronychus licas, H. arator, Anomala sp.*), trips (*Fulmekiola serrata*), midd (*Tetranychus urticae*), bladlus (*Sipha flava*). DNA-polymorfi analyse viste små genetiske forskjeller mellom populasjonene av *C. partellus* og *B. fusca*. Tilsammen 16 plantevernmidler ble brukt til å bekjempe skadegjørerne. Disse plantevernmidlene er fra svak til moderat giftig for mennesker, 50% er svært giftige for bier og 70% kan forurense miljøet. De som påførte disse plantevernmidlene i sukkerørfeltet brukte minimalt med verneutstyr og minst 65% hadde opplevd hudirritasjon, hodepine, hoste og rennende nese som følge av eksponering av disse midlene. Klimaendringer og klimavariasjon vil endre behov og bruk i forhold til mengde og type plantevernmiddel. Økte temperaturer vil sannsynligvis redusere effektiviteten av mindre giftige plantevernmidler, spesielt cypermetrin. Videre vil utlekking av plantevernmidler som bindes sterkt til jord øke ved høy nedbørintensitet og økt frekvensen av flom.

Nyttesopp innen slektene *Beauveria, Metarhizium* og *Isaria* ble identifisert fra jord- og sukkerrørprøver samlet fra sørlige Malawi. Flere isolater (81,7%) ble samlet fra jord enn fra planter (36,7%). De fleste av disse isolatene (72%) var *Beauveria*-arter. Ved hjelp av molekylær identifikasjon og fylogenetisk analyse ble *Beauveria* isolatene identifisert *til artsnivå og alle viste seg å være B. bassiana. De* var nært relatert til *B. bassiana* AFNEO_1-clade isolert fra barkebillen *Hypothenemus hampei* i kaffefelt i Sør-Amerika og i Afrika. *B. bassiana* isolatene fra Malawi var tydelig delt inn (clustered) i ulike grupper (clades). Dette er den første rapporten om *B. bassiana* og *Isaria* spp. som endofytt i sukkerrør og også første rapport om *B. bassiana, Metarhizium sp* og *Isaria* sp i sukkerrørfelt i Malawi.

Resultater fra forsøk med inokulering av *B. bassiana* i ulike deler av sukkerrørplanten viste at *B. bassiana* effektivt kunne inokuleres både ved sprøyting av blader, vanning av jord/ røtter og stammeinjeksjon. Stammeinjeksjon var svært effektiv med henholdsvis (75%) sammenlignet med sprøyting av bladvverk (43%) og jord/rotvanning (25%). Etter inokulering ble *B. bassiana* gjenfunnet i både gamle og nye blader og stammevev, selv om grad av gjenfinning ble redusert med tiden. Imidlertid var planter som hadde fått stammeinjeksjon mye kortere enn planter som hadde blitt inokulert gjennom blad og jord/ røtter.

Resultatene fra surveys og forsøk med nyttesopp vil være nyttig i forbindelse med kontroll av skadedyr i sukkerrør og også i andre kulturer.

List of papers included in the thesis

This PhD thesis contains the following papers:

I. **Trust Kasambala Donga**, Richard Meadow, Bishal K. Sitaula and Ole M. Eklo 2018. Impact of climate variability on use and exposure of pesticides used in sugarcane production. Manuscript.

II. **Trust kasambala Donga** and Ole Martin Eklo. 2018. Environmental load of pesticides used in conventional sugarcane production in Malawi. Accepted: Crop Protection, 108: 71-77.

III. **Trust Kasambala Donga** and Richard Meadow. 2018. Determination of genetic diversity in *Chilo partellus, Busseola fusca* and *Spodoptera frugiperda* infesting sugarcane in Southern Malawi using DNA barcodes. Accepted: Insects, 9(3), 74.

IV. **Trust Kasambala Donga**, Richard Meadow, Nicolai V. Meyling and Ingeborg Klingen. 2018. Occurrence and diversity of fungal endophytes of sugarcane (*Saccharum officinarum*) tissues and insect pathogenic fungi in sugarcane fields in Malawi. Manuscript.

V. **Trust Kasambala Donga**, Fernando E. Vega and Ingeborg Klingen. 2018. Establishment of the fungal entomopathogen *Beauveria bassiana* as an endophyte in sugarcane, *Saccharum officinarum*. Accepted: Fungal Ecology.

1. General introduction

1.1. Importance of sugarcane

Sugar! Is the most preferred natural sweetener and energy source worldwide! While the healthy benefits of sugar is a source of constant debate in the developed countries (Ruxton *et al*., 2010), it is a source of livelihood to millions of people and is integral to the economic development program of sugar producing countries (Hess *et al*., 2016). About 80% of the world's sugar is derived from sugarcane (*Saccharum officinarum*: Poaceae) while the remaining 20% is from sugar beet (*Beta vulgaris*: Amaranthaceae; FAOSTAT, 2018). In addition to sugar, sugarcane is also used to produce ethanol, bagasse, and molasses and press mud (Solomon, 2011). The crop sugarcane is cultivated in about 100 countries in the tropics and subtropics (FAOSTAT, 2018). In 2016, worldwide sugarcane production was estimated at 1.89 billion tonnes (FAOSTAT, 2018). The Americas is the largest producer of sugarcane. African countries contribute 5.9% to the global production (FAOSTAT, 2018). Malawi produces approximately 2.1 million tonnes per year, representing 14.11% of the total production in southern Africa (ILLOVO, 2017; FAOSTAT, 2018).

1.2. Sugarcane production in Malawi

It grows well where there are long periods of sunlight (12-14 hrs.), temperature range is between 20°C and 35°C and, humidity is high, 80-85% (DAFF, 2014). The crop requires a minimum of 1,100mm of rain per year or equivalent water from irrigation during the main growth phase (AgriFutures Australia, 2017). However, ripening requires a dry period (DAFF, 2014). Well-drained, fertile sandy to clay soils with a pH between 6.0 and 7.7 are ideal for sugarcane growing (DAFF, 2014; AgriFutures Australia, 2017).

Sugarcane for milling into table sugar and associated products is grown along the shores of Lake Malawi and the Shire River Valley. In the Shire River Valley, mean annual precipitation fluctuates between 400 and 700 mm; minimum temperatures are between 14°C in July and 23°C in February; mean maximum temperatures are between 27°C and 37°C in June and October; and maximum temperatures of 43°C are not unusual in October (Phiri and Saka, 2009). The lakeshore districts of Nkhata Bay and Nkhotakota are high altitude areas with average annual

rainfall of 1490mm, minimum temperatures of 21°C in July and 24°C in January, and mean maximum temperature are 32°C in October.

Sugarcane is vegetatively propagated using cane setts. Cane setts are sugarcane stems cut into small sections having 3-6 internodes. The recommended seed cane rate is 8-10 ton per hectare. Recommended varieties include MN1, N14, N19, N25, NcO 376 and R570. Initial planting of rainfed sugarcane is usually done at the beginning of the rainy season i.e. October to January. A row spacing for irrigated sugarcane is 1.5m and 1.0m for rain fed cane. Ridges are made in such a way as to conserve water. Cane setts are planted end-to-end in furrow either 1.5 sticks or double sticks. The initial sugarcane planted is referred to as plant cane and subsequent crop arising from remnants of harvest of this initial crop is called ratoon cane. Within four days of post-planting irrigation, a pre-emergent herbicide is applied. Fields are dried off for 30 days before being burned and manually harvested. The crop is harvested yearly for 3-15 years without replanting.

Historically, sugarcane has been grown in commercial estates located at Dwangwa in Nkhota Kota and Nchalo in Chikwawa districts with the involvement of smallholder farmers determined by Acts of Parliament (Chinsinga, 2017). Since 2010, the Government of Malawi has been promoting sugarcane production among smallholder farmers as a means of fighting rural poverty (Chinsinga, 2017). This resulted in an increase in area under sugarcane cultivation between 2011 - 2012 (Fig. 1). These farmers are organized into farmer associations. Depending on background of the association - formed either by an African Development Bank or European Union with Government of Malawi support, or by a grouping of farmers acting independently; sugarcane grown by these farmers is either irrigated or rainfed (Chinsinga, 2017). However, despite the increase in hectares, Figure 2 indicate that the amount of sugarcane crushed and sugar produced in Malawi has been decreasing since 2014. High pest pressure and greater climate uncertainties i.e. changing onset and duration of rainfall season, increased drought risk and reducing available water supplies may be contributing factors to this yield decline (Knox *et al*., 2010; Kusangaya *et al*., 2014).

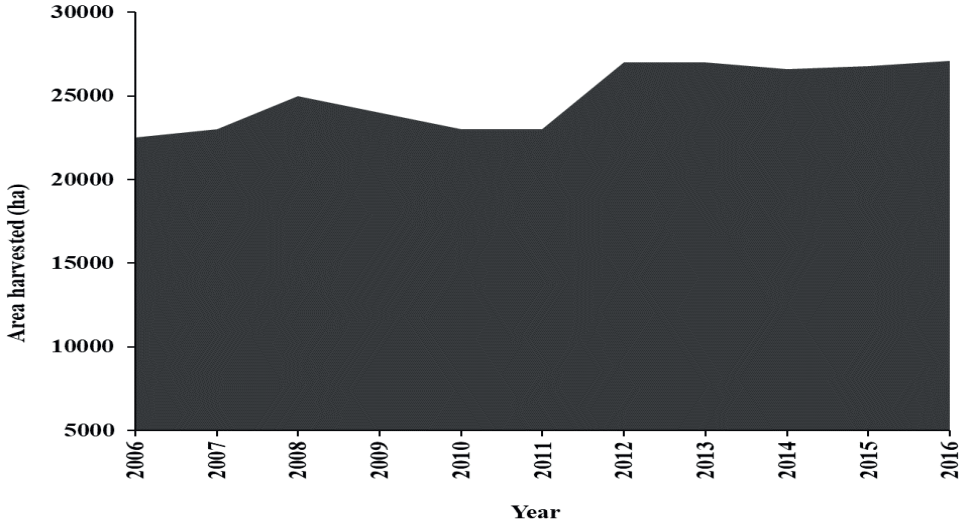

Figure 1. Trends in area under sugarcane production in Malawi for the period 2006-2016 (Source: FAOSTAT, 2018).

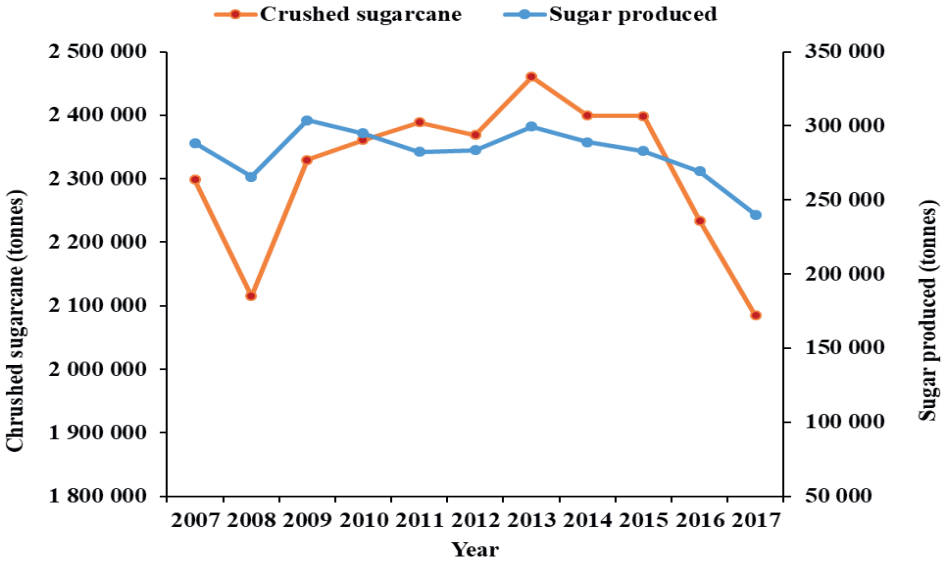

Figure 2. Amount of sugarcane crushed and sugar produced in Malawi for the period 2007-2017 (Source: ILLOVO Malawi, 2009; 2013; 2017).

1.3. Sugarcane production constraints in Malawi

1.3.1. Pest of sugarcane in Malawi

The sugarcane phytobiome includes over a thousand arthropod pests, numerous pathogens and weeds (Strong *et al*., 1977). Worldwide, the orders of insects that contain species that are economic pests of sugarcane are Lepidoptera (mainly stemborers), Hemiptera (aphids), Orthoptera (grasshoppers, locusts), Coleoptera (larvae of beetles commonly called white grubs), and Isoptera (termites), (Meager, undated). However, pest status and composition varies with geographical region. In Brazil, the sugarcane pest complex includes the spittlebug, *Mahanarva fimbriolata*; the curculionid *Sphenophorus levis* and sugarcane borer, *Diatraea saccharalis* (Dinardo-Miranda and Fracasso, 2013). In addition to *D. saccharalis*; the yellow sugarcane aphid (YSA), *Sipha flava*; the corn wireworm, *Melanotus communis*; the whitegrub, *Tomarus subtropicus*; and the lesser cornstalk borer (LCB), *Elasmopalpus lignosellus* are economic pests of sugarcane in United States of America (Cherry *et al.,* 2015). In the Indian subcontinent, the early shoot borer *Chilo infuscatellus*, the internode borer *Chilo Sacchariphagus* and the top borer *Scirpophaga excerptalis* cause significant yield losses (Nrip and Gaikwad, 2017). Much of the knowledge on the biology and management of sugarcane pests in Africa is derived from research conducted in South Africa. However, the research is focused on pests that are of economic importance to South Africa. Differences in pest composition and status, climate and crop management practices, may affect pest biology and behaviour in other countries. In South Africa, the African stalk borer, *Eldana saccharina*; white grubs, *Schizonycha affinis* and *Hypopholis sommeri* and thrips, *Fulmekiola serrata* are examples of important pest species (Way *et al*., 2011a, 2011b; Leslie *et al*., 2013). In Mozambique, *Chilo Sacchariphagus* is a pest of concern to the sugar industry (SASRI, 2014).

There is little information on the diversity and richness of pest species in Malawi. This is partly due to dependency of the Malawi sugarcane industry on South Africa for research and crop management practices. Since the climate of South Africa is different from that of Malawi, some aspects of pest biology and ecology may vary from those in agroecological zones of Malawi. It only in the recent two decades that independent (not sponsored by government of Malawi initiated grants) and outgrowers have been allowed to grow sugarcane for milling. These farmers

do not have access to South African research outputs. In addition, compared to other cash crops such as tea and tobacco, research and extension structures aimed at addressing the needs of these farmers are non-existent.

Previous studies indicated the occurrence of white grubs, (*Heteronychus licas* and *H. arator*, *Anomala* sp.); thrips, *Fulmekiola serrata*; unidentified stemborer species, termites (*Macrotermes* spp), red spider mites (*Tetranychus urticae)* and aphids, (*Sipha flava and Melanaphis sacchari*) infests sugarcane in Malawi (Agricane, 2013; Conlong and Ganeshanshow, 2016; Koloko, 2014) White grubs are soil dwelling larvae of numerous beetle species (Curculionidae) that feed on the base of young sugarcane stalk and suck nutrients (Spaull, 2011). Their feeding on the roots results in stunted growth and sometimes crop failure (Way *et al*., 2011b). Thrips, red spider mites and aphids suck plant sap and their infestation results in sooty moulds, leaf necrosis, interfere with nutrient transport and may vector plant pathogens such as sugarcane mosaic virus. (Spaull, 2011; SASRI, 2014; Way *et al*., 2010a). Stemborers cause 'dead hearts' in young sugarcane plants while infestation in older plants renders the crop very susceptible to lodging (SASRI, 2014; Conlong *et al*., 2016). Hence, if not properly managed, arthropod pest infestation directly contributes to sugarcane yield loss.

1.3.2. Climate variability

Climate variability refers to 'variations in the mean state and other statistics (such as standard deviations, statistics of extremes, etc.) of the climate on all temporal and spatial scales beyond that of individual weather events' (IPCC, 2007). Climatic factors particularly precipitation and temperature affect both sugarcane and pest growth and development, but also farm operations. Generally, frost and drought negatively affect sugarcane growth. Increased severity of pests particularly aphids and thrips due a prolonged dry season has been reported in Mozambique, Swaziland, Zambia and Southern Malawi (ILLOVO climate change report, 2015; Koloko, 2016). Farm operations such as application of pest control measures and harvested are inhibited by prolonged flooding. On the other hand, a significant reduction in the amount and poor distribution of rainfall because of severe droughts or rising temperature affects availability of water for irrigation, resulting in poor crop yields (Emmet *et al*., 2013).

1.4. Management of sugarcane pests

1.4.1. Cultural control

Cultural control is based on the principle of creating an unfavourable environment for pest species through manipulation of normal agronomic practices. Cultural practices includes tillage, planting of pest-free materials and growing healthy plants that can withstand pest infestation. Quarantine regulations are in place reduce the risk of exchanging plant pathogens through the common practice of plating materials between sugarcane growing countries (Bailey, 2011). It is an international standard to subject seedcane to a hot water treatment (at 50°C for 2hours) followed by a dip in a fungicide to control seed borne pests (Davis and Bailey 2000). Roguing and burning of sugarcane showing signs of pathogen infection is also practiced. In South Africa, cultural control is the most viable option for managing the indigenous stemborer, *Eldana saccharina* as biological control using insect parasitoids is ineffective (Spaull, 2011). *E. saccharina* is also controlled through variety, nutrient and habitat management (Keeping and Meyer, 2002; Pillay and Ramouthar, 2015; Conlong *et al*., 2016). In Mexico and the United States of America, the Mexican rice borer, *Eoreuma loftini* is control by using resistant varieties (Showler and Castro 2010). Rotary tillage is used in the management of white grubs, *Dasylepida ishigakiensis* in Japan while deep ploughing is used in South Africa to manage high incidences of *Hypopholis sommeri, Schizonycha affinis, Adoretus fusculus*, *Astinopholis* sp, *Anomola* sp, *Heteronychus licas* and *Maladera* sp. (Kijima and Tarora 2010; Spaull, 2011). Varietal resistance, early planting during main season and nutrient management i.e. avoiding excessive nitrogen fertilization, are used for managing stemborers in Malawi (Koloko, 2014; Conlong *et al*., 2016).

1.4.2. Biological control

Biological control is defines as the practice of managing pest populations through the use of the pest's natural enemies and usually involves human intervention (Waage, 2007). It is an ecological approach for pest management. Examples of natural enemies are predators (lady beetles), parasitoids (numerous wasp species) and pathogens (bacteria, fungi and viruses). When large numbers of these natural enemies are released for control of a pest within a short period of time, it is called inundative biological control. Inoculative biological control involves periodic or season releases of natural enemies with the purpose of enhancing the efficacy of natural enemies

already present in the field (Hoy, 2008). Several natural enemies targeting various stages of insect pests have been identified.

Cotesia sesamia, C*otesia flavipes* and *Trichogramma* sp. are known to parasitoids of *B. fusca*, *C. partellus*, *Diatraea* sp. and *Scirpophaga excerptalis* (Ashraf and Fatima 1996; Botelho *et al*., 1999; Calatayud *et al*., 2011; Rutherford and Conlong, 2010; Goble *et al*., 2017). *Lydella minense, Paratheresia claripalpis* and *P. claripalpis* are also used to control *D. saccharalis* South America (Rossi and Fowler 2003; Willink *et al*. 1991). *Trichogramma Chilonis* provides effective control of *Chilo sacchariphagus* on Reunion Island (Goebel *et al*. 2010). A Granulovirus (ChiGV) and a bacterial strain, *Bacillus thuringensis* subsp. *Kurstaki* (Btk) are used against *Chilo infuscatellus* in Indian sugarcane (Kesavan *et al*., 2003; Rachappa *et al*., 2000). Biological control based on entomopathogenic fungi *Metarhizium anisopliae, Beauveria bassiana* and *B. brongniartii* is used to manage soil-borne pests like white grubs and termites, sucking insect pests such as aphids and spittle bugs, some stalkborers infesting sugarcane in Australia, Brazil, India, Indonesia, Pakistan, Reunion Island, South Africa, Thailand and United States of America (Arthurs and Dara, 2018; Li, 2010; Goble *et al*., 2017; Sallam, 2009). In Malawi, biological control (using egg parasitoids) are used on a very small scale at one estate to manage white grubs and stemborers, respectively (Koloko, 2014).

1.4.3. Chemical control

Chemical control involves the use of pesticides. World Health Organization (WHO) defines pesticides as '… chemical compounds that are used to kill pests, including insects, rodents, fungi and unwanted plants (weeds).' Pesticides are inherently toxic (hazardous) to man and the environment. The risk from a pesticide to man or environmental depends on the quantity used (exposure) and its toxicity. Pesticides risk is higher in developing countries and a large proportion of farmworkers suffer from pesticides poisoning (Kishi and Ladou, 2001). Several factors including poor regulatory and enforcement mechanisms; use of banned, highly toxic and obsolete pesticides; poor pesticides handling and storage, and lack or limited personal protective or spraying equipment contribute to higher pesticide risk (Thundiyil *et al*., 2003). Pesticides poisoning can occur via dermal (skin) contact, ingestion (mouth) and inhalation (Spaull, 2011). Pesticides poisoning may be acute (short-term) but also chronic (long-term) while organ failure and eventual death results from chronic pesticides exposure (Thundiyil *et al*., 2003). Pesticides

also contaminate soil, surface and underground water; kill beneficial organisms such as pollinators, pest's natural enemies, birds and bees (Aktar *et al*., 2009). Considering that that continuous and repeated use of a synthetic pesticides results in development of insecticide resistance and, toxicity of pesticides to humans and the environment, documenting the toxicity of and finding alternatives to chemicals currently used in sugarcane production in Malawi is of utmost importance.

2. The thesis

This thesis is on sugarcane production in Malawi and focuses on identification of Lepidopteran stemborer, pesticides used for managing sugarcane pests and the potential for biological control using beneficial fungi. The thesis is made up of 5 five manuscripts denoted as paper I – V. The manuscripts are based on literature and field survey, greenhouse experiment and laboratory analysis. A summary of project justification, methods used, main findings and conclusions are presented in sections below. Complete information on materials and methods, results, discussion are indicated in each respective paper.

2.1. Project justification

Insect pests are the most injurious pests and are responsible for about 50% crop losses in Africa (CABI, 2018). Management of these pests is currently biased towards insecticides use (Sheahan *et al*., 2017). The sugarcane industry in Malawi is the third largest consumer of pesticides in Malawi (GoM, 2013). Although pesticides help in reducing crop losses, the benefits are temporary. The continuous use of pesticides put humans and the environment at a greater risk of pesticide exposure (Lehtonen and Goebel, 2009; Lobin *et al*., 2017). Hence, identification of pesticides that carry a low risk, adoption of cultural practices that are known to significantly suppress pest populations and identification of alternatives to pesticides and integrating them in existing pest control programs can greatly contribute to reduction the risks arising from pesticides use. It is accepted the world over that this objective can be achieved by developing and implementing integrated pest management, IPM, approach (Parsa *et al*., 2014).

Implementation of an effective IPM program requires a good foundation (Orr and Ritchie, 2004; Parsa *et al*., 2014). The foundation is based on accurate identification of pest species present in the agroecosystem and availability of viable pest control alternatives (Overholt *et al*., 2001). Accurate pest identification is vital for making informed management decision. Morphological markers have long been used to identify organisms. Body length; antenna features; wing venation; setae and leg structure and arrangement are some of the morphological characteristics used to separate insect species. Analyzing these characters one by one requires a good technical training in insect taxonomy, it is time consuming and may not be practical where large numbers of insects are involved (Jalali *et al*., 2015). In addition, some morphologically similar species display variations in geographical distribution, behaviour, host preference and response to

control measures just to mention a few (Aseffa *et al*., 2006a; 2006b; Sezonlin *et al*, 2006a; 2006b). Molecular identification based on small fragments of mitochondrial DNA (mtDNA) or chloroplast DNA; isozymes and proteins markers that delimits species as pedigrees overcomes most of the problems associated with morphological and other classification systems (Hebert *et al*. 2003; Sreedevi *et al*., 2015). The maternally inherited mitochondrial gene, cytochrome oxidase I (Cox I) is widely used for distinguishing insects (Hebert *et al*. 2003; Jalali *et al*., 2015; Wang *et al*., 2016). However, amount of polymorphism identified and the statistical reliability of the results differ among the molecular markers (Sreedevi *et al*., 2015). Hence, since early 2000, integrating morphological and molecular markers has become accepted in insect taxonomy (Yang *et al*., 2012; Wang *et al*., 2016).

The second step in establishing an IPM program is documenting existing pest management measures employed by farmers. In IPM, the use of synthetic pesticides is minimal, as a last resort and is limited to less harmful pesticides. Therefore, quantifying the risks associated with current management options is crucial in helping farmers and policy makers adopt better pest management decisions that are environmentally benign (FAO, 2008). Several pesticides risk indicators or models have been developed. These models are mathematical equations that considers a variety of input data such as active ingredient toxicity, rate and frequency of application, chemical properties of the pesticides and farm size (FAO, 2008). Risk indicator models include the environmental impact quotient (EIQ), chemical hazard evaluation for management strategies (CHEMS1),multi-attribute toxicity factor (MATF), Norwegian environmental risk indicator (NERI), pesticides environmental risk indicator (PERI), environmental indicator model (SYNOPS), environmental potential risk indicator for pesticides (EPRIP), system for predicting the environmental impact of pesticides (SyPEP), environmental yardstick for pesticides (EYP) and the World Health Organization (WHO) classification of pesticides by hazard (Kovach *et al*., 1992; Levitan, 1997; WHO, 2009). Based on their inherent toxicity, WHO (2009) groups pesticides into 5 classes namely Ia: extremely hazardous; Ib: highly hazardous; II: moderately hazardous; III: slightly hazardous and U: unlikely to present acute hazard in normal use. The WHO (2009) classes mean that chemical identified as highly hazardous are more lethal and have a higher greater risk of poisoning than those that are slightly hazardous. The EIQ model is widely used in selecting the most benign pesticides (Kovach *et al*., 1992; Kniss and Coburn, 2015).

It is also used to compare the introduction of genetically modified organisms i.e. GMOs and is also recommended by Food and Agricultural Organization (FAO) of the United Nations for measuring the effect of introducing IPM (Eklo *et al*., 2003; Teng *et al*., 2005; Kromann *et al*., 2011; Brookes and Barfoot, 2015; Perry *et al*., 2016). The EIQ includes health risk and exposure of farmers, bystander, consumers and the environment. That means the WHO classes are included in the EIQ index. The EIQ model summarize all pesticide used during the season thus giving a total score for the environmental pesticide load/concentration (Kovach *et al*., 1992). The lower the EIQ value, the least hazardous the pesticides is. The EIQ model is also easier to use and requires only a few input data.

Managing pests with minimal environmental pesticide load requires the availability of effective non-chemical pest control alternatives (Lehtonen and Goebel, 2009). Deliberate actions aimed at enhancing the multiplication of biocontrol agents and improving soil health may significantly reduce the amount of pesticides used in sugarcane but also cost of control. For example, chemical control of *Diatraea centrella*, *Diatraea saccharalis* and *Castniomera licus*, the main pests of sugarcane in Guyana has been abandoned (Richards-Haynes, 2007; Lehtonen and Goebel, 2009; Guyana Sugar Corporation, 2017). Parasitism by the *Metagonistylum minense* and improved drainage and management practices provides effective control of these pests (Guyana Sugar Corporation, 2017). In Brazil, integration of an insect pathogenic fungi *Metarhizium anisopliae*-based biopesticide in the control regime of *Mahanarva fimbriola* results in effective control of the pest but at a 10 times less cost of synthetic insecticides (Ereno, 2002).

There are limited published studies focused on characterization of pest and beneficial organism in sugarcane production in Malawi. Few studies were conducted at Nchalo and Dwangwa Estates evaluating the performance of South African varieties under Malawi conditions (Isyagi and Whitbread, 2002; Khembo *et al*., 2005). A monitoring study initiated in 2002 on the spread of *C. sacchariphagus* found that the pest was not present at Dwangwa and Nchalo sugar estates (Way *et al*., 2004). Another study reported the occurrence of *Metarhizium* spp. on white grubs (4 isolates were identified from 154 cadavers) infesting sugarcane from undisclosed location in Malawi (Ngubane *et al*., 2012). All these studies were conducted in few commercial estates. No studies have been conducted on pest and insect pathogenic fungi occurrence, and pest management practices sugarcane under traditional farmers and outgrowers fields in Malawi.

Therefore, it is necessary to characterize the main Lepidopteran pests, document pesticide use and exposure, and find prospect for viable alternatives to pesticides.

Another factor to consider when developing an IPM program is feasibility or applicability of the pesticides alternatives to the actual implementers of the IPM strategy. Almost half of the Malawi population is illiterate and illiteracy is high in rural areas where the majority of the population lives (NSO, 2012; IMF, 2017). This means that the majority of farmers are illiterate. Therefore, they may fail to grasp and adopt technologies that require new skill acquision. In addition, these farmers use hand-operated knapsack and jecto sprayers are the main pesticide application equipment (Singa, 2007).

Finally, any IPM program to be adopted in Malawi needs to be presented to farmers in the context of reducing production costs and improving yields (Orr and Ritchie, 2004). The benefits and, how to deal with the risks (e.g. a minor pest becoming an economic pest; Ereno, 2002; van Antwerpen *et al*., 2008) associated with IPM need to be clearly define to farmers as they impact adoption (Pangapanga *et al*., 2012; Ward *et al*. 2016). This is especially important in the era of climate change where farmers need to make strategic decisions that enhance their ability to adapt to and mitigate the effects of climate change. Future climate projections under different scenarios suggest an increase in maximum temperatures for Malawi (Saka *et al*., 2012; Zinyengere *et al*., 2014). However, projection on precipitation indicate greater uncertainty and variations with locations (McSweeney *et al*., 2010; Saka *et al*., 2012; Gama *et al*., 2014). The northern and central part of the country is projected to have a 200-400 mm compared to increase in mean yearly precipitation a 50-200mm for southern Malawi (Fig. 1; Saka *et al*., 2012). This projected mean annual increase will be due to an increase in the proportion of rainfall that falls in heavy events of up to 19% occurring during December through February (McSweeney *et al*., 2010). Although it is difficult to determine to what extent climate change/variability will impact sugarcane production in Malawi, studies from elsewhere indicate that these projections will have a significant impact on moisture availability and will alter the biology of both host plants and/or associated arthropod species and pesticides use (Biggs *et al*., 2013; Delcour *et al*., 2015; Ewald *et al*., 2015; Gawander, 2007; Hallmann *et al*., 2017; Munguira *et al*., 2015; Noyes *et al*., 2009; Zhao and Li, 2015). In addition, there is still a lack of knowledge on how extreme climate events

such as droughts and floods will affect farmers' behaviour and practices pertaining to pesticides they use to control various crop pests.

2.2. Study objectives

The main aim of this study was to provide basic data required for development of integrated pest management strategies in sugarcane in Malawi, thereby contributing to reduced environmental pesticide load.

Specific objectives

i. To determine how sugarcane farmers in Malawi will respond in terms of pesticides use to climate variability and how the response will affect their exposure to pesticides using secondary data (Paper 1).
ii. To document existing pest control measures used by sugarcane farmers in Malawi and determine their corresponding environmental load (Paper II).
iii. To characterize the main Lepidopteran pests infesting sugarcane in Malawi (Paper III).
iv. To document and characterize the natural occurrence of potential beneficial fungal endophytes in sugarcane plant and insect pathogenic fungi in soils from sugarcane fields in Malawi that can be used as alternatives to inorganic pesticides (Paper IV).
v. To evaluate inoculation methods for establishing an entomopathogenic fungus (*Beauveria bassiana*) as an endophyte in sugarcane, and assess whether the inoculations affects plant growth (Paper V).

2.3. Materials and methods

All field surveys were conducted in Malawi in Nkhata Bay, Nkhota Kota, Chikwawa and Nsanje Districts, respectively. Laboratory experiments were conducted at Lilongwe University of Agriculture and Natural Resources' (LUANAR) Bunda Campus in Lilongwe and at Bvumbwe Agricultural Research Station at Bvumbwe in Thyolo District, respectively. Molecular analysis were conducted at Sugarcane Research Institute (SASRI), Mount Edgecombe, KwaZulu-Natal, South Africa and at Norwegian Institute for Bioeconomy Research, Ås, Norway. The field surveys and laboratory work were conducted between 2015 and 2018.

2.3.1. Insect collection and identification

About 221 insect samples were collected from sugarcane plants between June 2016 and March 2017, from 9 locations in Chikwawa and Nsanje Districts belonging to 5 agricultural extension planning areas of the Shire Valley Agricultural Development Division (Paper III). All larvae were preserved in 70% alcohol in 30 mL sealed vials and were kept at +4°C until morphological and molecular identification analysis. Morphological identification was based on descriptions provided by Meijirman and Ulenberg (1996) and FAO (2018). GeneJet Genomic DNA Purification kit (Thermo Scientific, Waltham, MA, USA) was used to extract DNA for use in molecular identification according to the manufacturer's instructions. Amplification of the partial cytochrome oxidase subunit I (Coi I) gene region was performed to confirm results of morphological identifications following the methods described by Folmer *et al*. (1994).

2.3.2. Pesticide and secondary data collection

Data on pesticide use and handling practices, and health effects experienced while handing pesticides were collected using a questionnaire survey between June 2015 and January 2016 from 55 individual sugarcane farmers and 6 key informants representing 1474 sugarcane farmers in Nkhata Bay, Nkhota Kota and Chikwawa districts, respectively (Paper I and II). The pesticide data from this survey was inputted into an online EIQ calculator available on Cornell University website (NYSIPM, 2017). Ecotoxicological data pertaining to the reported pesticides (Paper I and II) were obtained from the pesticides properties database of the University of Hertfordshire and WHO (2009). A review of published data on impact of climate change/variability on drivers of pesticides exposure was done using the pesticides used in sugarcane production in Malawi as a case study (Paper I).

2.3.3. Soil and sugarcane sample collection, and mycological analysis

Soil samples (10 per field, n = 60) and sugarcane plants (10 per location, n = 60) were collected with the help of a garden spade from 6 locations in Chikwawa District (Paper IV). The garden spade was disinfested between collection points by dipping in 70% alcohol to prevent cross-contamination (Klingen *et al*., 2002). Five heat-conditioned *G. mellonella* larvae were used to bait entomopathogenic fungi (Meyling and Eilenberg, 2007) from soil following procedures outlined by Clifton *et al.* (2015). Each fungal infected *G. mellonella* larvae was considered an isolate. Using a sterile scalpel, each plant was dissected into 3 separate parts: leaf, stem and root.

These plant sections were surface sterilized by passing them in household bleach (1% sodium for 3 min) and ethanol (70% for 1 min) followed by triple rinsing in sterile distilled water. The sterilized section were plated on Sabouraud Dextrose Agar (SDA, Oxoid) and incubated in the dark at 25±5°C.

Fungal growth ensuing from the edges of the sterilized plant sections and from *G. mellonella* larvae were identified morphologically to genus level by examining sporulation structures and conidia shape under the dissecting and light microscopy (Humber, 2012). Extraction of DNA was accomplished using DNeasy Plant Mini kit (Qiagen, Germany) following manufacturer's instruction (Goble *et al*., 2012). Molecular identification was based on amplification of Bloc intergenic region using primer pair B22U (5′-AGATTCGCAACGTCAACTT-3′) and B822L (5′-GTCGCAGCCAGAGCAACT-3′; Rehner *et al*., 2011). Sequencing for fungal isolates was done by GATC Biotech (in Germany) while SASRI (in South Africa) did for insect samples, respectively.

2.3.4. Phylogenetic analysis

Phylogenetic analysis were carried out for insect samples and fungal isolates (Paper III and IV) DNA sequences were edited and assembled using CLC Main workbench v7.0.1 (QIAGEN, Hilden, Germany) and aligned using ClustalW (Thompson *et al*., 1997) in BioEdit 7.2.5 (Hall, 1999). Published sequences available from GenBank were also downloaded for phylogenetic comparisons. Neighbor-Joining (NJ) and maximum likelihood (ML) analyzes based on K-2 parameter model (Kimura, 1980) with complete gap deletion and 1000 bootstrap replications were conducted in Mega6 (Tamura *et al*., 2013). Based on model selection results (lowest Bayesian Information Criterion value), Tamura 3-parameter with discrete Gamma distribution (T92+I) was the best-fit substitution model for the insect samples data while Kimura 2-parameter 80 with discreet Gamma distribution (K2+G) was the best-fit model for fungal isolates (Tamura *et al*., 2013). Separate phylogenetic analyses using the best-fit model were performed for *C. partellus* (n = 50), *B. fusca* (n = 11), *S. frugiperda* (n = 11) and *B. bassiana* (n = 80) in Mega6 with 1000 bootstrap replications. DNA polymorphism analyses were done using DnaSP v5 (Librado and Rozas, 2009).

2.3.5. Establishment of insect pathogenic fungi as a sugarcane endophyte

A greenhouse experiment was conducted to determine the best method for inoculating sugarcane (variety MN1) with an insect pathogenic fungi, *B. bassiana* (strain GHA) at Bvumbwe Agricultural Research Station (BARS; 15°55'27.1"S 127 35°04'12.5"E, 1174 m.a.s.l) located in Thyolo district, southern Malawi (Paper V). Three methods of inoculating plants with a fungus were employed in this study i.e. foliar spray, stem injection and soil drench (Wagner and Lewis, 2000; Posada *et al.,* 2007; Tefera and Vidal, 2009). Plants were inoculated 7 days after the emergence of the primary shoot using soil drench, stem injection and foliar sprays. Fungal colonisation was evaluated 7-10 and 14-16 days post inoculation (DPI) using the fragment plating method surface sterilizing plant tissue sections, and plating the sterilized sections on selective growth (Torres *et al*. 2011; Vega, 2018). Effects of fungal inoculations on plant growth was evaluated at the end of the experiment.

2.4. Main results and discussion

2.4.1. Impact of climate change on pesticides used in sugarcane production

In general, high temperature as predicted in current climate change scenarios will favour pests' proliferation (Chandiposha, 2013; Das *et al*, 2011; Matthieson, 2007). As ectotherms, temperature influences insect feeding, metabolism, reproduction, development and dispersal. Higher temperature will enhance the multiplication of insects through reduced development time resulting in shortened life cycles. The spittlebug (*Neophilaenus lineatus*) is predicted to increase its host range in the United Kingdom (Whittaker and Tribe 1996). Shortening of generation time and increased pest activity has been reported for *Plutella xylostella* in Southern Africa (Nguyen *et al*., 2014; Ngowi *et al*., 2017). Natural enemies especially parasitoids may become less efficient if host species emerge earlier and there is rapid development of susceptible stages. The dominance of *Chilo partellus* over indigenous stemborers in Africa has been attributed in part to asynchrony with its natural enemies (Mutamiswa *et al*., 2017). A recent study by Machekano *et al*. (2018) found that due to differences in basal temperature responses between *P. xylostella* and its parasitoid *Cotesia vestalis*, the co-evolved host-parasitoid synchrony may be offset. These temperature induced changes may result in increased frequency of pest outbreaks forcing farmers using biological control to resort to pesticide use in order to minimize crop losses.

Projected higher temperatures will affect pesticide efficacy. For instance, pyrethroids such as cypermethrin is very toxic at temperatures below 26°C while organophosphates such as profenofos are more toxic at higher temperatures (Jegede *et al*., 2017; Noyes *et al*., 2009). However, organophosphates are generally more toxic to humans and the environment compared to pyrethroids. Because of the loss in efficacy of pyrethroids, farmers will resort to using more organophosphates, inadvertently increasing their pesticides exposure risk. In addition, more insecticides will be applied to combat pest outbreaks as evidenced by the recent Government of Malawi and sugarcane estates responses to outbreaks of fall armyworm (*Spodoptera frugiperda*) and yellow sugarcane aphid (*Sipha flava*) outbreaks during 2016-2017 and 2013-2014 cropping seasons, respectively.

Climate scientists predict an increase in amount of rainfall received over short periods resulting in increased risk of flooding (Challinor *et al*., 2007; Gilbert *et al*., 2007). There is a greater risk of pesticides contamination of groundwater and surface water bodies through leaching and erosion of sorbed pesticides at higher rainfall intensities (Bloomfield *et al.,* 2006; Camenzuli *et al.,* 2012; Probst *et al.,* 2005; Silburn *et al.,* 2013). On the contrary, the degradation of pesticides is expected to be higher in conditions of higher temperatures, resulting in reduced environmental contamination (Dong and Sun, 2017; John *et al.,* 2016).

2.4.2. Incidence and management of sugarcane pests in Malawi

As with the rest of sugar producing countries, traditional farmers grow sugarcane for household consumption and trade in local markets. Usually, the crop is row intercropped or grown in rotation with maize and various vegetables. On the other hand, commercial estates grow the crop for processing into sugar, ethanol and other related products. These commercial estates also outsource some of the sugarcane from smallholder farmers called outgrowers. In Malawi, outgrowers may belong to a farmer association or may be independent (Paper II and IV). The farmer association acts as a broker i.e. negotiating the contracts and acquiring input materials on credit on behalf of the outgrowers. Some farmer associations such as Dwangwa Smallholder Farmers and Kasinthula Cane Growers Association also perform agronomic operations such as pesticides application and harvesting on behalf of farmers.

Farm surveys we conducted in 2015 and 2016 showed that plant pathogens, weed and insect pest infestation were the main sugarcane production constraints (Paper II). Weed were categorized

into 4 groups: grasses (monocotyledons), broad-leafed (dicotyledons), sedges (monocotyledons) and mosses. Before canopy closure, weeds compete with plants for water, nutrients and light (Turner, 2011). Insect species belonging to 15 different genera were found infesting sugarcane (Paper II and III). *C. partellus* was the main stemborer pest.

C. partellus is an exotic pest originating from Asia while *B. fusca* is a native of Africa. It has been present in Malawi for almost 90 years (Tams, 1932). *S. flava* (a native of the Americas) is a recent introduction to Africa. It was detected attacking sugarcane for the first time in Malawi in during 2013-2014 cropping season in Chikwawa district. During 2015-2016, outbreaks of the fall armyworm, *Spodoptera frugiperda* (also a native of the Americas) were reported on maize (*Zea mays*) in several African countries (FAO, 2017; Goergen *et al*., 2016). We found this pest infesting sugarcane in Chikwawa district (Paper III).

Management of weeds and insect pests was highly dependent on pesticides (Table 1, Paper II). Information detailing how each specific pesticide should be handled is provided on a pesticide label. Pesticide labels for all the pesticides we documented in this study were in English. We found that only 10% or our respondents understood the information on the pesticide label. The pesticides used in the commercial estates and in some outgrowers' fields were sources from South Africa. However, the rest of the farmers bought the pesticides from local agro input dealers. A permit obtained from the Malawi Pesticides Control Boards (PCB) is required for all agro input dealers to store and sell pesticides. Agro input dealers are required to have knowledge about toxicity and risks, associated with pesticides use handling and how to minimize the risks. The problem is that there are no official tests that can be taken to document agro input dealers' pesticides knowledge. Moreover, there is limited enforcement of pesticide regulations in Malawi due to several factors including financial constraints and low number of qualified personnel.

Table 1. Pesticides used by sugarcane farmers in Malawi

Trade name	Active ingredient	Target pests
Aceta, Acetamiprid	acetamiprid	Aphids, red spider mites
Agromectin	Abamectin	Red spider mites
Ametryn	Triazine	Annual broadleaf weeds and grasses
Atrazine	atrazine and other triazines	Annual broadleaf weeds and grasses
Chlorpyrifos	Chlorpyrifos	Larvae (white grubs) and adults of black maize beetles
Cypermethrin	cypermethrin	Aphids, stemborers
Diuron	Diuron	Weeds and mosses
Dimethiote	dimethoate	Aphids, thrips
Dichlorvos		Aphids, thrips
Harness	Acetochlor	Annual grasses
Bandit	Imidacloprid	Thrips
MCPA	2-methyl-4-chlorophenoxyacetic acid	Broadleaf weeds and certain grasses
Metolachlor	S-metolachlor	Broad-leafed and annual grassy weeds
Marshall	carbosulfan	
MSMA	monosodium methanearsonate	Grass, sedges, broad-leafed weeds
Profenothrin	profenofos + cypermethrin	Red spider mites
Roundup	Glyphosate)	Most annual grasses

Herbicides were commonly used in our study areas in fields measuring 2ha or more. About 60% of outgrowers in Nkhota Kota and in all commercial estates regularly applied herbicides, although application rates varied greatly (Paper II). About 44% of the outgrowers in Nkhota Kota applied herbicides as cocktails containing 2 or 3 active ingredients. Ametryn and glyphosate were some of the frequently used herbicides (Table 1). Non-chemical weed control methods included hand weeding i.e. uprooting weeds by hands only and hand hoeing i.e. uprooting weeds with the help of a hoe. Hand weeding and hand hoeing was also employed to supplement chemical control in the large estates. This is standard practice in sugarcane weed management (Takim and Suleiman, 2017; Turner, 2011).

We also documented insecticides which are chemicals used against insect pests. Acetamiprid, chlorpyrifos, cypermethrin and imidacloprid are some of the insecticides (Table 1, Paper II). Application rates for acetamiprid and cypermethrin varied greatly among traditional farmers. No

insecticides were applied to control stemborers during the study period even though recommended pesticides were available.

Because of their feeding behaviour, stemborers are difficult to control with insecticides. The most damaging stage (larvae) feed in leaf whorls for a short period of time before penetrating the stem where they live until pupation. Therefore, there is short period of time where foliar insecticides can be applied because once the stemborer enter the stem, they cannot come in contact with the insecticides. This means that only systemic insecticides can be used against stemborers. Hence, non-chemical methods like early planting during main season and avoiding excessive nitrogen fertilization are employed. Scraps from tobacco (*Nicotiana tabacum*) stems were also use in managing maize black beetles and white grubs.

Even though fungal disease called smut caused by *Sporisorium scitamineum* was reported by 35% of respondents, no fungicides were used. Pest free sugarcane planting materials (seedcane) are dipped in 50°C hot water for 2 hours, roguing and burning of infected plants and sterilizing of harvesting equipment are used to manage the disease. This practice is employed throughout the sugar industry worldwide.

The problems of poor pesticides handling are not limited to sugarcane farmers nor Malawi as a country. Coffee and tobacco farming are the top 2 consumers of pesticides imported into Malawi (GoM, 2013). Orr and Ritchie (2004) reported that vegetable farmers in southern Malawi used highly hazardous insecticides, usually applied at above recommended doses and applied these insecticides more than 10 times. In years where there is a higher pest pressure (e.g. *Tuta absoluta* infestation in tomato during 2016-2017 crop season), farmers applied insecticides just before harvest (T. Kasambala Donga, personal observation). In West Africa, calendar application of pesticides to vegetable farmers was very common (Williamson *et al*., 2008). In Ethiopia, farmers used a mixture of a highly toxic insecticides (malathion) and another chemical banned for agricultural use worldwide (DDT) for control of weevils that infest maize in storage (Williamson *et al*., 2008). In Zimbabwe, vegetable farmers did not follow recommended application rates for insecticides (Sibanda *et al*., 2000),

2.4.3. Risks associated with pesticides used in sugarcane production in Malawi

Pesticides application help to reduce pest populations within short periods. However, there are several problems associated with herbicides and insecticides. In Paper II, we made use of the EIQ model, Pesticide Properties Database (PPDB, 2017) and WHO (2009) classification of pesticides by hazard to identify pesticides that posed a higher environmental and health risk to man.

We found that two commonly used insecticides agromectin and dichlorvos belonged to WHO (2009) highly hazardous class while the rest were either moderately or slightly hazardous. Based on highest application rate reported, MCPA had the highest environmental risk (EIQ value = 5025.2) while acetamiprid had the lowest (EIQ value = 153.8). Except S-metalochlor, all the pesticides used are moderately or highly toxic (oral toxicity) to humans. About 50% of the pesticides are highly toxic to bees and birds, while 30% are highly toxic to aquatic life. About 70% of the pesticides used have a higher probability of contaminating the environment (PPDB, 2017).

Pesticide exposure in human occurs via absorption through the skin (dermal contact), ingestion and inhalation of pesticide fumes. The main pesticides exposure routes for the farmers involved in this study were during pesticide handling (loading into sprayers and during spraying) and storage (pesticides were stored within the house). All farmers we interviewed knew the possible negative effects associated with pesticides. However, this awareness was not enough to compel them to invest in personal protective equipment (PPE). This is evident as two thirds of farmers wore plastic boots and cotton overalls to protect themselves from pesticides but only 9% had equipment meant to protect the head region. Similar results have been reported in other developing countries. In Côte d'Ivoire, over 75% cotton farmers corrected understood information on pesticide relating to the need for protecting eyes, nose and mouth; pesticide applicators did not wear any piece of PPE during 53% of times of pesticide applications (Ajayi and Akinnifesi, 2007). In northern Greece, 99% had knowledge of adverse effects of pesticides on human health but 46% of tobacco farmers did not use any PPE (Damalas *et al*., 2006). Therefore, it was not surprising that most of the farmers had experienced multiple acute symptoms due to pesticide exposure (Table 2). The most common symptoms were skin irritation, headache, coughing and running nose.

Table 2. Common acute health symptoms reported by farmers exposed to pesticides during pesticide handling and storage

Health symptom	Frequency of report (%)* of specific symptom (n = 55)
skin irritation	78
Coughing	67
Running nose	67
Headache	67
Skin rash	22
Other (chest pain, fever, dizziness and diarrhoea)	11

*Multiple responses allowed.

There are a number of factors that influence improper pesticides handling among farmers in developing countries including Malawi that render them highly vulnerable to pesticides exposure. Illiteracy among farmers is one of the primary driver of pesticides abuse (Ajayi and Akinnifesi, 2007). Because of illiteracy, farmers have limited understanding application and safety instructions contained on the pesticide label. Where is there is limited agricultural extension support, it is difficult for farmers to extrapolate application rates given on pesticides labels (normally given in hectares or acres) to very small farm sizes (Ajayi and Akinnifesi, 2007; Bon *et al*., 2014). The second driver is governments, farmer association, or buyers' policies regarding pesticides. In Malawi, registered sugarcane farmers get pesticides (mostly herbicides) as part of inputs package on credit every year. Farmers growing cotton on contract with cotton ginning companies are also in a similar situation. In Côte d'Ivoire, free pesticides are given to cotton farmers (Ajayi *et al*., 2011). Lack of effective alternatives to pesticides is also a contributing factor. For instance, the Government of Malawi has a document (GoM, 2013) outlining IPM strategies for minimizing pesticides use in agriculture and ensuring environmental protection, but few effective non-chemical pest control technologies have been developed. Therefore, farmers have no choice than resorting to synthetic pesticides to manage the high pest

pressure and ensure they harvest something. High poverty levels among the population of Malawians (The World Bank Group, 2017) may account for the lack of investment in PPE.

The risk of environmental pesticide exposure can be reduced by addressing factors that contribute to poor pesticides handling and developing effective pest management strategies that have the least impact on human health and the environment. IPM is accepted worldwide as best approach in ensuring sustainable agriculture (FAO, 2014). There is a great need for suitable training of farmers themselves, farmer association pesticide applicators and agricultural extension personnel in good pesticides handling and disposal procedures. Farmer associations are very important in ensuring safe use of pesticides. We found that farmers belonging to farmer associations were provided inputs on credit and herbicides were included in the input package regardless of farm size or understanding of situation on the ground. A deliberate policy can be put in place that requires farmers to purchase a PPE set on becoming a member. Pesticide application should be based on economic thresholds in all sugarcane plantains and not just in large estates. Deliberate efforts need to be put in place to generate viable non-chemical pest control methods that can be used to replace certain harmful pesticides and may be integrated in existing pest control programs.

2.4.4. Natural occurrence of beneficial fungal endophytes entomopathogenic fungi in sugarcane fields in Malawi

Entomopathogenic fungi (EPF) in the genera *Beauveria*, *Metarhizium* and *Isaria* (order Hypocreales) are ubiquitous in soil and are also known to occur as endophytes of plants (Clifton *et al*., 2015; Fisher *et al*., 2011; Gurulingappa *et al*., 2010; Lacey *et al*., 2015; Reay *et al*., 2010; Vega *et al*., 2008). In paper IV, we isolated from soil and sugarcane in 12 sugarcane fields in southern Malawi *Beauveria* sp., *Isaria* sp. and *Metarhizium* sp. Isolates were collected from soil by *Galleria mellonella* insect bait and can be considered entomopathogenic fungi. We also identified *Beauveria* sp. and *Isaria* sp. from surface sterilized sugarcane tissue, so it is probable that the isolates were endophytes. More isolates were collected from soil (81.7%, n = 60) than from sugarcane (36.7% n = 180). These results are consistent with previous findings that show a higher proportion of entomopathogenic fungi recovered from soil than from plant tissue (Ramos *et al*., 2017; Klingen and Haukeland, 2006). *Beauveria* was the most dominant genera as it was isolated from all locations and occurred at a higher frequency (72%) compared to *Isaria* (19%)

and *Metarhizium* (9 %). Molecular identification based on the Bloc intergenic region of 50 *Beauveria* isolates from soil and sugarcane indicated that the isolates were *Beauveria bassiana.*

Endophytic *B. bassiana* has been reported in over 20 plant species distributed across 12 families including Fabaceae, Solanaceae, Malvaceae, Poaceae, Cucurbitaceae, and Euphorbiacea (Jaber and Ownley, 2018). However, our findings in the first report of *B. bassiana* occurrence as an endophyte of sugarcane. This also the first reports of *Isaria* sp. and *Metarhizium* sp. occurrence in sugarcane, and in agricultural soils in Malawi, respectively.

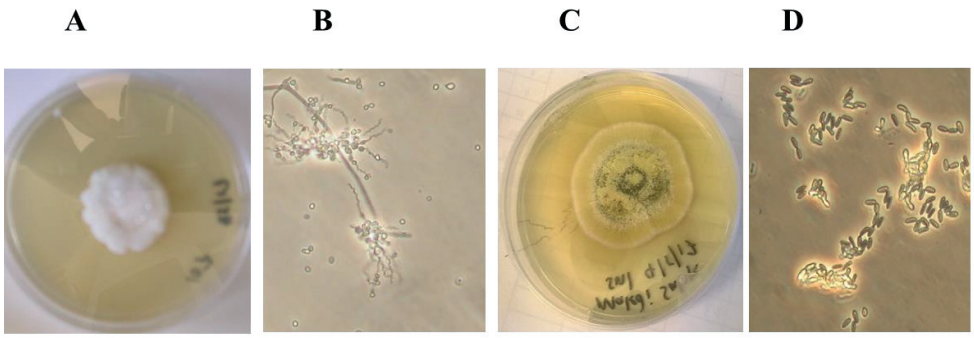

Figure 3: Colony appearance of *Beauveria* sp. and *Metarhizium* sp. on Sabouraud dextrose agar (A and C) and under the microscope (x400 magnification, B and D).

Phylogenetic analysis based on Bloc intergenic region of 50 *Beauveria* isolates indicated that the isolates were *Beauveria bassiana* (Paper IV). We identified a single clade that aligned closely with *B. bassiana* AFNEO_1 clade which comprises *B. bassiana* isolated from the coffee berry borer, *Hypothenemus hampei* (Coleoptera: Curculionidae) in coffee fields of South America and in Africa (Rehner *et al*., 2006). Analysis of DNA polymorphism showed little genetic differentiation among the isolates. This may indicate gene flow among the locations. Gene flow is an important element in the maintenance of genetic diversity as it provides a way in which new genes are introduced in a population. However, high rate of gene flow reduces genetic differentiation between population as genes are exchanged (APSNET, 2018). Gene flow between *B. bassiana* populations can occur via wind currents (Hajek, 1997) and possibly through

exchange of seedcane. The low genetic diversity among the *B. bassiana* populations can also be attributed to the geographical scale considered. All isolates were collected from one agroecological zone.

This is clearly different from agroecosystems in Denmark where *B. bassiana* isolated from soil, plant surfaces and insects at a single location were highly diverse (Meyling *et al*., 2009).

Molecular characterization of the remaining isolates will be done before submitting this manuscript for publication. Molecular characterization will also help us to identify the presence of entomopathogenic species within our *Isaria* isolates since unlike *Beauveria* sp. and *Metarhizium* sp. (Fig. 3), not all *Isaria* spp kill insects.

2.4.5. Inoculation of sugarcane by an entomopathogenic fungus, *Beauveria bassiana*

Our greenhouse experiment demonstrated for the first time that *B. bassiana* has the ability to colonise sugarcane following soil drench, stem injection and foliar sprays of sugarcane plants (Paper V). *B. bassiana* recovery was significantly higher in plants that had stems injections (75%) and foliar sprays (43%) than soil drench (25%), respectively, in line with previous reports (Quesada-Moraga *et al*. 2007; Tefera and Vidal 2009; Guesmi-Jouini *et al*. 2014; Russo *et al*. 2015; Jaber and Enkerli 2017). Poor persistence in soil may be the reason for the low recovery of the unformulated *B. bassiana* (Vänninen *et al.* 2000). In addition, inoculation of sugarcane with *B. bassiana* using soil drenches and foliar sprays was not detrimental to the plant but enhanced plant growth during the 16-day experimental period. Several studies have reported similar findings (Posada *et al.* 2007; Lopez and Sword 2015; Jaber and Enkerli 2017). However, as reported in similar studies conducted on crops in the same family as sugarcane (Inglis *et al*. 1993; Renuka *et al*. 2016), the amount of *B. bassiana* recovered in this study decreased with time. This means that multiple application of *B. bassiana* may be required to derive maximum benefits from the endophytic interaction.

Since we isolated and characterized *B. bassiana* in Paper IV, future effort will focus on evaluating the persistence and pathogenicity of the Malawi isolates for use in biological control of pest species and contribute to reduction of pesticide risks in Malawian agriculture. *B. bassiana* is recorded to infest over 700 insect species distributed across the following insect families Aleyrodidae, Curculionidae, Cercopidae, Scarabaeidea, Aphididae, and Thripidae (de Faria and

Wraight, 2007; Inglis *et al*., 2001). Hence, aphids, thrips, mites and white grubs will be the target pests since they have a wide host range and are economic pests of several food crops grown in Malawi. Since stem injections are not practical on a large scale, we will focus on soil and foliar sprays (Akello *et al*., 2008; Meyling and Eilenberg, 2007; Posada *et al*., 2007; Hajek and Meyling, 2018). In addition, *Beauveria* species are considered safe (Zimmermann, 2007). We will also explore the potential of endophytic *B. bassiana* in controlling stemborers systematically as has been reported in the United States of America (reviewed in Jaber and Ownley, 2018).

2.5. Conclusion and future perspectives

2.5.1. Conclusion

Our results indicate the main serious pests of sugarcane in Malawi are sucking insects (*Sipha flava*, *Fulmekiola serrata* and *Tetranychus urticae*), soil borne pests (*Heteronychus licas* and *H. arator*, *Anomala* sp.) and stemborers (*Chilo partellus* and *Busseola fusca*). We also found *Spodoptera frugiperda* sp. 1 infesting sugarcane. There was low genetic differentiation among population of *C. partellus* and *B. fusca* in southern Malawi. Management of these insect pests and also weeds were depended on synthetic pesticides. Climate variability via above average temperature will influence the amount and type of these pesticides. There is a great risk of environmental contamination and pesticides exposure among sugarcane independent sugarcane farmers.

Two widely entomopathogenic fungi *Beauveria bassiana* and *Metarhizium* sp. were found occurring naturally in sugarcane and soils collected from sugarcane fields in Malawi. Our *B. bassiana* isolates were closely related to *B. bassiana* from Africa and South America collected from the coffee berry borer, *Hypothenemus hampei.* The Malawian *B. bassiana* were characterized by low genetic variation. Foliar and soil drenches of a commercial formulation of *B. bassiana* were effective in inoculating sugarcane. These results provides the starting point for exploring entomopathogenic fungal-based biological control of crop pest in Malawi.

2.5.2. Future perspectives

The use and poor handling of highly and extremely hazardous pesticides indicate that there is high potential of environmental contamination and development of pesticides exposure-related chronic illnesses among farmers. There is great need for pesticide awareness campaigns targeting farmers, agro-dealers, farmer associations and extension workers. We greatly recommend providing pesticide labels in vernacular languages. There is also a need to conduct further studies to determine which pesticides applied in sugarcane fields are leaching and contaminating the environment.

One important research topic is examining pesticide residue levels in groundwater wells used by communities surrounding sugarcane estates. It is also important to track pesticide residues in nontarget organisms such as birds nesting in grasses and reeds, and fish in water bodies draining through sugarcane fields. The fact that fall armyworm was found on sugarcane indicate that it is pertinent to monitor this pests and its impact on sugarcane.

Assessment of occurrence of entomopathogenic fungi in sugarcane and other cropping systems in all the major agroecological zones of Malawi will continue. Bioassays will have to be conducted first in the laboratory to identify most virulent isolates and later field trials will be implemented. Pilot IPM trials will also be conducted.

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4. Papers I – V

Impact of climate variability on use and exposure of pesticides used in sugarcane production

Trust Kasambala Donga[1a], Richard Meadow[a], Bishal K. Sitaula[a] and Ole M. Eklo[a,b]

[a]Norwegian University of Life Sciences, P.O. Box 5003, NO-1432 Ås, Norway
[b]Norwegian Institute of Bioeconomy Research, P.O. Box 115, NO-1431 Ås, Norway

[1]Corresponding author email: trust.kasambala.donga@nmbu.no

Abstract

It is widely accepted that climate change will affect sugarcane production and its associated pests. Our aim with this paper was to review the impact of climate change on factors and processes affecting environmental exposure of pesticides used in sugarcane production in Malawi. We indicate that changes in temperature and rainfall will have a dual effect on pesticide risk. Temperatures (30-35°C) will affect pesticide toxicity although effects will vary with pesticide-pest combination. Rapid degradation of pesticides e.g. acetamiprid and atrazine is expected at temperatures above 30°C. Higher temperature may increase the incidence and severity of pests such as red spider mites prompting farmers to use more pesticides. On the other hand, the amount and timing of rainfall in relation to pesticide application is important in pesticide fate in the environment. There is a higher likelihood of pesticide transport to surface (through runoff) and percolating to groundwater at higher rainfall intensity. A higher soil water content will result in increased pesticide degradation. There a need to determine occurrence of pesticides residues in sugarcane cropping and aquatic systems surrounding sugarcane plantations. The sugar industry should consider the possibility of crop residues retention.

Keywords

Sugarcane, climate change, weather variability vulnerability, pesticide exposure, risk

1. Introduction

Worldwide, Africa is the most vulnerable region to climate change (Challinor *et al.,* 2007; Dasgupta *et al.,* 2014). However, spatiotemporal variation in terms of vulnerability and susceptibility exists among and within African countries (Adhikari *et al*., 2015). Vulnerability to climate change - 'the degree to which geophysical, biological and socio-economic systems are susceptible and unable to cope with, adverse impacts of climate change' (IPCC, 2007). Brooks *et al.,* (2005) outlined socioeconomic factors that determine a nation's vulnerability and adaptive capacity to climate change. These factors include economy, health and nutrition, literacy rate, infrastructure, geography and demography and dependence on agriculture (Brooks *et al.,* 2005). Malawi is one of the world's poorest countries with a gross national income (GNI) per capita of USD320 (The World Bank Group, 2017). The majority of the population live in rural areas. About 55% of females are literate compared to 73% of males. The HIV/AIDS prevalence rate is 9.2% (The World Fact Book, 2016). Since 2013/2014, food insecurity has been increasing (SADC/VAC, 2016). Poverty rates are highest in southern Malawi and it is at a higher risk of flood or water borne diseases (The World Fact Book, 2016; Mwale *et al.,* 2015). Hence, Malawi is very vulnerable to climate change impacts.

There is a consensus among scientists that climate change (increased atmospheric carbon concentration and surface temperatures, and variation in precipitation) will significantly affect agriculture (Delcour *et al.,* 2015; Aktar *et al.,* 2009; Noyes *et al.,* 2009; USAID, 2007). Changing onset and shortening of the rainfall season, increased frequency of riverine and flash floods, droughts, temperature and heat waves are evidence of climate change impacts in Malawi (Zulu *et al.,* 2012). McSweeney *et al.,* (2010) and Wood and Moriniere (2013) observed that it is difficult to isolate climate change from normal climate variability because of the variability of

Malawi's climate brought about by three external atmospheric drivers. Malawi's climate is greatly influenced by (1) the El Niño Southern Oscillation (ENSO), an Indo-Pacific phenomenon that modulates circulation (2) the Indian Ocean Dipole (IOD), an equatorial pattern that affects rainfall and (3) the Subtropical Indian Ocean Dipole (SIOD), which may be linked to higher than normal rainfall in southern Africa. Understanding how climate change/weather variability affects specific components of the agricultural sector is important for development and effective implementation of mitigation and coping strategies.

Many studies have focused on the impact climate change will have on various aspects of sugarcane production (Jones *et al.,* 2015; Zhao and Li, 2015; Marin *et al.,* 2014; Chandiposha, 2013; Fabio *et al.,* 2013; Knox *et al.,* 2010; Gawander, 2007; Deressa *et al.,* 2005). Overall, these studies indicate that projected future temperatures will have no significant effect on sugarcane growth since the projected temperature increases are within the crop's optimum range (30-32°C). High temperature scenarios will enhance sugarcane growth and yield (Gawander, 2007). However, temperatures higher than 35ºC will negatively affect sugarcane germination and internode development (Rasheed *et al.,* 2011; Bonnett *et al.,* 2006). Higher temperature will also lead to high evapotranspiration resulting in increased irrigation demands to minimise crop losses. In addition, temperature under current climate change scenarios will favour insect pests, weeds and certain fungal diseases (Das et al, 2011; Matthieson, 2007). Although, the occurrence of pests under changing climate is discussed in the literature, little attention has been given to implications of climate change on pesticide exposure in sugarcane production. Chandiposha (2013) provided an account of how climate change would influence pest occurrence and distribution but did not explain how the corresponding pesticides used to control such pests would be affected. Hence, the main objective of this review is to bring into focus the impact of

climate change on the sugarcane industry and the amount and exposure to pesticides used in sugarcane production in Malawi.

2. Theoretical framework

The risk from pesticide exposure is a function of pesticide toxicity and the probably of non-target organisms encountering it. Prevailing climate, soil condition and management influence the concentration (exposure) of a pesticide in the environment (Delcour *et al.,* 2015; Kerle *et al.,* 2007; Fig. 1).

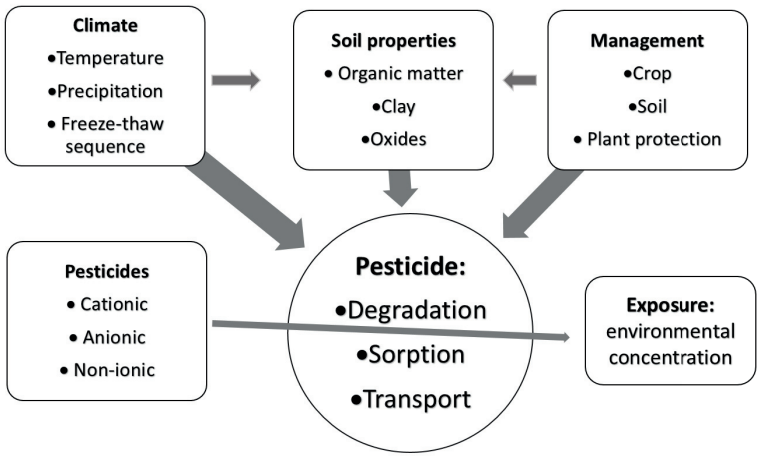

Figure 1: Factors and processes influencing exposure of pesticides in the environment (Eklo, 2018).

In this paper, we focus on how projected climate change will affect risk from pesticides used in sugarcane production using Malawi as a case example. We obtained information on pesticides, climate change and its effects on agriculture from published literature available on the internet, books, official and private documents. We first describe environmental properties of pesticides approved for use in sugarcane in Malawi. A detailed description of possible effects of rising temperatures and changing precipitation patterns on these pesticides afterwards.

3. Pesticides used in sugarcane production in Malawi

In order to minimize yield losses from weeds, arthropod pests and diseases; different types of pesticides are used in sugarcane production. In Malawi, herbicides and insecticides are the main types of pesticides used in the sugar industry (Kasambala Donga and Eklo, unpublished). Solubility in water, persistence in soil (measured as soil half-life), potential for adsorption to soil particles and mobility (K_{oc}) and dissociation (pKa) are considered key properties when determining how a pesticide or its metabolites behave in the environment (Kerle *et al.,* 2007). Water movement is important for transport of water-soluble pesticides, whereas wind transport is important for volatile pesticides.

Table 1 provides details on various aspects of pesticides used by sugarcane farmers in Malawi. Solubility values of pesticides in Table 1 indicate that agromectin, chlorpyrifos and cypermethrin are less soluble in water, while acetamiprid, dimethoate, monosodium methanearsonate (MSMA) and 2-methyl-4-chlorophenoxyacetic acid (MCPA) are highly soluble. Plants easily absorb pesticides that are highly soluble (Kerli *et al.,* 2007). Pesticides with less than 30 days soil half-life are nonpersistent. Moderately persistent pesticides such as glyphosate and cypermethrin have a soil half-life between 31 and 100 days. MSMA is the most persistent pesticide listed in Table 1. In Table 1, pesticides such as abamectin, chlorpyrifos, cypermethrin, fluazifop-P, glyphosate and profenofos have high K_{oc} values. This implies that they are sorbed strongly to soil particles and remain concentrated on the application site. Soil half-life values range from 1-7 days for acetochlor to 200 days for MSMA. Some of the pesticides such as atrazine, ametryn and diuron have a high potential for contaminating groundwater through leaching. Glyphosate, MCPA and MSMA readily dissociate in solution (high solubility values) but differ in their degradation and organic carbon sorption constant. Profenofos, diuron, cypermethrin and chlorpyrifos do not

readily ionize but have a high propensity for adsorption onto soil particles. There is a high probability that runoff will contain these chemicals. There is high risk of surface and groundwater contamination from pesticides with low sorption coefficients such as acetamiprid, acetochlor, metolachlor, ametryn and atrazine.

4. Climate effects on pesticides exposure

4.1. Pest occurrence

Climate induced changes will alter both the pest and/or host biology. Wet and humid conditions favour the proliferation of fungal and bacterial diseases. Climate induced dry weather may increase the incidence of ratoon stunt disease and smut (Matthieson, 2007). Although these are important diseases of sugarcane in Malawi, increase in their incidences will not affect pesticide exposure since these diseases are controlled using cultural methods.

Higher temperatures may also increase the incidence and severity of insect pests. The severity of red spider mites infesting sugarcane in Chikwawa is closely linked to periods of dry hot weather, low humidity and high evapotranspiration (Koloko, 2016). A highly toxic pesticide, fipronil was used to manage an outbreak of African migratory locusts in the Lower Shire River Valley. During the 2014/2015 cropping season, additional amounts of acetamiprid and cypermethrin were sprayed to manage an outbreak of yellow sugarcane aphids.

Table 1: Overview table describing commonly used active ingredients, target pests, application rates and key environmental factors. Sources: PPDB, 2017; Kerli *et al.,* 2007; EU, 2004; Kasambala Donga and Eklo, unpublished; EXTOXNET, 94

Substance group Active ingredient (a.i.)	Target pests	Typical application rates (g a.i. ha^{-1})	Mode of action	Solubility in water (mg L^{-1}) [1]	Half-life in soil (DT50: days)[2]	Organic carbon sorption constant (K_{oc})[3]	Dissociation constant at 25°C (pKa)
Avermectins (abamectin)	Arthropod pests: aphids (Sipha flava), thrips and red spider mites	21.6	Stimulate the chloride channels that are regulated by the neurotransmitter glutamate	Insoluble	1-7	4,000	-[4]
Organophosphate (dimethoate)	Aphids	2.2	Acetylcholinesterase (AChE) inhibitor	39800	2.6		No dissociation
Organophosphate (chlorpyrifos)	Soil and foliage arthropod pests	750	Acetylcholinesterase (AChE) inhibitor.	1.05	21	8151	-
Pyrethroid (cypermethrin)	Broad spectrum of pests especially Lepidoptera and aphids	300	Sodium channel modulator	0.009	69	156250	-
Ethanimidamide (acetamiprid)	Hemiptera spp. especially aphids	300	Acetylcholine receptor (nAChR) agonist.	2950	3	200	0.7 strong acid
Phosphonoglycine (glyphosate)	Weeds and grasses	1080 –	Inhibition of EPSP synthase	10500	23.79	1424	2.34 strong

[1] Highly soluble pesticides have large solubility values.

[2] Soil half-life: < 30 days implies nonpersistent, 30-100 days means moderately persistent and > 100 days shows pesticide is highly persistent.

[3] The higher the K_{oc} value, the more strongly the pesticide is sorbed.

[4] "-" indicates data not available.

		3570					acid (pKa2) 5.73
Chloroacetamide (S-metolachlor)	Grasses and some broad-leaved weeds	1536	Inhibition of VLCFA (inhibition of cell division)	480	21	110-369	-
Organometal (organic arsenical)	Sedges, Grasses and broad-leaved weeds	2160	Inhibition of VLCFAs (Inhibition of cell division)	580000	200	-	9.02 weak acid
Phenoxyacetic acid (MCPA)	Annual and perennial weeds	1080	Synthetic auxin	29390	25	-	3.73 weak acid
Phenylureas (diuron)	Weeds and mosses	1600	Inhibits photosynthesis	35.6	89	813	-
Organophosphate (profenofos)	Lepidopteran pests and mites	440	Acetylcholinesterase (AChE) inhibitor	28	7	2016	-
Triazines (ametryn) (atrazine)	Most annual and broad-leaved weeds	900-1200 1125-1350	Inhibits photosynthesis (photosystem II)	200 35	37 29	316 100	10.07 very week acid 1.7 very weak base
Phenoxyaliphatic Acids (Fluazifop-p-butyl)	Ripener	55.5	Inhibits acetyl-CoA carboxylase	0.93	8.2	3394	-
Ethylene generator (ethephon)	Flower suppressant	480	Plant growth regulator with systemic properties	1000000	13.4	-	2.82
Phenylpyrazole (fipronil)	Various insect pests and mites		Broad-spectrum with contact and stomach action. GABA-gated chloride channel antagonist	3.78	142	-	No dissociation

Fipronil is highly toxic to terrestrial and aquatic life, does not dissociate and has high potential for bioaccumulation (PPDB, 2017). These few examples illustrate the impact of climate induced pest outbreaks on pesticides use and exposure. Farm workers and local communities are at increased risk of pesticide exposure through pesticide drift into canals renders (Wilson *et al.,* 2004) as they use water in irrigation canals for bathing and other household chores.

4.2. Pesticide toxicity

Higher temperatures will affect the toxicities of pesticides on their target pests although the effects will vary with pesticide-pest combination (Fishel, 2015; Noyes *et al.,* 2009; Donahoe, 2001). Temperature extremes affect pesticide efficacy through improper storage. Higher temperatures may cause pesticides to expand and also to volatilize and spill out upon opening of the container. Farmers lacking proper chemical stores and storing pesticides within their homes are at greater risk of pesticide exposure. Sadly, this is the case in many developing countries (Mengistie *et al.,* 2015; Stadlinger *et al.,* 2010; Kasambala Donga and Eklo, unpublished). Organophosphates tend to be more toxic to arthropod pests at 26-28°C than at 20°C while pyrethroids are more toxic at lower temperatures (Jegede *et al.,* 2017; Noyes *et al.,* 2009). Maximum temperatures in the sugarcane growing areas of Malawi range between 27°C-37°C (Phiri and Saka, 2008) are higher than temperatures used in pesticide toxicity studies (Jegede *et al.,* 2017; Noyes *et al.,* 2009). Since cypermethrin is widely used in Malawi to control a range of insect pests infesting sugarcane, a reduction in efficacy is likely to result in either increased frequency or amount of pesticide application.

4.3. Pesticide degradation

As shown in Fig. 1, temperature strongly influences the degradation of a pesticide and several reports exist on its effects on some of the pesticides examined in this study (de Beeck *et al.,*

2017; Jegede *et al.,* 2017). The rate of degradation of atrazine increased with increasing temperature (Dong and Sun, 2017). Higher temperature also enhances the activities of microorganisms that degrade pesticides. At 30°C and pH 7, bacteria degraded 90% of chlorpyrifos and profenofos within 8 days (John *et al.,* 2016). Acetamiprid degradation was rapid in soils with higher temperatures (Vela *et al.,* 2017). The sugarcane growing districts in Malawi experience considerably high temperatures (above 30°C) during most of the year. Hence, we expect the estimate of risk of pesticide exposure to be significantly lower under rising temperature assuming all other degradation factors remain constant.

Soil moisture is also an important factor in pesticide degradation (Chai *et al.,* 2013; Sebaï *et al.,* 2010). Except for rainfed sugarcane (less than 20%), irrigation is essential to meet the crop's water demand. Under current climate scenarios, the demand for irrigation will rise. Irrigation may cancel high-temperature induced drought effects on pesticide degradation (Gonczi, 2016).

4.4. Pesticide transport

The pesticides currently used in sugarcane production in Malawi use water as a solvent. High temperatures will result in an increase in volatilization of highly- and semi-volatile pesticides through evapotranspiration of pesticides and their metabolites to the atmosphere (Bloomfield *et al.,* 2006). However, most of the pesticides in use are less volatile (Kasambala Donga and Eklo, unpublished). Water-based pesticides such as MSMA and its metabolites show some persistence in soil and sediments because they tend to move slower than water and remain concentrated in shallow soil depths (Mahoney *et al.,* 2015; Bloomfield *et al.,* 2006) increasing the possibility of pesticide contamination in the environment after initial applications. A study in Australian forests found residues of atrazine and its metabolite desethylatrazine in 1.8m deep groundwater (Kookana *et al.,* 2010).

Rainfall is a key factor influencing the transport of pesticides in the environment. The onset of the rainy season is around October to November in most parts of Malawi, with the highest rainfall occurring around February to March or early April, especially in the north. The rains tail off in late April and May when winter begins. Amount and timing of rainfall in relation to pesticide application is a much more important factor than average annual rainfall and temperature (Wang *et al.,* 2018). For Malawi, the observed and predicted increases in the proportion of rainfall that falls in heavy events and during the wetter months of January and February affect the following pesticides pathways: leaching to surface and ground water, runoff and erosion. There is a high probability of pesticide movement to surface and groundwater at higher rainfall intensities since wetter soils have higher hydraulic conductivities (Bloomfield *et al.,* 2006). The hydraulic conductivity varies with soil type and the water content of a particular soil. The soils in the main sugarcane growing areas are chiefly alluvial in Nkhota Kota, and alluvials and vertisols in Chikwawa. The water holding capacity of vertisols is high when compared to alluvials. This implies that there will be higher likelihood of pesticide-rich water percolating to groundwater in areas with vertisols in situations of higher rainfall intensities. On the other hand, a higher soil water content will result in increased degradation rate of pesticides (Jebellie, 1996) and hence, lower the pesticide risk estimate.

Increased rainfall intensities may also result in flooding and runoff. Runoff will directly influence the fate of pesticides through an increased erosion of soil particles and transport of sorbed pesticides (Bloomfield *et al.,* 2006). Increased precipitation may enhance runoff contamination by pesticides (Silburn *et al.,* 2013; Probst *et al.,* 2005). Rainwater and floodwater runoff account for transport of a quarter of the diuron applied yearly to sugarcane in Australia (Camenzuli *et al.,* 2012). Approximately 19% of the rainfall received in Malawi is lost through

surface runoff (GoM, 2008). It is possible therefore, that a significant proportion of pesticides currently used in agriculture in Malawi is lost through this pathway. Therefore, in the event of increased precipitation and floods, the concentration of pesticides such as acetamiprid and metalochlor is expected to be high if these episodes occur immediately after their application. About 33% of Malawians do not have access to potable water (WHO and UNICEF, 2015). They depend on surface- and groundwater for drinking and other household chores (Chidya *et al.,* 2016; Chimphamba and Phiri, 2014) and are at greater risk of pesticide exposure.

4.5. Pesticide sorption

Soil management practices influence sorption - the distribution or partitioning of a pesticide in an environment. Sorption reduce risk of pesticide leaching but can also reduce pesticide degradation rate as the pesticides are not available for the microorganisms. Dinisio and Rath (2016) reported high sorption of abamectin occurring in soils rich in organic matter. In another study, metalochlor and atrazine sorption increased in soils amended with biochar (Deng *et al.,* 2017; Trigo *et al.,* 2016). Biochar have some of the same effects like sugarcane burning after harvest and thereby increasing sorption. Adsorption of atrazine and endosulfan were better in soils covered with rice husks (Rojas *et al.,* 2014). Leaching of MCPA was significantly reduced in Mediterranean agricultural soils amended with olive oil mill wastes (Peña *et al.,* 2015). These results show that efforts aimed at improving soil fertility have a significant influence on the exposure of pesticides to the environment through enhancement of pesticide degradation and sorption.

Crop management is also an important factor in pesticide sorption. In Malawi as in many of the sugarcane producing countries, sugarcane is burned prior to harvesting. Some ashes from burning plant residues are blown away from the site while some ashes remain on the sugarcane

field. These ashes contribute to pesticide sorption in soils (Yang and Sheng, 2003). For instance, soils amended with ashes from rice and wheat crop residues had higher sorption for diuron (Yang and Sheng, 2003). Sugarcane burning strongly influence the adsorption of substituted ureas and s-trianzines (Hilton and Yuen, 1963). However, the practice can lead to reduced effectiveness of pesticides. Annual burning of cereal fields also reduces the efficacy of chlorpyrifos, dimethoate and clomazone (Xu *et al.,* 2008; Kamm and Montgomery, 1990). In addition, the practice negatively affects the population of microbes and total organic matter (Souza *et al.,* 2012), very essential components in microbial degradation of pesticides. Thus, burning reduces pesticide risk through increased pesticide sorption. At the same time, it may also increase pesticide exposure risk due to increased demand for inputs (fertilizer and herbicides).

Increases in rainfall coupled with intensive farming using nitrogen fertilizers and burning of crop residues can result in acidification of soils. The pH of a soil and the ionic state of the pesticide influence pesticide fate. For example, at pH 4, part of ametryn (pKa = 4.10) exists as a positively charged conjugate acid (de Paula *et al.,* 2016). The electrostatic interaction between the ametryn conjugate and the ionised soil particles are enhanced. As a result, ametryn is more persistent in acidic soils (de Paula *et al.,* 2016). According to Meyer and Heathman (2015), the soils under intensive sugarcane production in Chikwawa have become acidic. Increasing temperature coupled with frequent irrigation or flooding may have contributed to the soil acidification through soil mineral leaching. In addition, excess cations contained in plant material necessary for balancing anions on organic molecules that could have neutralised the soil acidity upon decomposition are not available (Rengel, 2011). This implies that there will be accumulation of residues of weak acids such as MSMA, MCPA, glyphosate and ametryn and non-dissociating

pesticides. This scenario will increase the probability of soil contamination and negatively affect soil-dwelling non-target organisms.

In conclusion, timing and amount of rainfall, and temperature will continue to influence degradation, sorption and transport of pesticides used in sugarcane production. Higher temperature will negatively affect pesticide toxicity prompting farmers to use more and/or change pesticides. There is greater risk of pesticides contaminating water bodies through runoff and erosion of sorbed pesticides. Persistence of pesticides such as ametryn and glyphosate may be higher in the acidic soils. There a great need to determine occurrence of pesticide residues in sugarcane cropping and aquatic systems surrounding sugarcane plantations. The sugar industry should consider the possibility of crop residue retention.

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Environmental load of pesticides used in conventional sugarcane production in Malawi

Kasambala Donga, Trust*[1] and Eklo, Ole Martin[1,2]

[1]*Nowergian University of Life Sciences, Campus Ås, Universitetstunet 3, 1433 Ås*

[2]*Norwegian Institute of Bioeconomy Research P.O. Box 115, NO-1431 Ås, Norway*

Corresponding author email: Email:* trust.kasambala.donga@nmbu.no or trustkasambala@yahoo.co.uk

Abstract

The sugarcane industry is the third largest user of pesticides in Malawi. Our aim with this study was to document pesticide use and handling practices that influence pesticide exposure among sugarcane farmers in Malawi. A semi-structured questionnaire was administered to 55 purposively selected sugarcane farmers and 7 key informants representing 1474 farmers in Nkhata Bay, Nkhotakota and Chikwawa Districts in Malawi. Our results indicate that herbicides and insecticides were widely used. Fifteen moderately and one extremely hazardous pesticide, based on World Health Organization (WHO) classification, were in use. Several of these pesticides: ametryn, acetochlor, monosodium methylarsonate and profenofos are not approved in the European Union because of their toxicity to terrestrial and aquatic life, and/or persistence in water and soil. Farmers (95%) knew that pesticides could enter the human body through the skin, nose (53%) and mouth (42%). They knew that pesticide runoff (80%) and leaching (100%) lead to contamination of water wells. However, this knowledge was not enough to motivate them to take precautionary measures to reduce pesticide exposure. Farmers (78%) had experienced skin irritation, 67% had headache, coughing and running nose during pesticide handling. Measures are in place to reduce pesticide exposure in the large estates and farms operated by farmer associations. Smallholder farmers acting independently do not have the resources and capacity to minimize their exposure to pesticides. There is need to put in place pesticide residue monitoring programs and farmer education on commercial sugarcane production and safe pesticide use as ways of reducing pesticide exposure.

Keywords

Sugarcane, pesticides exposure, environmental load, EIQ.

1. Introduction

Sugarcane is the second most valuable crop after tobacco contributing 9-12% of Malawi's foreign exchange earnings (FAO, 2015). In 2017, large estates contributed 83% to national production compared to 17% for smallholder farmers (ILLOVO, 2017). The Government of Malawi supports smallholder production of sugarcane as a sustainable way of reducing poverty (Chinsinga, 2017). Hence, the number of smallholder sugarcane famers also known as outgrowers has been increasing since 2011. However, since 2014, the amount of sugarcane processed at sugar mills from smallholder farmers has been decreasing while it has remained constant for the estates (ILLOVO, 2017). There are many contributing factors to the low sugarcane tonnage by smallholder farmers. Pest occurrence and poor crop management may be some of the factors (Tena et al., 2016).

Pesticides are widely used throughout the sugar industry. The industry consumes 10-15% of pesticides imported in Malawi (GoM, 2017). Herbicides recommended for use in sugarcane production in Malawi include ametryn, atrazine, monosodium methylarsonate (MSMA), 2-methyl-4-chlorophenoxyacetic acid (MCPA), s-metolachlor, pendimethalin, diuron, acetochlor and glyphosate (GoM, 2017; Agricane, 2011). Glyphosate is a pre-emergent herbicide for the control of emerged annual and perennial weeds, and for crop/ratoon eradication. It is a recommendation that farmers apply glyphosate when the land is lying in fallow. Atrazine and pendimethalin are also pre-emergent herbicides for the control of annual broadleaf and some grass weeds. Application of these herbicides is at the time of planting/ratooning and before weed emergence. Ametryn and MSMA are post-emergent herbicides for control of most annual and broadleaf weeds. Some herbicides such as acetochlor, atrazine and glyphosate are both pre -and post-emergent herbicides. Several insecticides including chlorpyrifos and profenofos have government approval (GoM, 2017).

The undesirable effects of pesticides on the environment and human health are widely recognized. Pesticides can pollute the environment through pesticide runoff, drift, leaching and bioaccumulation (Mostafalou and Abdollahi, 2013; Wang et al., 2011; Weichenthal et al., 2010). The pesticide dichlorvos is an organophosphate fumigant pesticide that has no approval in the European Union (EU). It is highly toxic, has a high tendency to bioaccumulate (PPDB, 2017). Even though glyphosate is considered to have low mammalian toxicity (Tarazona et al., 2017),

its intensive use leads to groundwater contamination, herbicide resistance and inhibition of plant growth (Cederlund, 2017; Schryver et al., 2017; Van Stempvoort, 2016). Glyphosate is highly discussed in the EU because of possible carcinogenetic potential (EC, 2017). Glyphosate has approval for use in the EU until 2022 (PPDB, 2017).

The Government of Malawi acknowledges that pollution of waterbodies, air, soil and food due improper handling, storage and disposal of pesticides is of high concern (GoM, 2010). Hence, there are laws and policies for regulating pesticides. The Pesticides Act No. 12 of 2000 regulates the management of import, export, manufacture, distribution, storage, disposal and use of pesticides in Malawi. The integrated pest management plan (IPM) set in 2013 seeks to promote the use of environmentally friendly practices in major crops (GoM, 2017). IPM 'means careful consideration of all available plant protection methods and subsequent integration of appropriate measures that discourage the development of populations of harmful organisms and keep the use of plant protection products and other forms of intervention to levels that are economically and ecologically justified and reduce or minimize risks to human health and the environment. IPM emphasizes the growth of a healthy crop with the least possible disruption to agro-ecosystems and encourages natural pest control mechanisms' (EU Directive 2009/128/EC). Only pesticides with the least potential for environmental contamination can be included in IPM programs (FAO, 2014). The major problem in implementing successful IPM programs in Malawi is a lack of, or insufficient data on environmental pesticides load – toxicity resulting from pesticides. Hence, the main objectives of this work was to determine the environmental and health effects associated with pesticides used in sugarcane production in Malawi.

2. Materials and methods

2.1. *Sugarcane production in Malawi*

Sugarcane is vegetatively propagated using cane setts (stem cutting having 3-6 internodes). The recommended seed cane rate is 8-10 ton per hectare. Row spacing for irrigated sugarcane is 1.5m and 1.0m for rain fed cane. Either 1.5 or double cane setts are planted end-to-end in furrow. The initial sugarcane planted is plant cane and the subsequent crop arising from remnants of harvest of this initial crop is ratoon cane. Herbicides are applied on a calendar basis. Insecticides and acaricides are applied based on action thresholds.

Fields are allowed to dry for 30 days before being burned and manually harvested. The act of burning sugarcane concentrates sucrose and drives away snakes and crocodiles.

There is a sugar mill at Dwangwa Estate in Nkhotakota and in Nchalo Estate in Chikwawa owned by ILLOVO Sugar Malawi Limited. Associated with these mills are smallholder farmers growing rainfed or irrigated sugarcane on contracts. These farmers acquire farm inputs on credit from registered farmer associations (Agricane, 2011). It is important to note that some associations perform agricultural operations such as herbicide applications, and pest and disease scouting on behalf of their members at a cost. In some associations, the farmer has the liberty of carrying out all the farm activities himself. These differences have consequences on farm practices among the various smallholder farmers.

2.2. *Description of study sites*

In Malawi, sugarcane is intensively cultivated in the Nkhata Bay, Nkhotakota, and Salima and Chikwawa districts (Fig. 1). The Nkhata Bay and Nkhotakota districts are high altitude areas with average annual rainfall of 1490 mm received mostly between December and April. The crop is rainfed in Nkhata Bay. The major source of irrigation to the sugar industry in Nkhotakota is Dwangwa River that drains into Lake Malawi. Chikwawa is a low altitude area (<150 masl) with half of the average rainfall received in Nkhotakota. Water is drawn from the Shire River that flows out of Lake Malawi. Because of the topography of Chikwawa, the district is prone to annual flooding from water movement from the Shire Highlands and groundwater discharge into the river (Meyer and Heathman, 2015). In addition to sugarcane, many agricultural activities involving the use of pesticides take place on the catchments of the Dwangwa and Shire rivers, and Lake Malawi.

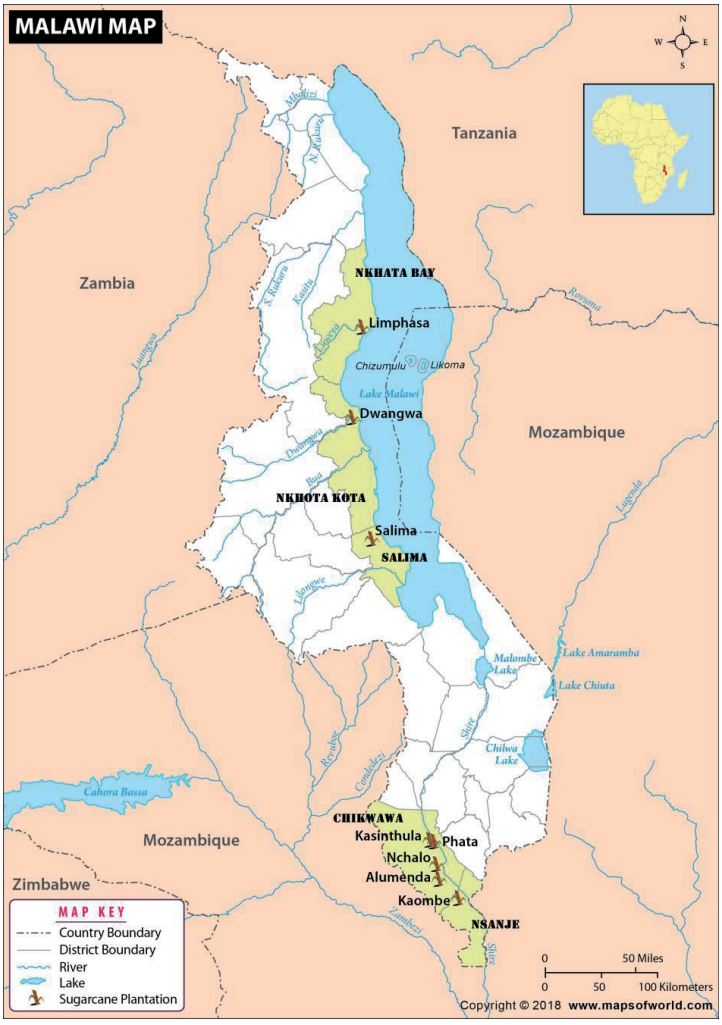

Figure 1: Map of Malawi showing location of sugarcane plantations and study sites in Nkhata Bay, Nkhotakota and Chikwawa Districts.

2.3. *Study population*

We conducted the survey between June 2015 and January 2016 in Nkhata Bay, Nkhotakota and Chikwawa (Fig. 1). We used purposive sampling to identify respondents from association membership lists and/or with the help of local agricultural extension officers. As of 2015, there were 2039 registered smallholder sugarcane farmers belonging to 18 associations in Malawi. Only farmers belonging to associations who had applied pesticides themselves during 2014/15 were included in the survey. We also interviewed the farm/section/estate/agriculture managers for Dwangwa and Nchalo Estates; Kabadwa Cane Growers Association, Dwangwa Smallholder Cane Growers Association and Independent Cane Growers in Nkhotakota; Limphasa Sugar Corporation Limited in Nkhata Bay; and Kasinthula Cane Growers' Association in Chikwawa. These represented 1474 smallholder farmers and served as key informants. A pre-coded and pre-tested semi-structured questionnaire was interviewer-administered to capture information practices and knowledge related to pesticides. 'Yes' and 'No' were the allowable responses to closed questions. There were also questions with four to six factors per question and respondents were required to choose the most important. Respondents were politely requested to provide their demographic details, pesticide application history and the source of money used for buying pesticides.

2.4. *Sugarcane pests and pesticides used to control pests*

During the above-described interviews, farmers were requested to give information on incidence and severity of pests on their sugarcane farms. Another question required the farmers to rank the pests in order of importance. A pesticide knowledge section of the questionnaire collected information on whether the farmers knew the names of recommended pesticides, their application rates (quantity of pesticide mixed a specific water volume in a sprayer) and frequency. A series of closed questions helped the interviewer to capture data on type and timing of pesticide application. The questionnaire had questions also on effectiveness of the pesticides they have used.

2.5. *Environmental pesticide load*

Except in commercial estates, the majority of farmers in Malawi do not keep pesticides records (Tebug et al., 2012). This limited our choice of pesticide risk assessment models. Therefore, environmental pesticide load was determined using the environmental impact quotient

(EIQ) model. The EIQ model is easier to use and requires only a few input data. The EIQ model is widely used for comparing different pesticide strategies and the environmental impact of pesticides used in agriculture (Kromann et al., 2011; FAO, 2008; Eklo et al., 2003). The EIQ model summarizes all pesticides used during the season, thus giving a total score for the environmental load (Kovach et al., 1992). Pesticide data: active ingredients (a.i.) quantity (in grams, g), application rates (g.a.i.) per hectare (ha) obtained from the questionnaire survey was entered into the EIQ model. Pesticide data pertaining to farmers who could not remember the quantities of pesticides they had used in 2014/15 were excluded in the calculation of environmental load. We used the online EIQ calculator on the Cornell University website (NYSIPM, 2017). In the online calculator, the application rate was given in g.a.i per $100m^2$. We also consulted the World Health Organization (WHO) recommended classification of pesticides by hazard and guidelines to classification published in 2009.

2.6. *Effects of pesticides used on human health*

During the questionnaire survey stated above, respondents were asked to report acute effects of pesticides they had experienced. Knowledge about how pesticides could enter the human body, ground wells and food were also evaluated. Farmers' handling of obsolete pesticides, pesticide storage and disposal of pesticide containers was also documented.

2.7. *Data analysis*

All statistical analyses were conducted in the Statistical Package for the Social Sciences (SPSS) version 24. Descriptive statistics used were means and percentages. Cross tabulations and chi-square test (χ^2) were used to show how different groups of respondents answered the survey questions (Punch, 1998). For example, age and education level could affect a respondent's ability to apply the correct application rate of a pesticide. For each farm, the environmental impact (EI) of each active ingredient per hectare was calculated using the formula shown below:

EI per ha = EIQ x application rate (g. a.i. per ha) x % active ingredient x number of applications.

3. Results

3.1. *Study population*

We interviewed 42 smallholder farmers in Nkhotakota and 13 in Chikwawa districts and 6 key informants. The 13 farmers interviewed in Chikwawa do not sell their sugarcane to any sugar mill in Malawi. The majority of respondents had completed primary school (Table 1). About 79% fully depended on farming for income while 12% owned businesses. The most common sugarcane variety grown was MN1 (45.0%) seconded by R570 (32.0%). None of the farmers had attended training on sugarcane production. All key informants were above 40 years, had training in agronomy and over 10 years of experience in sugarcane cultivation. Income source was the main determinant of planting date ($\chi^2 = 8.383$, $df = 3$, $p = 0.039$), October-December for rainfed cane and April-September for irrigated cane. Harvesting took place 12-15 months later.

3.2. *Sugarcane pests and pesticides used to control pests*

Considering pests together, weed infestation was a major pest in all the respondents' farms. Herbicides were applied in all the estates and 60% of the smallholder farms in Nkhotakota. No herbicides were applied on the farms of farmers we interviewed in Chikwawa. The fungal disease smut caused by *Sporisorium scitamineum* was the most reported pest (35%) followed by sugarcane mosaic virus disease (17%).

Table 1: Farmer's demographic data (n = 55).

Characteristic Age (years)	No. respondents	% respondents
20-29	5	9.1
30-39	9	16.4
> 40	41	74.5
Education		
None	1	1.8
Primary	38	69.1
Secondary	11	20.0
Tertiary	5	9.1
Sugarcane farming experience (years)		
< 5	35	66.0
5-10	7	13.2
> 10	11	20.8

Farm size (ha)		
< 5	43	78.2
5-10	8	14.5
> 10	4	7.3

Rusts (*Puccinia melanocephela, P. fulvous sp.* Nov. and P. *kuehnii*) and ratoon stunt (*Leifsonia xyli* subsp *xyli*) diseases were mentioned by less than 5% of the respondents. Stemborers were the main insect pests (16%) reported followed by white grubs (10%, larva of *Heteronychus* spp). Termites (*Macrotermes* spp) and aphids (yellow sugarcane aphids, *Sipha flava*) were reported by less than 10% of the respondents. The incidences of these pests varied with production system. Outgrower farmers in Nkhotakota reported sugarcane mosaic virus disease as the main sugarcane disease. Smallholder farmers in Chikwawa frequently mentioned the incidence of Lepidopteran stemborers.

Key informants confirmed the occurrence and identity of the pests reported by smallholder farmers. They also provided the situation on the estates and smallholder farms managed by farmer associations and a list of recommended pesticides. In addition to the pests reported by farmers, the following pests occurred on the estates: unidentified species of mealy bug (Pseudococcidae), leaf roller moth larvae (Lepidoptera: Noctuidae), earth pearl or margarodes scale (Margarodidae), scale insects (Coccidae) and grasshoppers; nematodes; sugarcane aphid (*Melanaphis sacchari*) sugarcane thrips (*Fulmekiola serrata*), and red spider mites, RSM (*Tetranychus urticae*). Only half of these were considered economic pests and warranted induction of control mechanisms. The incidence of yellow sugarcane aphids was highest in Chikwawa (Nchalo Estate and farms belonging to the Kasinthula Cane Growers Association). No insect pests or fungal diseases were reported at the Limphasa Sugar Company in Nkhata Bay.

The farmer's decision to start using pesticides was based on advice of extension workers 52%, pesticide label 26% and their own judgement 19%. However, the decision to apply herbicides was dependent on farm size ($\chi^2 = 8.000$, $df = 3$, $p = 0.046$). Only half of the farmers with secondary school education could understand the information indicated on the pesticide

label ($\chi^2 = 35.616$, $df = 12$, $p = 0.000$). Those with primary education relied equally on extension workers and fellow farmers on pesticides related issues ($\chi^2 = 32.716$, $df = 3$, $p = 0.000$). Nevertheless, pesticide(s) a farmer actually used was dependent on pesticide availability ($\chi^2 = 7.700$, $df = 3$, $p = 0.006$). Timing of pesticide application was based on pest occurrence ($\chi^2 = 27.543$, $df = 16$, $p = 0.036$).

Although all respondents reported sugarcane diseases, no pesticides were used to manage them. Instead, cultural methods such as varietal resistance, use of disease free seed, sterilizing cutting equipment and manual removal of diseased plants were employed. Insecticide were applied on large estates and farmers' fields in Chikwawa. The insecticides acetamiprid and cypermethrin were used to manage aphids while four different insecticides controlled thrips. The organophosphate chlorpyrifos was used to control black maize beetles (Table 2).

Smallholder farmers we interviewed in Chikwawa did not spray any herbicides on their farms. Herbicides were routinely applied in 60% of outgrowers' fields in Nkhotakota, large estates and association-managed farms. Forty-four percent of these farmers applied herbicides as cocktails containing 2 or 3 herbicides. Commonly used herbicides were ametryn, atrazine, MSMA, MCPA and glyphosate (Table 2). Herbicide application rates for planted and ratoon sugarcane were different. For instance, for planted cane, the recommended rate for ametryn is 2.40L/ha compared to 1.8L/ha for ratoon cane. Atrazine has three application rates (L/ha): 2.70 for planted cane, 2.40 and 2.25 for ratoon cane, respectively. Application rates of ametryn (mean = 1710.00, $p = 0.000$), MSMA (mean = 2259.49, 1372.369, $p = 0.000$) and MCPA (mean = 768.00, $p = 0.012$) differed significantly among the smallholder farmers in Nkhotakota. According to key informant interviews, glyphosate and acetochlor was used to terminate weeds from waterways, spot and perimeters, and for crop eradication. Fusilade forte 150 EC (fluazifop-p-butyl 150g/L) is a ripener while ethrel 480 EC (ethephon 480g/L) is a flower suppressant used on large estates. All respondents used 20L knapsack and 15L jacto sprayers.

Table 2: Pesticides used by sugarcane farmers in Malawi and their target pest.

Pesticide type	Active ingredient	Target pest(s)
Insecticide	Abamectin	RSM, thrips, aphids
	Acetamiprid	Aphids
	Carbosulfan	Stemborers
	Chlorpyrifos	Larvae and adult black maize beetles
	Cypermethrin	Aphids, stemborers
	Dichlorvos	Aphids, thrips
	Dimethiote	Aphids, thrips
	Profenofos	Thrips and RSM
	Imidacloprid	Thrips
Herbicides	Acetochlor	Annual grasses
	Ametryn	Annual broadleaf weeds and grasses
	Atrazine	Annual broadleaf weeds and grasses
	Diuron	Weeds and mosses
	Glyphosate	Most annual grasses
	MCPA	Broadleaf weeds and certain grasses
	MSMA	Grass, sedges, broad-leafed weeds
	Pendimethalin	Annual broad-leafed weeds
	S-metolachlor	Broad-leafed and annual grassy weeds

We found that large estates had some elements of IPM in place for managing arthropod pests. Based on key informant interviewed, there are action thresholds for insecticide application. To minimize spider mites infestations, trash/tops remaining after cane burning and haulage is practiced at Nchalo Estate. The egg parasitoid *Trichogramma chilonis* (at a rate of 2.5 c.c ha^{-1}, six releases in a growing season beginning from 4th month onwards at 15 days interval) is used to control stemborers. Scrap tobacco stems were used to manage maize black beetles. For management of all pests, each variety has less than 30% in the disposition. Monitoring of pests in time, space and varieties is routine.

3.3. *Environmental pesticide load*

The calculated EI per ha values for commonly used pesticides in sugarcane production in Malawi are indicated in Table 3. The range of a.i. EIQ values was 12.5-59.5 with lowest EIQ value for s-metalochlor and highest for profenothrin. EI per hectare for an active ingredient was a function of application rate. Agromectin and acetamiprid had the lowest EI per hectare (12.0 and 12.3-153.8) while dichlorvos and MCPA and MSMA had the highest EI per hectare values (7129.0, 5025.5, 4120.0 and 4044.4) respectively. Based on WHO (2009) classification of pesticides, 70% of the pesticides used by farmers were moderately hazardous while the rest were slightly hazardous (Table 3).

3.4. *Effects of pesticides used on human health*

Potential pesticide exposure pathways for farmers were pesticide storage, mixing, spraying and working in sprayed fields. Farmers preferred to store pesticides within the house (75%). The majority except of those with tertiary education lacked suitable personal protective equipment (PPE). Knee-length plastic boots and cotton overalls were the most widely used PPE (72%). All farmers recognized pesticides as poisons that can cause health problems. About 95% of them knew that pesticides could enter the human body through the skin, nose (53%) and mouth (42%). They knew that pesticides runoff (80%) and leaching (100%) lead to contamination of water wells. Food contamination through pesticide handling close to kitchens and spray droplets were recognized by over 80% of the farmers. All farmers in this study had knowledge of acute effects of pesticides. The most felt effects were skin irritation, 78%; headache, coughing and running nose (67%); skin rash (22%); fever, dizziness, chest pain and diarrhoea (11%). Vomiting and diarrhoea were mentioned only by female farmers ($F = 8.980$, $p = 0.005$).

Table 3: Active ingredients, WHO toxicity class and EIQ values for pesticides used by sugarcane growers in Malawi

Pesticide (active ingredient)	WHO toxicity class[a]	Application rate (a.i. g ha^{-1}) range	a.i. EIQ	EI per ha
Agromectin 18 EC (Abamectin 18g/L)	Ib	21.6	34.7	12.0
Acetamiprid (acetamiprid 200g/L)	II	24-300	28.7	12.3-153.8
Marshal 250 EC (25% v/v carbosulfan)	II	281.25	50.7	304.7
Chlorpyrifos 500 EC (500 g/L chlorpyrifos)	II	750	26.9	898.3
Cypermethrin 200 EC (200g/L cypermethrin)	II	37.5-600	36.4	24.3-389.2
Dichlorvos EC (organophosphate 1000g/L)		1500	53.3	7129.0
Dimethiote 40EC (400g/L dimethoate)	II	224	33.5	267.7
Profenothrin 440 EC (40% of profenofos + 4% cypermethrin)	II	440	59.5	934.8
Bandit 350 SC (350g/L Imidacloprid)	II	700	36.7	802.4
Harness 960 EC (960g/L acetochlor)	III	1152-1600	19.9	1959.5-2721.6
Ametryn 500 SC (500g/L triazine)	II	465-3750	24.2	501.6-4044.9
Atrazine 500 SC (485g/L atrazine + 15g/L other triazine)	III	750-1800	22.9	764.5-1834.8
Diuron 800 SC (diuron 800 g/L)	III	1350	26.5	2550.5
Roundup (510g/L glyphosate)	III	324-3570	15.3	159.5-2490.2
MCPA (400g/L phenoxyacetic acid)	II	480-3840	36.7	628.2-5025.2
MSMA 720 SL (720g/L organic arsenical)	II	670-3564	18	774.7-4120.9
Metolachlor 960 EC (s-metolachlor)	III	1080	12.5	1156.2
Pendimethalin 330 EC (dinitroaniline 330 g/L)	II	742.5	30.2	659.5

[a] Ib: highly hazardous; II: moderately hazardous; III: slightly hazardous (WHO, 2009).

Pesticides that no longer have regulatory approval or are under restricted use in the European Union (EU) were still approved by the Government of Malawi. Atrazine belongs to triazines and is an herbicide that does not have approval in the European Union (EU, PPDB, 2017). Ametryn is also a triazine herbicide that does not have regulatory approval in the EU due to its persistence in soil and water under certain conditions (PPDB, 2017). MSMA is not widely approved for use in the developed world due to its toxicity and persistence in soils (PPDB, 2017). Profenofos has high potential for bioaccumulation and is highly toxic to birds, fish and aquatic invertebrates (PPDB, 2017). Imidacloprid, acetamiprid, chlorpyrifos and cypermethrin are approved for restricted use in the EU since they are moderately to highly toxic to birds, honeybees and fish (Table 4).

Table 4: Ecotoxicology parameters of pesticides used by sugarcane growers in Malawi

Active ingredient	Approval status in the EU	Mammalian toxicity (oral) level	Toxicity to Honeybees	Birds	Aquatic life
Abamectin[a]	√				
Acetamiprid	√	M	H	H	H
Carbosulfan	x[4]	H	H	H	H
Chlorpyrifos	√	H	H	H	H
Cypermethrin	√	M	H	L	H
Dimethiote	√	M	H	H	M
Profenofos	x[3]	M	H	H	H
Imidacloprid	√[2]	M	H	H	M
Acetochlor	x[3,4]	H	M	M	M
Ametryn	x[4]	M	L	L	M
Atrazine	x[3,4]	M	M	L	M
Diuron	√	M	L	M	L
Glyphosate	√	M	M	M	M
MCPA	√	M	L	M	M
MSMA	x[3,4]	H	M	L	M
S-metalochlor	√	L	L	M	M

√: yes; x: no; L: low, M: moderate, H: high (University of Hertfordshire Pesticides Properties Database)

[a]No specific ecotoxicology data is available for this product. Toxic to water birds, fish and bees (Abamectin MSDS, 2013).

[2] Approved with restrictions on certain flowering plants

[3] Approved in the United States of America

[4] Approved in Australia

4. Discussion

In this study, we report that pesticides are widely used to control weeds and arthropod pests infesting sugarcane cultivation in Malawi. We have also documented significant variation in pesticide application rates among smallholder farmers, a result consistent with previous findings elsewhere (Jallow et al., 2017; Schreinemachers et al., 2017). Only one of the 16 active ingredients reported in our study was extremely hazardous based on (WHO) classification. However, the majority are as moderately or slightly hazardous (PPDB, 2017). Although measures are in place to reduce human and environmental exposure to pesticides on the large estates and farms operated by farmer associations, smallholder farmers acting independently do not have the resources and capacity to minimize their exposure to pesticides.

We found that farmers relied on fellow farmers and extension workers for pesticide choice and handling. In addition, income did not influence farmers' pesticide choice. Our results partly agrees with the findings of Jallow et al. (2017). They found that other farmers were an important source of pesticide information for vegetable farmers in Kuwait. However, pesticide retailers significantly influenced Kuwaiti farmers' decisions to initiate pest control using pesticides, while pest occurrence was main determining factor for farmers in our study. The reason for these differences is that farmers in the study by Jallow et al. (2017) procured pesticides on a cash basis unlike the majority of smallholder farmers in our study, who got their pesticides on credit from the farmer association. In addition, only a few pesticides such as acetochlor, cypermethrin, acetamiprid and glyphosate are readily available from retailers in our study area. Farmers can access MSMA, MCPA and triazines only through the farmer association.

Herbicide cocktails (some with similar active ingredients and/mode of action) were used by more than a third of farmers in Nkhotakota. Since the crop is mostly rainfed in this area, many farmers were prompted to combine herbicides to combat high weed proliferation. In addition, some of these farmers grow cane in seasonal wetlands where difficult to control weed species such as C*ynodon* and *Cyperus* are the dominant species. However, over time this pesticides abuse (under- or over-dosing and using herbicide cocktails) could lead to development of herbicide resistance and other negative effects on the environment (El-Nahhal and Hamdona, 2017; Vencill et al., 2012; McCoy, 2010).

We also found that plant and ratoon cane have different recommended rates of herbicides in Malawi. The likelihood of an illiterate farmer remembering the specific application rates for each growth stage are minimal. Even those who were able to read the pesticide label did not fully understand the information recorded on the label. As long as the herbicides are effective at the lower application rates, from a farmer's point of view, there is no compelling reason to adopt the recommended application rates. Disregarding pesticide label instructions increases the risk of pesticides poisoning, the development of herbicide resistance and environmental contamination.

We used the EIQ model to identify pesticides or pest management systems with a low environmental impact (Kromann *et al*., 2011; Eklo et al. 2003; Kovach et al., 1992). Pesticides with low EI per ha are considered to be more environmentally benign and can be integrated in IPM programs. Based on the EI, we recommend agromectin, acetamiprid, cypermethrin and dimethiote for insect pest control and a ban on dichlorvos. The use of some herbicides such as acetochlor and triazines need to be restricted to reduce negative impact on humans and other non-target organisms. However, the EI per hectare value does not provide actual quantitative meaning on the nature of impact of a pesticide on the environment (Peterson and Schleier, 2014; Dushoff et al 1994). Hence, we obtained pesticide ecotoxicology data from the pesticides properties database of the University of Hertfordshire and WHO (2009) recommended classification of pesticides by hazards. Based on these two sources, we found that almost half of the pesticides reported in this study have potential to contaminate aquatic systems even at low concentrations (Olivier et al., 2013; Stoner and Eitzer, 2012). About 73% of the pesticides are also known to be highly toxic to honeybees, birds, fish and aquatic life (PPDB, 2017; Sanchez-Bayo and Goka, 2014; Ventura et al., 2008). The fact that there are no restriction on use of such pesticides is of great environmental concern. This is especially critical considering most of the rivers in the north and south of the country drain into Lake Malawi (GoM, 2010). Rare species of birds in southern Africa and endemic fish species inhabit the shores and marshes of Lake Malawi, and the Dwangwa and Shire Rivers (Anonymous, undated; Avibase, 2003). It is importance therefore, to establish pesticide monitoring programs.

Four pesticides namely chlorpyrifos, acetochlor, MSMA and carbosulfan used by sugarcane farmers in Malawi are highly toxic to mammals (PPBD, 2017). In this study, we only documented acute symptoms of pesticide exposure. However, farmers are also at a greater risk of developing pesticide-related chronic diseases through continued pesticide use, poor pesticide handling practices, dietary exposure, and drinking and using pesticide-contaminated water (Van der Werf, 1996; Ouedraogo et al., 2014; Mostafalou and Abdollahi, 2013; Saadi and Abdollahi, 2012; Wang et al., 2011; Weichenthal et al., 2010). Farmers exposed to the organophosphates chlorpyrifos and profenofos are at greater risk of neurotoxication (PPBD, 2017). The chloroacetamide acetochlor is a mutagen, organ toxicant and affects the reproductive system. Atrazine is a carcinogen and may cause coma, respiratory collapse, gastric bleeding and renal failure (PPBD, 2017).

We find that all respondents interviewed knew the harmful effects of pesticides. They also had knowledge of pesticide exposure routes in humans, groundwater and food. However, they did not take precautionary steps to reduce their exposure or use recommended application rates. These findings are in line with similar studies done elsewhere (Jallow et al., 2017; Schreinemachers et al., 2017; Anang and Amikuzuno, 2015). Either smallholder farmers did not have full understanding of the health risks posed by pesticides or did not consider personal protective equipment a priority considering the majority could not understand the pesticide label and had minimal financial capacity. The decision by some sugarcane farmer associations to perform all pesticide related activities for the farmers is critical in reducing farmers' exposure to and environmental contamination by pesticides. Otherwise, associations may consider giving personal protective clothing and equipment as part of inputs given to farmers on credit.

Reducing pesticide exposure risk among sugarcane producers can be achieved by following IPM principles. The IPM package for weeds could include the following: a) preventative measures aimed at reducing infestation and spread of weeds such as field sanitation, weed control along field margins and trenches, and equipment disinfestation after each use. b) Enhancing the ability of the plant to outcompete weeds. This can be achieved through varietal selection, observing seeding rates, row spacing, and fertilizer rates and placement. c) Herbicide

rotations and application at recommended application rates. This is a very crucial aspect considering that farmers did not follow the approved application rates.

Some key pests, e.g. aphids can be managed by using fungal entomopathogens alone or in combination with insecticides (Wraight et al*.,* 2016; Akbari et al., 2014; Tefera and Pringle, 2004). Kasambala et al (unpublished) are documenting the occurrence of and characterizing fungal entomopathogens in sugarcane cropping systems in Chikwawa. They are also evaluating the potential efficacy of *Beauveria bassiana* (Hypocreales: Ascomycota) foliar sprays against aboveground arthropod pests of sugarcane under field conditions at the Nchalo Estate.

5. Conclusion and recommendations

Our results indicate the environmental and health risks associated with pesticides currently used for controlling weeds and arthropod pests infesting sugarcane in Malawi. We show that there is a need for training both farmers and extension personnel in sugarcane production. There is a need for pesticide awareness campaigns targeting farmers, agro-dealers, farmer associations and extension workers. We greatly recommend providing pesticide labels in vernacular languages. There is also a need to conduct further studies to determine which pesticides applied in sugarcane fields are leaching and contaminating the environment. One important research topic is examining pesticide residue levels in groundwater wells used by communities surrounding sugarcane estates. It is also important to track pesticide residues in non-target organisms such as birds nesting in grasses and reeds, and fish in water bodies draining through sugarcane fields.

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insects

MDPI

Article

Determination of Genetic Diversity in *Chilo partellus*, *Busseola fusca*, and *Spodoptera frugiperda* Infesting Sugarcane in Southern Malawi Using DNA Barcodes

Trust Kasambala Donga [1,2,*] and Richard Meadow [1]

[1] Norwegian University of Life Sciences, P.O. Box 5003 NMBU, NO-1432 Ås, Trondheim 7439, Norway; richard.meadow@nmbu.no
[2] Lilongwe University of Agriculture and Natural Resources, P.O. Box 219 Lilongwe, Malawi
* Correspondence: tkasambara@bunda.luanar.mw; Tel.: +47-99-879-013

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Abstract: Sugarcane is one of the most valuable crops in the world. Native and exotic Lepidopteran stemborers significantly limit sugarcane production. However, the identity and genetic diversity of stemborers infesting sugarcane in Malawi is unknown. The main objectives for this study were to identify and determine genetic diversity in stemborers infesting sugarcane in Malawi. We conducted field surveys between June 2016 and March 2017 in the Lower Shire Valley district of Chikwawa and Nsanje, southern Malawi. Molecular identification was based amplification the partial cytochrome oxidase subunit I (COI) gene region. Phylogenetic trees for sequences were generated and published GenBank accessions for each species were constructed. We found that Malawi *Busseola fusca* (Lepidoptera: Noctuidae) specimens belonged to clade II, *Spodoptera frugiperda* sp. 1 (Lepidoptera: Noctuidae) and *Chilo partellus* (Lepidoptera: Crambidae) were infesting sugarcane. Interspecific divergence ranged from 8.7% to 15.3%. Intraspecific divergence was highest for *B. fusca*, 3.6%. There were eight haplotypes for *B. fusca*, three for *S. frugiperda* and three for *C. partellus*. The importance of accurate species identification and genetic diversity on stemborer management is presented.

Keywords: Sugarcane; Lepidoptera; Noctuidae; Crambidae; population genetics; COI gene

1. Introduction

Sugarcane is an important cash crop throughout the tropics. Southern Africa has the lowest yields of sugarcane (hg/ha), 82% less than the world average [1,2]. For over 50 years, sugarcane has been grown for processing purposes in Malawi. Production is intense, year-round, and under irrigation in estates. Smallholder farmers contribute 20% to the national production [3,4]. Some of these farmers grow sugarcane under irrigation while others solely depend on rainfall. Some farmers grow the crop either as an intercrop or as a monocrop or border crop. The crop is row intercropped with maize (*Zea mays* L.), sorghum (*Sorghum bicolor* L. Moench), vegetables, or a combination, during the dry season (May to November). Due to continuous monocropping on the large commercial estates, pest prevalence is high. In addition, continuous pest refugia are provided by intercropping or rotating sugarcane with cereals such as maize and sorghum.

A myriad of arthropod pests infests sugarcane. About 50 species of Lepidopteran moths belonging to three families, namely Noctuidae, Crambidae, and Pyralidae, infest sugarcane [5,6]. Within the family Pyralidae, *Eldana saccharina* Walker, a native of Africa is considered a serious pest of sugarcane [6]. It is widely distributed in sub-Saharan countries [7]. The species of *Chilo* (Crambidae), namely *C. partellus* and *C. sacchariphagus*, are also economic pests of sugarcane in eastern and southern

Africa [8]. *C. partellus* is an invasive pest that was introduced from India to Africa. Sugarcane is also a host for *C. orichalcociliellus* [9]. *Sesamia calamitis*, *S. creta*, and *Busseola* (Noctuidae), although considered as main pests of maize and sorghum [9,10], can also infest sugarcane. The larvae of these moths bore into and feed internally on stem tissue. The larval entry points on the stem provide entrance for fungal diseases. In younger plants, larval feeding results in death of the apical meristem, a condition called 'dead hearts.' In older plants, feeding damage results in increased risk of lodging. In addition, the quality and quantity of yield (sucrose) is also affected.

Multiple stemborer species may infest a field or individual plants [11,12]. However, variation exists in the pest status of these pests on sugarcane in Africa [7]. In South Africa and Zimbabwe, *E. saccharina* Walker is a major pest [13]. In Mozambique, the main stemborer species attacking sugarcane is *C. sacchariphagus* Bojer [14,15], while in Botswana it is *Chilo partellus* Swinhoe [16]. Although *E. saccharina* and *Sesamia calamistis* Hampson are present in Ethiopia, they are not economic pests on small-scale sugarcane farmers' fields [6]. Outbreaks of the fall armyworm, *Spodoptera frugiperda* (J.E. Smith) were first reported in Africa in 2016 [16,17]. During the 2016–2017 cropping season, *S. frugiperda* was reported to infest maize in several African countries. Although *S. frugiperda* prefers maize, it can also infest sugarcane [16].

The cytochrome c oxidase subunit 1 (COI) mitochondrial DNA (mtDNA) gene is widely used in identification and determination of insect population structure [18,19]. Genetic diversity in *B. fusca* populations is well documented. *B. fusca* populations cluster into three clades namely West Africa (W), Kenya I (KI), and Kenya II (KII) [20–22]. Clade KII comprises *B. fusca* species from eastern and central Africa [19,20]. On the contrary, studies establishing genetic differentiation in *C. partellus* in Africa are limited. A study by Sezonlin M. et al. [19] found that *C. partellus* populations collected from maize and sugarcane fields in South Africa and Swaziland were genetically similar. In that study, 11 *C. partellus* larvae from South African sugarcane were analyzed. The sequences generated in that study were not compared with sequences from other countries to determine genetic variations. Also, there are significant differences in the climate and geography of Malawi from that of South Africa. It has been suggested that gene flow between organisms of the same species might be restricted by physical barriers such as mountains and major rivers which may lead to speciation overtime [18].

Lack of knowledge of pest species identity and composition makes it difficult to properly address the problem in the context of integrated pest management. Published records indicate the occurrence of *C. partellus*, *C. orichalcociliellus*, and *B. fusca* in Malawi [23–25]. An unknown species of *Chilo* and *C. sacchariphagus* are reported in unpublished records of sugar estates in Chikwawa, southern Malawi. There is no record of *E. saccharina* occurrence in the country even though the pest occurs in neighbouring Mozambique [7]. Currently, stemborer management is based on varietal mixtures. Chemical control is less effective because of the cryptic nature of the pests. Biological control using the egg parasitoid *Trichogramma chilonis* is also recommended. Research on occurrence of fungal pathogens with insect control potential began in 2015. The success of such efforts hinges on correct pest identification and characterization, which is currently lacking. Our aims in this study were to accurately identify stemborer infesting sugarcane in Chikwawa and Nsanje Districts, southern Malawi using the COI gene, and determine diversity and relatedness among stemborer species with published reference sequences from GenBank. Results of this study will contribute to effective management of stemborers in the Malawi sugarcane industry.

2. Materials and Methods

2.1. Survey Sites

Sugarcane is grown in the Nkhata Bay, Nkhota Kota, Salima, Chikwawa, and Nsanje districts (Figure 1). There are several estates in Chikwawa, namely: Kasinthula, Sande, Nchalo, and Alumenda Estates. Kaombe Estate is located in Nsanje District. In addition to estates, smallholder farmers typically grow sugarcane in seasonal low-lying wetlands (locally called 'dimba') under rainfed conditions and

residual moisture. No fertilizers or manure or pesticides are applied. The Shire River provides water for irrigation in Chikwawa and Nsanje districts, respectively.

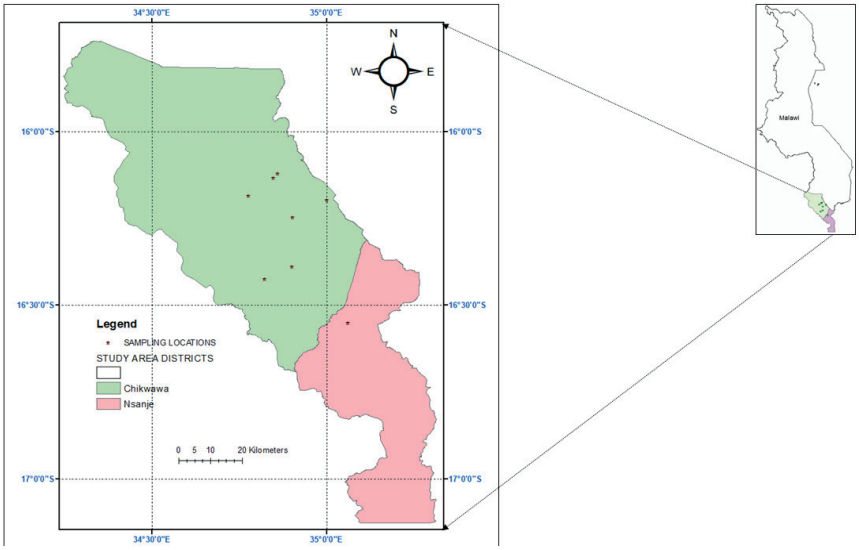

Figure 1. Map of localities where *Busseola fusca, Chilo partellus*, and *Spodoptera frugiperda* were sampled in Chikwawa and Nsanje districts, southern Malawi.

2.2. Survey Methodology

Commercial sugarcane production in Malawi dates back to 1968 [26]. Surveys were conducted in 48 fields belonging to Kasinthula, Nchalo, Alumenda, Kaombe, and Sande Estates, and smallholder fields located in agricultural extension planning areas (EPA) of Mbewe, Kalambo, Livunzu, and Mikalango in Chikwawa and Nsanje districts in southern Malawi from June 2016 to March 2017. All larvae collected were stored in 70% alcohol in 30 mL sealed vials and kept at 4 °C. The vials had labels corresponding to a datasheet that had the following information: collection date, location, plant damage, life stage, and number of larvae collected. The samples were shipped to the South African Sugarcane Research Institute (SASRI), Mount Edgecombe, KwaZulu-Natal, South Africa and the Norwegian University of Life Sciences, Ås, Norway for identification and molecular characterization, respectively.

2.3. Morphological and Molecular Identification

Morphological identification of the collected larvae to genus or species level, or both, was based on external anatomy (chaetotaxy and crochet arrangement) based on identification keys provided by Meijirman and Ulenberg [27]. Fall armyworm samples were identified using FAO [28] descriptions of the pest. A dissecting microscope was used in examining the larval specimens. Larvae were allocated to three species namely: *Busseola fusca*, *Chilo partellus*, and *Spodoptera frugiperda*. Molecular tools described below were used to confirm species and identify unknown species.

2.4. DNA Extraction and Amplification

A total of 217 larvae were morphologically identified to species level, two specimens to genus level and two to order level, respectively. At least one larval specimen from each of the identified species/genera/order and from each of the 48 fields sampled were sent for DNA based identification at the South African Sugarcane Research Institute (SASRI), Mount Edgecombe, KwaZulu-Natal, South Africa. DNA was extracted from whole insects (if very small) or a body part, using the GeneJet Genomic DNA Purification kit (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions. The DNA was quantified using a NanoDrop Spectrophotometer (Thermo Scientific, Waltham, MA, USA). PCR amplification was conducted using the KAPA 2G Robust PCR Kit (Kapa Biosystems, Cape Town, South Africa) with approximately 50 ng DNA template. The final reaction conditions were as follows: 1x Kapa2G Buffer A, 0.2 mM dNTP mix, 0.5 µM each HCO 2198 and LCO 1490 and 0.5 units Kapa2G Robust DNA Polymerase. The DNA primer sequences used were HCO 2198 (5′ TAAACTTCAGGGTGACCAAAAAATCA 3′) and LCO 1490 (5′ GGTCAACAAATCATAAAGATATTG 3′) [29].

PCR reactions were conducted in an Applied Biosystems Veriti Thermal Cycler (Applied Biosystems, Marina Bay, Singapore). The thermal cycling profile was 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 50 s and 72 °C for 90 s. Final extension was at 72 °C for 10 min. PCR products were purified using a DNA Clean and Concentrator kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions.

2.5. DNA Sequencing

DNA sequencing was conducted using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Sequencing reactions were conducted in an Applied Biosystems Veriti Thermal Cycler using the BigDye Terminator v3.1 kit recommended thermal cycling profile. Sequencing products were purified using the BigDye XTerminator Purification Kit (Applied Biosystems, Foster City, CA, USA) according to manufacturer's instructions. DNA sequences were analysed by capillary electrophoresis using the ABI3500 Genetic Analyser (Applied Biosystems, Foster City, CA, USA) following standard operating protocols.

2.6. Sequence Analysis

DNA sequences were trimmed on the 5′ and 3′ ends to remove poor quality sequences using CLC Main workbench v7.0.1 (QIAGEN, Hilden, Germany). The putative identities for each sequence were established by comparison with the DNA barcode sequence repository of the BOLD database. Sequences were aligned using ClustalW [30] with default settings in BioEdit 7.2.5 [31]. In addition, reference sequences from GenBank were downloaded (Table 1) and incorporated in phylogenetic study. A neighbor-Joining (NJ) and maximum likelihood (ML) analysis based on K-2 parameter model [32] with complete gap deletion and resampled with 1000 bootstrap replications were done using all sequences generated in the study and the reference sequences. We used the model selection option in Mega6 [33] to find the best-fit substitution model for our dataset. Based on the lowest Bayesian Information Criterion (BIC) value, Tamura 3-parameter with discrete Gamma distribution (T92 + I) [33] fit the dataset best. Maximum Likelihood (ML) was performed in using the best-fit model and clusters and 1000 bootstrap replications were used to support clusters. Separate phylogenetic analyses with reference sequences were performed for *B. fusca* (n = 11) and *S. frugiperda* (n = 11) in Mega6. DnaSP v5 [34] was used to calculate DNA polymorphism parameters: number of polymorphic (segregating) sites, S; number of haplotypes, h; haplotype (gene) diversity, Hd; and nucleotide diversity, Pi (π). All sequences produced have been submitted to GenBank.

Table 1. Description of reference sequences used in this study and their associated GenBank accession numbers.

Family	Genus	Species	Accession No.
Noctuidae	*Busseola*	*Fusca*	KY472246, KY472247, KM061945, KM061880, DQ337201, DQ337199
	Spodoptera	*frugiperda*	KY472240, KY472248, KY472250, KY472253, KY472255, GU095403 JQ547900, HM136602 HM136600, HM136599
	Sesamia	*inferens*	KC911715
Crambidae	*Chilo*	*partellus*	KX351380, HQ991218 KP233794, HQ990905 HQ991286, HQ991263 HQ990908, HQ991263

3. Results

3.1. Occurrence of Busseola fusca, Chilo partellus, and Spodoptera frugiperda in Sugarcane Fields

3.1.1. Morphological Identification

From 48 sugarcane fields (Table S1), 221 larvae were collected. Based on morphology, we identified 219 larvae as Lepidoptera and 2 as Diptera. The 219 Lepidopteran larvae belonged to four genera namely *Chilo*, *Busseola*, *Spodoptera*, and *Sesamia.* Morphologically, *Sesamia* spp could not be identified to species level. However, we identified the remaining Lepidopteran larvae as *Busseola fusca*, *Chilo partellus*, and *Spodoptera frugiperda* (Figure 2).

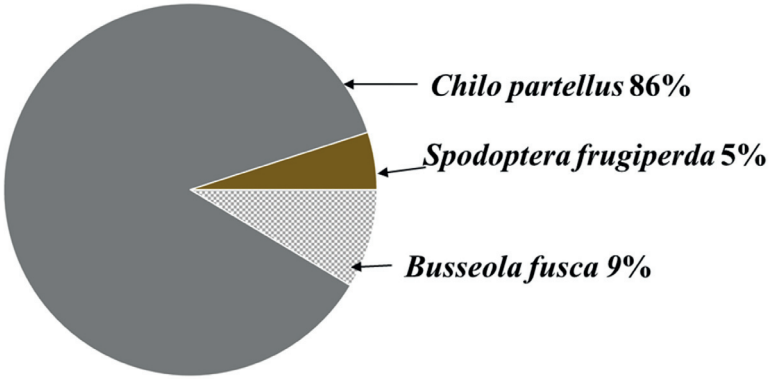

Figure 2. Percent distribution of *Busseola fusca*, *Chilo partellus*, and *Spodoptera frugiperda* (based on morphological) collected from sugarcane fields in Chikwawa and Nsanje districts, southern Malawi (n = 217).

3.1.2. DNA Based Identification

DNA was extracted from, amplified, and sequenced for 65 samples. Based on initial BOLD searches; 59 sequences were identified as *C. partellus*, 4 as *B. fusca*, 1 as *S. frugiperda* and *C. anus Curtonotum anus* (Curtonotidae: Diptera). Initial GenBank searches could not resolve the identity of the *Sesamia* larva as the top 20 searches showed 94.5% identity match as *S. inferens* and the same percentage to *B. fusca*. However, based on phylogenetic analyses, the sequence for this larva aligned with *B. fusca* with higher bootstrap branch support values (Figure 3).

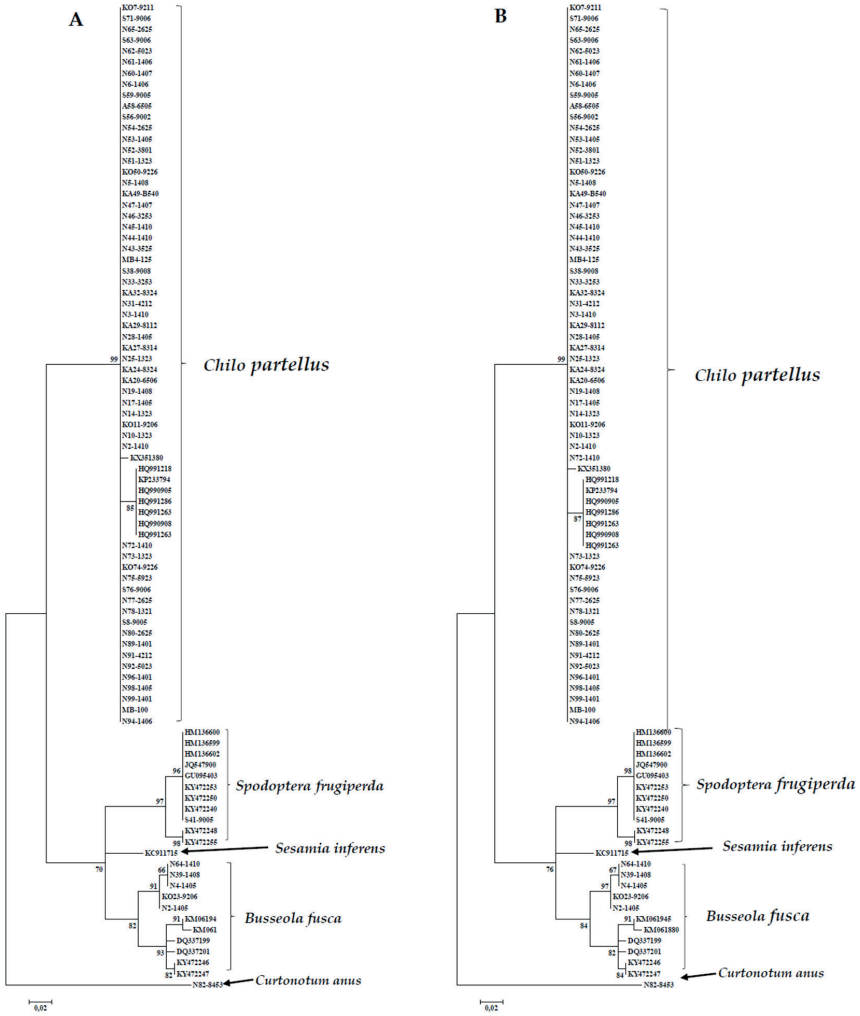

Figure 3. Phylogenetic tree inferred using the Maximum Likelihood (ML)) method of mtDNA CO1 region of *Busseola fusca*, *Chilo partellus*, and *Spodoptera frugiperda* sequences obtained from sugarcane fields in southern Malawi together with reference sequences from other African countries. (**A**) The tree is based on the Kimura 2-parameter method. (**B**) The tree is based on Tamura 3-parameter model with evolutionarily invariable (T92 + I). Both trees were resampled with 1000 bootstrap replicates. Bootstrap support values on the branches are given.

3.2. Sequence Analysis

Sixty-five sequences of varying length (average 585 bp) were generated for *B. fusca*, *C. partellus*, and *S. frugiperda*. Sequences were trimmed to 539 bp and used in analyses. A total of 25 sequences were downloaded from GenBank for comparisons and comprised *B. fusca* (n = 7), *C. partellus* (n = 8) and *S. frugiperda* (n = 10) (Table 2). A NJ and ML tree was produced for all sequences (n = 90) from this study and GenBank. Both NJ and ML trees had comparable topologies with clearly differentiated clades denoting distinct species (Figure 3). The first clade included all *C. partellus* specimens and their

corresponding reference sequences (Figure 3). The second clade consisted of *S. frugiperda* individuals and the third cluster had *B. fusca* samples (Figure 3).

Table 2. Haplotype number and diversity in *Busseola fusca*, *Chilo partellus*, and *Spodoptera frugiperda* populations.

Species	No. of Individuals (*n*)	No. of Polymorphic Sites (S)	No. of Parsimony Informative Sites (PI)	No. of Haplotypes	Haplotype Diversity (H_d)	Nucleotide Diversity (π)	Intraspecific Divergence (mean)
B. fusca	11	40	36	8	0.9273	0.036	0.037
C. partellus	70	3	2	3	0.220	0.003	0.003
S. frugiperda	11	9	8	3	0.473	0.005	0.009

Based on both NJ and ML analyses of the alignment of the alignment with COI gene sequences, we found that all *C. partellus* clustered with the reference sequences (Figure 3). The COI gene sequenced Malawian *C. partellus* samples formed one cluster which was strongly supported (bootstrap support value, 99%). As depicted in Figure 4, *B. fusca* individuals formed four distinct clusters corresponding to country of origin. Finally, the *S. frugiperda* sequence generated in this study aligned with *S. frugiperda* sp.1 from Ghana and the Americas (Figure 5). Mean between groups genetic distances were: *S. frugiperda* and *C. partellus*, 13.5%; *C. partellus* and *B. fusca*, 15.3%; *B. fusca* and *S. frugiperda*, 8.7%. Mean within group species divergence were 0.3% for *C. partellus*, 3.7% for *B. fusca*, and 0.9% for *S. frugiperda.* Intraspecific divergence for individuals within *B. fusca* ranged between 0.1% and 1.9%; 0.9% and 1.6% *S. frugiperda;* 0.0 and 2.1% *C. partellus* (supp. file S1).

Haplotype analysis using DnaSP identified three different haplotypes for *S. frugiperda*, eight for *B. fusca* and three for *C. partellus*, respectively (Table 3). *S. frugiperda* COI sequence data had nine polymorphic sites (1.73%) of which eight (1.54%) were parsimony informative (Table 3). Similarly, the sequence data for *B. fusca* contained 40 segregating (7.78%) and 36 parsimony informative (7%) sites, respectively (Table 3). *C. partellus* had three polymorphic (2.09%) and two parsimony informative (1.40%) sites. Based on the sequence statistics shown in Table 3, nucleotide diversity (π) for each of the three species indicate very low genetic diversity. Haplotype distribution for all three species is shown in Table 3. All *C. partellus* specimens from Malawi were in the most common haplotype, H-3 (Table 3). There were two haplotypes (H-1 and H-2) that had *B. fusca* individuals from Malawi (Table 3).

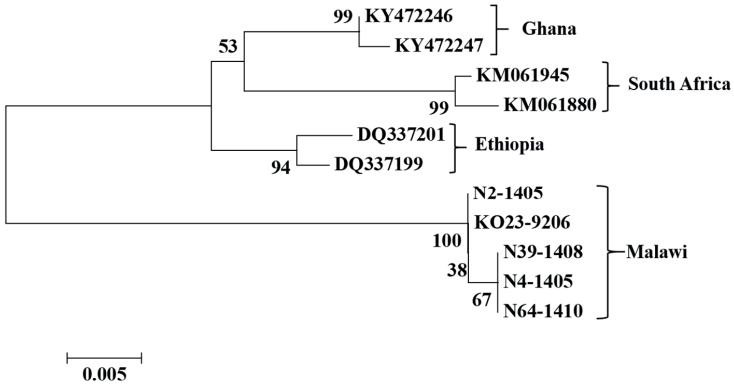

Figure 4. Phylogenetic tree inferred using the Neighbor-Joining (NJ) method of 11 mtDNA CO1 region of *Busseola fusca* sequences obtained from sugarcane fields in southern Malawi together with reference sequences from other African countries. The tree is based on the Kimura 2-parameter method. The tree was resampled with 1000 bootstrap replicates. Bootstrap support values on the branches are given.

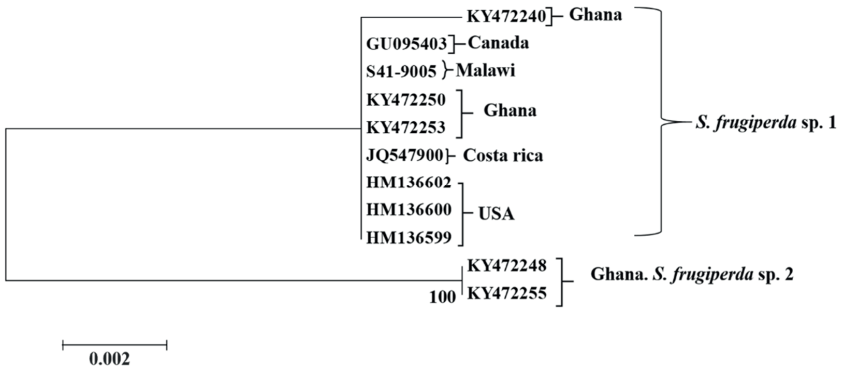

Figure 5. Phylogenetic tree inferred using the Neighbor-Joining (NJ) method of 11 mtDNA CO1 region of *Spodoptera frugiperda* sequences obtained from sugarcane fields in southern Malawi together with reference sequences from other African countries. The tree is based on the Kimura 2-parameter method and 1000 bootstrap duplications.

Table 3. Distribution of *Busseola fusca*, *Chilo partellus*, and *Spodoptera frugiperda* into respective haplotypes.

Species	Haplotype	No.	Individuals
~~1-4~~ *B. fusca*	H-1	3	N4-1405, N64-1410, N39-1408
	H-2	2	KO23-9206, N2-1405
	H-3	1	KY472246
	H-4	1	KY472247
	H-5	1	KM061945
	H-6	1	KM061880
	H-7	1	DQ337201
	H-8	1	DQ337199
S. frugiperda	H-1	8	S41-9005, KY472250, KY472253, GU095403, JQ547900, HM136602, HM136600, HM136599
	H-2	1	KY472240
	H-3	2	KY472248, KY472255
C. partellus	H-1	1	KX351380
	H-2	7	HQ991218, KP233794, HQ990905 HQ991286, HQ991263, HQ990908, HQ991263
	H-3	58	N2-1410, N10-1323, KO11-9206, N14-1323, N17-1405, N19-1408, KA20-6506, KA24-8324, N25-1323, KA27-8314, N28-1405, KA29-8112, N3-1410, N31-4212, KA32-8324, N33-3253, S38-9008, MB4-125, N43-3525, N44-1410, N45-1410, N46-3253, N47-1407, KA49-B540, N5-1408, KO50-9226, N51-1323, N52-3801, N53-1405, N54-2625, S56-9002, A58-6505, S59-9005, N6-1406, N60-1407, N61-1406, N62-5023, S63-9006, N65-2625, KO7-9211, S71-9006, N72-1410, N73-1323, KO74-9226, N75-5923, S76-9006, N77-2625, N78-1321, S8-9005, N80-2625, N89-1401, N91-4212, N92-5023, N96-1401, N98-1405, N99-1401, MB100, N94-1406

4. Discussion

The cytochrome oxidase (COI) gene of the mitochondrial DNA is generally used to identify biotypes and study population genetics in insects [18–22]. In this study, based on phylogenetic

analyses of the COI gene, larvae of Lepidopteran species infesting sugarcane in southern Malawi were identified as *Busseola fusca*, *Chilo partellus* and *Spodoptera frugiperda* (Figures 3–5).

There are two cryptic species within *S. frugiperda* known as 'species 1 or rice' and 'species 2 or maize or corn' strains [35]. Both races occur in Africa [36]. The two races differ in their susceptibility to chemical and biological agents [36]. Phylogenetic analysis based on the COI gene sequence, the *S. frugiperda* sample we collected aligned with *S. frugiperda* sample from Florida in the United States of America (USA). This indicated that the *S. frugiperda* specimen was of American origin. Moreover, the *S. frugiperda* DNA sequences sample from Kaombe closely aligned *S. frugiperda* spp. 1 or 'rice' strains (Figure 5) from Ghana where first reports of *S. frugiperda* introduction in Africa were from [17]. DNA polymorphism analysis for this pest showed very low genetic diversity alluding to its recent introduction in Africa.

S. frugiperda is an invasive species that was recently introduced in Africa [16,17]. It has a strong preference for grasses [16]. Since the 2016/2017 cropping season, *S. frugiperda* has been proving to be a serious pest of maize in Malawi. So far, the Government of Malawi's efforts on managing this pest are chiefly curative. The Food and Agricultural Organization (FAO) of the United Nations recommends the use of pheromone traps for detecting the incidence and severity of *S. frugiperda* [37]. Accurate identification of pest species is essential for effectiveness of pheromones traps as a monitoring tool [38]. Our results indicate that *S. frugiperda* infesting sugarcane in the Lower Shire Valley is the 'rice strain.' There is a need to ascertain if the 'rice strain' is the only *S. frugiperda* race infesting sugarcane in the Lower Shire Valley since both races are known to infest maize. Considering the availability of host plants throughout the year and the voracious nature of *S. frugiperda*, this species has the potential to become a serious pest of sugarcane if no effective measures are put in place to control its spread. It is also essential to determine the biology and species composition of *S. frugiperda* populations on major cereal crops of Malawi.

B. fusca specimens characterized in the study had 3.7% intraspecies divergence indicating the presence of geographical species [18,20–22]. The species had a higher haplotype diversity but low nucleotide diversity (Table 2). This indicates that there is low genetic differentiation in *B. fusca*. Our finding agrees with Assefa Y. and Dhlamini T. [18], and Peterson B.et al. [39] who reported limited sequence divergence for *B. fusca* in both Swaziland and South Africa. However, these authors did not determine genetic relatedness of their *B. fusca* insect specimens with those in other African countries. Phylogenetic analysis for *B. fusca* sequences generated in this study formed a distinct but closely related clade to *B. fusca* sequences from South Africa but was distantly related to *B. fusca* from Ethiopia and West Africa, Ghana [18,35,40]. This indicates that the *B. fusca* in southern Malawi is part of the Southern Africa population. This observation is in line with known *B. fusca* population expansion in Africa [20]. Sezonlin M. et al. [20] indicated that *B. fusca* populations in southern Africa belong to clade originate from Kenya and belong to *B. fusca* clade KII. The characteristic features for *B. fusca* clade KII are high haplotype diversity and low nucleotide diversity [20–22].

In this study, we have determined the identity of *Chilo* species infesting sugarcane in Southern Malawi using both morphological and the COI 1 gene barcode. It is *Chilo partellus* and not *C. sacchariphagus*. As an entire population, *C. partellus* samples sequenced in this study displayed low genetic diversity. Evidence of this is the low haplotype diversity (Hd) and nucleotide diversity (π) calculated for *C. partellus.* This agrees with previous studies done on *C. partellus* specimens from South Africa [19]. The current recommendation involving the use of the generalist egg parasitoid *T. chilonis* may be less effective. Instead, the larval parasitoid *Cotesia flavipes* commonly used in *C. partellus* classical biological control [41] should be employed.

Genetic variation within pest species may affect pest biology and the effectiveness of pest control tactics [42–44]. For instance, *B. fusca* morphotypes differ in their susceptibility to the main biological control agent, *Cotesia sesamiae* [20,21,41]. Similarly, genetic differentiation among *E. saccharina* populations is associated with the pest's host preferences and its natural enemy guild in different agroecological zones of Africa [45].

This study has shown that *C. partellus* (and not *C. sacchariphagus*) *and B. fusca* are the main stemborers of sugarcane in southern Malawi. We also found that the recently invasive fall armyworm *S. frugiperda* 'rice strain' infested sugarcane in southern Malawi. Genetic variability was low in *B. fusca* and the majority of *C. partellus* populations. Some *C. partellus* individuals demonstrated higher genetic diversity. Accurate pest identification is the key to sustainable and effective pest control. It is important to sequence cereal stemborer species and associated natural enemies (arthropod and microbial) from all agroecological zones of Malawi in order to improve current and offer prospects for future biocontrol using microbial pesticides.

Supplementary Materials: The following are available online at http://www.mdpi.com/2075-4450/9/3/74/s1, Table S1: Lepidoptera larvae sampling points in sugarcane fields located in Chikwawa and Nsanje districts, southern Malawi; supp. file S1: Sequences of representative larvae collected from sugarcane fields in Chikwawa and Nsanje District, Southern Malawi.

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Conflicts of Interest: The authors declare no conflict of interest.

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Natural occurrence of entomopathogenic fungi in the Hypocreales as endophytes of sugarcane (*Saccharum officinarum*) and in soil of sugarcane fields

Trust Kasambala Donga[a,b], Richard Meadow[a], Nicolai V. Meyling[c] and Ingeborg Klingen[c,*]

[a]Department of Plant Sciences, Nowergian University of Life Sciences (NMBU), Campus ÅS, Universitetstunet 3, 1433 Ås, Norway.

[b]Lilongwe University of Agriculture and Natural Resources (LUANAR), P.O. Box 219, Lilongwe, Malawi.

[c]Division for Biotechnology and Plant Health, Norwegian Institute of Bioeconomy Research (NIBIO), Høgskoleveien 7, 1431 Ås, Norway.

***** Corresponding author email address: ingeborg.klingen@nibio.no

Abstract

The occurrence of entomopathogenic fungi in the Hypocreales as endophytes in sugarcane (*Saccharum officinarum*) and in soil samples from sugarcane fields was evaluated in six location in southern Malawi. Fungi from soil were isolated by baiting 60 soil samples by the *Galleria mellonella* larvae method while fungal endophytes were isolated from 180 surface-sterilized plant tissue sections. Entomopathogenic fungi in the Hypocreales were isolated from all locations and fungi were found in 81.7% of the soil samples and in 36.7% in sugarcane plant tissue as endophytes. The genus *Beauveria* was most frequently isolated (83 isolates) but also *Metarhizium* (10 isolates) and *Isaria* (22 isolates) were collected. *Beauveria* spp. were more frequently obtained from soil samples than from sugarcane plant tissues (63.3% in soil compared to 25% from plant tissues, $\chi^2 = 67.383$, df = 15, $P < 0.001$). Phylogenetic analysis of 50 *Beauveria* spp. based on DNA sequencing of the Bloc intergenic region indicated that these isolates were *B. bassiana* and aligned with *B. bassiana* isolates of African and Neotropical origin previously denoted as AFNEO_1. However, the Malawi *B. bassiana* isolates formed a distinct clade with 99-100 bootstrap support values. To the best of our knowledge, this is the first report of *B. bassiana* and *Isaria* spp. occurring naturally as an endophyte in sugarcane. Further, it is the first report of *B. bassiana, Isaria* spp. and *Metarhizium* spp. in the soil of sugarcane fields in Africa.

Keywords

Entomopathogenic fungi, Beauveria bassiana, Metarhizium, Bloc gene region, Malawi, sugarcane

1. Introduction

Entomopathogenic fungi in the order Hypocreales are known to kill and infect arthropods, occur in soil, and as endophytes in some plants or (Vega *et al*., 2008; Gurulingappa *et al*., 2010; Reay *et al*., 2010; Fisher *et al*., 2011; Clifton *et al*., 2015; Lacey *et al*., 2015). Depending on their biology and ability to grow on artificial media, they may be used in biocontrol of plant pests (Lacey *et al*., 2015; Onwley *et al*., 2010). Entomopathogenic fungi may cause epizootics in soil-dwelling pest insects and mites (Pell *et al*., 2001). The genus *Beauveria* and *Metarhizium* and *Isaria* (Cordycipitacea: Hypocreales) are used in inoculative or inundation biological control of agricultural pests (Akello *et al*., 2008; Meyling and Eilenberg, 2007; Posada *et al*., 2007; Roy *et al*., 2010).

The natural occurrence and diversity of entomopathogenic fungi in arthropods, soils and plants may be affected by abiotic and biotic factors such as climate, habitat, soil properties, plant species, agricultural practices and sampling method (Bruck, 2010; Klingen *et al*., 2002; Klingen and Haukeland, 2006; Meyling and Eilenberg, 2007; Quesada-Moraga *et al*., 2007). Klingen and Haukeland (2006) suggest that the use of pesticides, especially fungicides but also herbicide may reduce the prevalence of entomopathogenic fungi in the soil. Further, Klingen *et al*. (2002) found more entomopathogenic fungi in organic arable fields than in conventional. Clifton *et al*. (2015) found that soils from organic soybean or cornfields had more entomopathogenic fungi than conventional and that the occurrence of entomopathogenic fungi was negatively affected by tillage, nitrogen content of soil, herbicide and fungicide application. Further, Ramos *et al.* (2017) found more *B. bassiana* in soil and roots from organic than conventional bean fields. Meyling and Eilenberg (2007) reported that *M. anisopliae* was the most prevalent species in soils collected from agricultural fields compared to undisturbed areas such as hedgerows while *B. bassiana* was frequently isolated from soils from the undisturbed areas. In addition, *Beauveria* and *Metarhizium* have both been isolated as endophytes from perennial woody plants such as coffee, pine and cocoa (Ganley and Newcomb, 2006; Posada and Vega, 2005; Vega *et al*., 2008) and non-woody plants such as beans and maize (Bing and Lewis, 1993; Parsa *et al*., 2016). The occurrence of entomopathogenic fungal endophytes varies with regard to plant tissue (Arnold and Herre, 2003).

Even though *B. bassiana* is known to be effective against arthropod pests that infest sugarcane (Cherry *et al*., 2004; Tefera and Pringle, 2004; Goble *et al*., 2012; Wu *et al*., 2014), few studies have focused on natural occurrence of entomopathogenic fungi in sugarcane cropping systems. Ngubane *et al*. (2012), however, isolated *Metarhizium anisopliae*, *Beauveria bassiana*, *B. brongniartii* and *Lecanicillium lecanii* from various insect cadavers collected from six sugarcane-growing countries in Southern Africa. To the best of our

knowledge, however, no studies on natural occurrence of entomopathogenic fungi in the Hypcreales as naturally occurring endophytes in sugarcane has been conducted. Based on this background, the aims of the present study were to investigate whether entomopathogenic fungi within the Hypocreales occur naturally in soils and as endophytes in sugarcane fields in Malawi and whether different sugar cane cropping systems affects their prevalence.

2. Materials and methods

2.1. Description of sugarcane production, location and sampling of plants and soil

Sugarcane is vegetatively cultivated short stem cuttings (referred to as plant cane) and from old growth (referred to as ratoon cane). As in many African countries, smallholder farmers grow sugarcane for home consumption but also sell raw sugarcane in local markets (Baucum *et al*., 2009). These farmers grow traditional cultivars or a mixture of cultivars and intercrop the sugarcane rows with other crops such as maize and vegetables. Sugarcane is grown in seasonal wetlands, valley bottoms called '*dambo*' and low-lying areas called '*dimba*.' For the purpose of this paper, we will refer to these as 'traditional' fields. Traditional farmers use a hoe for tilling the soil twice or more times per year and they irrigate the field as required. Insecticides are used without following economic thresholds by traditional farmers (Kasambala Donga and Eklo, 2018; Orr and Ritchie, 2003). Commercial estates owned by foreign multinational companies also grow sugarcane to process it into sugar and other sugarcane based product. These estates use irrigation and other cultivars that originate from both within and outside African. A third category of sugarcane farmers are referred to as 'outgrowers' and they grow sugarcane using the same varieties as the commercial estates either under rainfed conditions or irrigation. Outgrowers are supposed to follow production guidelines used in commercial estates and may belong to a farmer association that provides input packages (seed, fertilizer and herbicides) on credit or may act independently (Kasambala Donga and Eklo, 2018). Commercial estates and outgrowers sugarcane fields are ploughed once every 3.8 years. In commercial estate and outgrowers sugarcane fields, insecticides and herbicides are applied according to economic threshold levels (Kasambala Donga and Eklo, 2018) provided by ILLOVO Malawi agronomists based at Nchalo Estate. Sugarcane is harvested green in traditional fields but is burnt prior to harvesting in commercial estates and outgrowers' fields.

In this study, field surveys were conducted from July to December 2016 in six locations namely Mitole, Maseya, Phata, Kasinthula and Alumenda in Chikwawa District of southern Malawi. Within each location, two sites were randomly selected and 30 m x 30 m quadrat was used to establish sampling units (Fig. 1). We sampled plants that were less than 5 months-old. The average number of harvesting cycles (ratoons) for sugarcane production in Malawi is 3.8 years. Therefore, only sugarcane in the fourth to seventh cycle of

ratooning were sampled. Plants were sampled by carefully uprooting one plant form the center and from the four corners of the 30 m x 30 m quadrat. Collected plants (n = 60) were transported live in plastic bags in cooler boxes to the laboratory for subsequent assessment (within 24 h) for the presence of endophytic entomopathogenic fungi. Five soil samples were collected at a distance of 60 cm from the base of the collected plant and down to 15 cm depth by the use of a garden spade. The spade was sterilized in 70% alcohol between sampling to prevent cross-contamination. Soil samples were then placed separately in 1 L polyethylene bags and transported immediately in 40 L cooler boxes to the laboratory for processing.

2.2. Isolation of fungi

2.2.1. Isolation of endophytic fungi from plant samples

Upon arrival at the laboratory, the soil was carefully shaken off the plant roots and roots were washed with tap water. From each sampled sugarcane a 100 mm section of stem, leaf and root was cut out and surface sterilized as described by Parsa *et al*. (2013) by immersion them for 2 min in 3% sodium hypochlorite followed by 2 min in 70% ethanol and then rinsed thrice for 30 s in sterile distilled water. Effectiveness of the sterilization process was evaluated by plating 100 µl of the last rinse water on Sabouraud Dextrose Agar (SDA, Oxoid) with 1% antibiotics (0.2 g penicillin, 0.2 g chloramphenicol and 0.2 g tetracycline dissolved in 10 mL sterile distilled water, followed by filter sterilization through a 0.2 mm filter). No fungal growth from the last rinse of water indicated that sterilization was successful. The sterilized plant tissue sections were dried on sterile paper for 1 min and trimmed the edges so that the sections measured 60 mm. The 60 mm trimmed section were further dissected into five pieces and plated on SDA. After sealing with Parafilm, the Petri dishes were incubated in the dark for 14-21 days at 25±5 °C. Fungal growth emerging from the plant tissue were reisolated onto new SDA plates to obtain pure cultures. Mycelia and conidia from pure cultures were stored on silica gel at 25±5 °C and later used for morphological and molecular characterization.

2.2.2. Isolation of fungi from soil samples

In the laboratory, the five soil samples per site were thoroughly mixed to produce a 12 composite pooled soil samples. Soils were kept at 4 °C for until processing but never longer than for five days. All soil samples were sieved through a 2 mm mesh sieve to remove debris. Dry soil samples were slightly moistened with sterile water while wet soils were first air-dried to remove excess water and reduce the incidence of nematodes. The *Galleria mellonella* bait method described by Zimmermann (1986) was used to isolate entomopathogenic fungi from soil samples. Before used as baits, 4-5 week-old *G. mellonella* larvae were heat-conditioned as described by Woodring and Kaya (1988) by immersing in 56 °C sterile water for 15 s, followed by pouring cold water on top of the larvae for 30 s and then letting the larvae rest for 1 h to

recover. Five live heat-conditioned *G. mellonella* were then added to a 350 ml plastic container with aerated lid containing 300 g of the sifted soil sample and incubated for 14 days in the dark at 25±5 °C. The plastic containers were inverted once every two days to promote larval movement through the soil.

Containers with soil samples were checked daily and dead larvae were removed and surface sterilized by immersing them in 70% alcohol for 10 s, rinsed thrice in sterile water for 10 s and left to dry on a sterile paper towel. They were then individually placed in a moist chamber and incubated for 14 days at 25±5 °C. Dead lave were observed every 2 days for fungal growth and emerging fungi were isolated by placing them on SDA with 0.1% antibiotics and incubated as described above. A fungal culture obtained from a single larva was considered an isolate. Fungal isolates were stored in silica gel until morphological and molecular characterization.

2.3. Morphological identification of fungi

Entomopathogenic fungi in the Hypocreales were identified morphologically by examining under a 400X phase contrast microscope to genus level according to Humber (2012).

2.4. Molecular identification of fungi down to species level

To identify entomopathogenic fungi in the Hypocreales down to species level molecular techniques needs to be used (Bischoff *et al*., 2009). Molecular analysis of fungi down to species level in this study have until now, only been conducted on the 50 isolates that were morphologically identified to be in the genus *Beauveria*. We are presently working on molecular identification of the fungal isolates in the other genera as well.

2.4.1. DNA extraction, PCR amplification and sequence analysis

DNA extraction and PCR reactions were done at NIBIO, Ås, Norway. A few silica gel crystals from the stored fungal isolates placed onto SDA plates (9 cm diameter) and incubated in the dark for 14 days at room temperature (21-25 °C) in the laboratory at NIBIO. Mycelium and conidia were then harvested by scraping off a small portion of the fungus using a sterile scalpel. The harvested mycelium and conidia were then ground to a fine powder using a pestle and mortar in liquid nitrogen before extracting the genomic DNA using DNeasy Plant Mini kit (Qiagen, Germany) according to manufacturer's instruction (Goble *et al*., 2012).

PCR amplification targeting the Bloc intergenic region for 50 *Beauveria* isolates were carried out (Rehner *et al*., 2011) using Bio-Rad T100™ Thermal cycler. Amplification of the Bloc gene region was achieved with the primer pair B22U (5′-AGATTCGCAACGTCAACTT-3′) and B822L (5′-GTCGCAGCCAGAGCAACT-3′). The reaction volume of 50 µl contained 1.5µl Mm $MgCL_2$, 1 x PCR buffer, 4 µ 200 µM dNTPs, 1 µl of each primer (10µM), 0.1 µl 0.5U Platinum *Taq* DNA polymerase and 3µl genomic DNA. Cycling conditions were for Bloc gene regions were as follows: 5 min at 95 °C denaturation followed by a touch-down protocol with 30 s denaturation at 95 °C, 30 s at 70–60 °C (reducing annealing temperature by 1 °C per cycle), and 1 min at 72 °C. An additional 30 cycles were performed including 30 s at 95 °C, 30 s annealing at 60 °C, and 1 min at 72 °C followed by a final extension of 5 min at 72 °C.

The extracted DNA was quantified using gel electrophoresis - 1.0% agarose gel with TBE (45 mM Tris base, 45mM boric acid, 1mM EDTA pH 8.0). Staining of bands with ethidium bromide (Thermo Fisher Scientific, USA) was done to help with visualization of the amplified DNA through GelDoc EQ (Bio-Rad Laboratories, USA) gel imaging system equipped with PDQuest 2-D analysis software (Bio-Rad Laboratories, USA). The size of the PCR products was determined by comparing to a 100 bp DNA ladder (New England Biolabs, UK). PCR products were diluted (where necessary) in nuclease free water to acquire the right concentration (10-50 $ng^{-\mu l}$) recommended for sequencing. Sanger sequencing was done by GATC Biotech (Germany) using the B22U/ B822L primer pair.

2.4.2. Phylogenetic analysis

The sequences obtained from Bloc region were traced, edited and assembled using CLC Main workbench 7. Consensus sequences were aligned using ClustalW in BioEdit 7.2.5 (Hall, 1999). Published sequences (Goble *et al*., 2012; Kernasa *et al*., 2016; Rehner *et al*., 2006) for the identified genera were included in phylogenetic analysis. Intraspecific divergence was calculated using Mega6 (Kimura, 1980). DNA polymorphism was determined using DnaSP v.5 (Librado and Rozas, 2009). Preliminary Neighbor-Joining (NJ) and Maximum Likelihood (ML) trees were generated for the aligned sequences using Mega6 (Tamura *et al*., 2013). Both NJ and ML trees were based on Kimura 2-parameter model, K2P (Kimura, 1980). Using model selection option in Mega6, we found that Kimura 2-parameter 80 with discreet Gamma distribution (K2+G) was the best-fit model to our dataset based on lowest Bayesian Information Criterion (BIC) value. We used the best-fit model to generate ML analysis using 1000 bootstrap replications. Reference sequence of *Beauveria malawiensis* was included to root the phylogenetic tree.

2.5. Data analysis

Preliminary data exploration indicated that the data (frequencies of occurrence of soil and plant samples positive for *Beauveria* spp, *Isaria* spp. and *Metarhizium* spp. collected from soil and sugarcane plants) did not follow a normal distribution. Hence, frequency data were analyzed using non-parametric tests, independent samples option. Statistical analyses were carried out in SPSS version 25 (IBM® Statistics Software).

3. Results

3.1. Morphological identification

A total of 180 plant tissues collected from 60 sugarcane plant samples and 60 *G. mellonella* larvae were used to bait 12 soil samples in this study. Fungi were isolated from 66 out of 180 (36.7%) plant tissues collected while entomopathogenic fungi were isolated from 49 out of 60 (81.7%) *G. mellonella* baited from soil. The entomopathogenic fungi found belonged to three genera: *Beauveria*, *Metarhizium* and *Isaria*. Irrespective of location and ecological habitat (soil or plant tissues), the mean proportion (±SE) of *Beauveria* isolates was significantly higher ($\bar{x}$ = 0.61±1.081, χ^2 = 70.390, df = 15, P < 0.001) than that of *Metarhizium* spp. ($\bar{x}$ = 0.05±0.277, χ^2 = 18.089, df = 10, P = 0.0) and *Isaria* spp. ($\bar{x}$ = 0.17±0.579, χ^2 = 27,127, df = 15, P = 0.028). The distribution of these fungal isolates were significantly different across location (χ^2 = 30.611, df = 5, P < 0.001) and field (χ^2 = 36.770, df = 11, P < 0.001; Fig. 2). *Beauveria* spp. and *Isaria* spp. were isolated from all fields (except in Maseya where no *Isaria* spp. was isolated) while no *Metarhizium* spp. were isolated from Nchalo, Kasinthula and Phata (Fig. 2). There was no significant difference in the occurrence of *Beauveria* spp. in commercial estates and outgrowers fields but between these two field types and traditional fields (for each cropping system n = 80, P < 0.05). *Beauveria* spp., *Metarhizium* spp. and *Isaria* spp. also occurred naturally as endophytes of sugarcane (Fig. 2). *Beauveria* spp. were more frequently obtained from soil than from plant tissue (45.7% in soil compared to 33.7% in leaves 15.6% in stems and 4.8% in roots; χ^2 = 67.383, df = 15, P < 0.001). From plant material, *Isaria* spp. were isolated more from leaves (χ^2 = 36.414, df = 15, P = 0.002) and stems (χ^2 = 20.571, df = 15, P = 1.150) than from roots. *Metarhizium* spp. were mostly isolated from soil with (χ^2 = 18.089, df = 10, P = 0.053).

3.2. Phylogenetic analysis

Sequences for all the 50 *Beauveria* isolates were generated and 29 Bloc sequences were downloaded from the GenBank for phylogenetic placement of the sequences. The alignment contained 845 positions. After eliminating gaps and missing data, 703 nucleotide positions were included in the final dataset. NJ and ML analyses based on the Bloc produced trees with similar topologies with well-resolved clusters representing isolates of five different species (Fig. 3). There were two main branches, one representing *B. bassiana* and *B. varroae* species and the other representing *B. pseudobassiana*, *B. brongniartii* and *B. malawiensis* species, respectively. All 50 *Beauveria* isolates sequenced in this study belong to *B. bassiana* (Fig. 3). The *B. bassiana* clade further separated into several branches. One branch contained all the 50 Malawian isolates and reference sequences of Africa (Cameroon, Côte d'Ivoire, Kenya, Togo) and the Neotropics (Brazil, Colombia, Costa Rica, Mexico, Nicaragua) denoted as AFNEO_1 (Rehner *et al*., 2006). The AFNEO_1 clade includes *B. bassiana* strains isolated from coffee or from the coffee berry borer, *Hypothenemus hampei* (Rehner *et al*., 2006). Within this branch, the Malawi isolates formed a distinct clade with 99-100 bootstrap support values (Fig. 3).

Inter- and intra-specific divergences were calculated for all Bloc isolates. Pairwise genetic distances was lowest (0.016) for Malawian *B. bassiana* and *B. bassiana* of AFNEO_1 origin. Genetic divergence between Malawian *B. bassiana* and *B. bassiana* of non-AFNEO_1 origin was 0.045 (Table 1). No mean intraspecific divergence within the Malawian *B. bassiana* isolates was observed, while it was 0.014 within the AFNEO_1, and 0.045 within the non-AFNEO_ 1 *B. bassiana* individuals (*B. bassiana* s.l. isolated isolates collected from insects in several orders and from countries not listed in the AFNEO_1 group (Table 2; Rehner *et al*., 2006). Table 2 also shows the results of haplotype analysis. A total of 23 haplotypes was found within *B. bassiana* species. The isolates from Malawi presented 3 haplotypes in which 1 was unique (110-A-S-C). The AFNEO_1 isolates presented 7 haplotypes. Description of individuals in each haplotype is presented in Table 3.

4. Discussion

To the best of our knowledge this is the first report of *B. bassiana* and *Isaria* spp. occurring naturally as an endophyte in sugarcane. *B. bassiana* and *Isaria* spp. isolated from sterilized sugarcane plant tissue may have originated from the naturally inoculated plant parts, soil or through infected insect hosts. This is supported by studies that recently demonstrated the ability of *B. bassiana* to experimentally establish as an endophyte of sugarcane (Kasambala Donga *et al*. (in press)). The higher incidence of *B. bassiana* in leaf tissue in our study could have been a result of aerial deposition of *B. bassiana* propagules (Hajek, 1997;

Meyling and Eilenberg, 2006). In addition, insects have the ability to transport *B. bassiana* to plant surfaces (Bruck and Lewis 2002a). It is also possible that virulent stage of *B. bassiana* infected sugarcane tissues through its endophytic relationship with the plant. As an endophyte, *B. bassiana* has been isolated from plant tissues of common bean, coffee and cocoa plants, faba beans, maize and pine needles (Akutse *et al*., 2016; Bing and Lewis, 1993; Ganley and Newcomb, 2006; Posada and Vega, 2005; Ramos *et al*., 2017; Vega *et al*., 2008; 2010). *Isaria* spp. (formerly *Paecilomyces*, Humber, 2012), has been reported as endophyte in rice (*Paecilomyces* sp.); mangrove (*Paecilomyces varioti*); banana (*Paecilomyces* sp.) and coffee plants (*Paecilomyces* cf. *fumosoroseus*, *P.* cf. *javanicus*; Ananda and Sridhar, 2002; Cao *et al*., 2002; Tian *et al*., 2004; Vega *et al*., 2008). Endophytism between entomopathogenic fungi such as *B. bassiana* (not all *Isaria* spp. are pathogenic to insects) and plants is considered to be detrimental to insect pests (Vega *et al*., 2010; Vega, 2018). The negative impact on insect pests may be through synthesis of herbivore-induced plant volatiles (HIPVs) and secondary metabolites (terpenoids) involved in plant defense against herbivory, and alteration of plant volatiles (kairomones) used by insects in host location (Lin *et al*., 2016; 2017; Price *et al*., 2011; Shrivastava *et al*. 2015; Vega, 2018).

We also report for the first time, the natural occurrence of *Beauveria* spp., *Isaria* spp. and *Metarhizium* spp. in soil from sugarcane fields in Malawi. The prevalence of *Beauveria* spp. was significantly higher in fields of commercial estates (Alumenda and Nchalo) than in fields of outgrowers (Kasinthula and Phata) or in traditional sugarcane fields (Maseya and Mitole). The difference in prevalence level may be due to variations in farm management practices (Geiger *et al*., 2010; Meyling *et al*, 2009). Although all sugarcane fields are subjected to convectional tillage i.e. turning over and loosening of soil after harvest, this practice is more frequent in traditional fields (multiple times in a year) compared to commercial estates and outgrowers' fields (done once in every 3.8 years). Convectional tillage has previously been reported to be negatively associated with abundance of entomopathogenic fungi in agricultural fields (Clifton *et al*., 2015; Oliveira *et al*., 2013). In traditional fields, insecticides are applied based on pest occurrence and not economic thresholds (Kasambala Donga and Eklo, 2018). As a result, more insecticides are used in traditional farmers' fields (Bon *et al*., 2014). Insecticides reduce insect populations in a field and maybe the endophytic inoculum in the plant due to fungally infected hosts and this may be important for the dissemination of entomopathogenic fungal inoculum between the soil and the phyllosphere (de Snoo, 1999; Klingen *et al*., 2002; Marshall and Moonen, 2002). However, several other biotic and abiotic factors that are known to influence the natural occurrence and diversity of entomopathogenic fungi in agroecosystems were not considered in this study (Clifton *et al*., 2015; Klingen *et al*., 2002; Klingen and Haukeland, 2006; Oliveira *et al*., 2013; Quesada-Moraga *et al*., 2007). To what extent these factors may have influenced our findings is currently unknown.

Our phylogenetic analysis placed the 50 *Beauveria* spp. isolates within *B. bassiana* clades. Malawian *B. bassiana* isolates found in our study were closely related to *B. bassiana* of other countries in African (Cameroon, Côte d'Ivoire, Kenya, and Togo) and Neotropical origin (Brazil, Colombia, Costa Rica, Mexico, and Nicaragua) referred to as AFNEO_1 and isolated from the coffee berry borer *Hypothenemus hampei* (Coleoptera: Cucurlionidae; Rehner *et al*., 2006). *B. bassiana* isolates characterized in our study belonged to a single distinct clade. The reason for this could be that all the AFNEO_1 isolates were from coffee berry borer but none of our isolates were isolated from this insect. Also, the low intraspecific divergence, haplotype and nucleotide diversity reported in this study show that there is gene flow within our *B. bassiana* populations. Therefore, we suggest that the *B. bassiana* population that we found in the six locations in Chikwawa district in southern Malawi could be considered as one structured population with a low genetic diversity. Our results are similar to Ramos *et al.* (2017) who found limited diversity among *B. bassiana* isolates from common bean fields in Cuba. It is also possible that we underestimated the diversity and richness of fungal endophytes isolated in our study as endophyte diversity and richness has been reported to increase with plant age in cacao (*Theobroma cacao*), Coccoloba (*Coccoloba cereifera*) and Lima bean (*Phaseolus lunatus*; Arnold and Herre, 2003; López-González *et al.* 2017; Sanchez-Azofeifa et al., 2012).

The present study is the first to report of *B. bassiana* and *Isaria* spp. as naturally occurring endophytic fungi in sugarcane. Further, it suggests that *B. bassiana* and *Isaria* spp. occupies a naturally occurring reservoir in soils and crop tissues of conventionally and traditionally grown sugar cane. It also highlights the importance of *B. bassiana* as a potential naturally occurring enemy of pests in sugarcane since *B. bassiana* is already known to be effective against arthropod pests that infest sugarcane (Cherry *et al*., 2004; Tefera and Pringle, 2004; Goble *et al*., 2012; Wu *et al*., 2014). Our molecular studies further suggest that the *B. bassiana* isolates we found in Malawi were closely related to *B. bassiana* of other countries in Africa and that these isolates are one structured population with a low gene flow. Future studies will focus on determining the effect of naturally occurring *B. bassiana* as and endophyte and in soil on sugarcane insect pest populations. Identification and analysing the rest of the isolates obtained in this study by the molecular methods described in this paper are underway and will be conducted before submitting this manuscript to a journal.

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Figure captions

Figure 1. Locations of the 12 sugarcane fields sampled in Chikwawa District, southern Malawi (A). Sampling scheme within a sugarcane field (B).

Figure 2. Number of fungal isolates in different genera (A). *Beauveria* spp., (B) *Metarhizium* spp., (C) *Isaria* spp. obtained from soils (n = 60) and as endophytes of sugarcane (*Saccharum officinarum)* tissues (root, n = 60; stem, n = 60; leaf, n = 60) from 2 commercial estates (Alumenda and Nchalo), 2 outgrowers fields (Kasinthula and Phata) and 2 traditional fields (Maseya and Mitole) in Chikwawa District, southern Malawi.

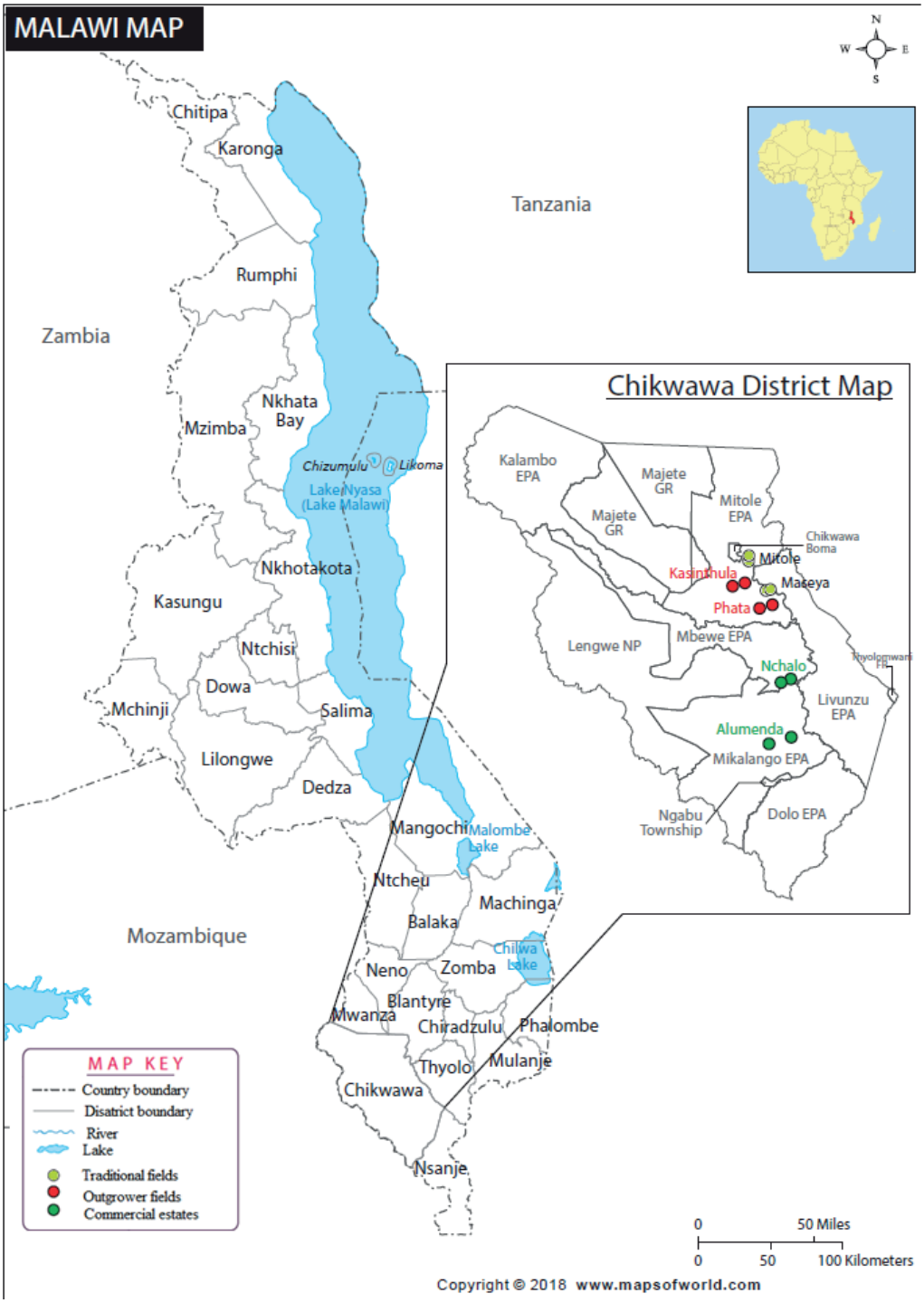
Figure 3. Phylogenetic tree of *Beauveria* indicating the position of Malawian isolates collected from sugarcane fields within the worldwide *Beauveria* genetic structure. The tree was inferred by (A) Maximum Likelihood (ML) method based on the Kimura 2-parameter model with a discrete Gamma distribution (K2+G) and (B) Neighbor-Joining (NJ) using the Kimura 2-parameter method of intergenic Bloc region of 50 Malawian and 30 reference sequences from GenBank (given with their associated accession number). For the Malawi isolates, code after isolate number denotes substrate: S = soil, P = plant; location: MS = Maseya, MT = Mitole, N = Nchalo, P = Phata, K = Kasinthula, A = Alumenda. Branch support was measured through 1000 bootstrap repeations.

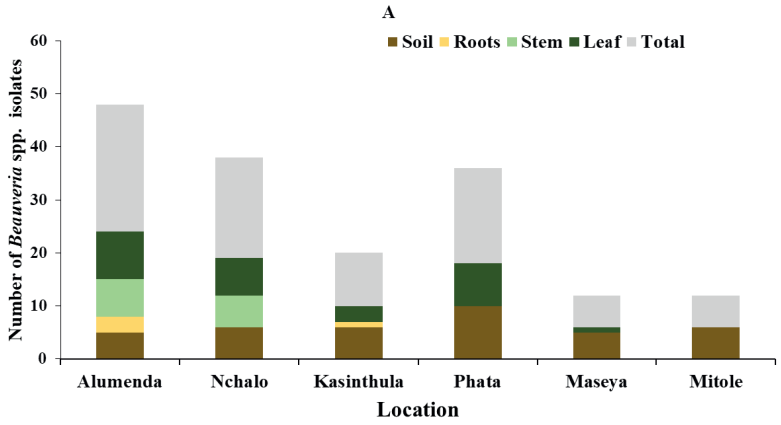
Table captions

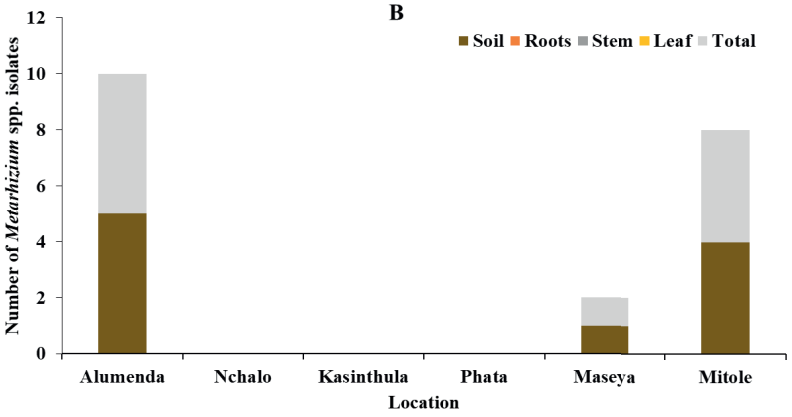
Table 1: Pairwise genetic distances (K2P) between *Beauveria* species estimated using Mega6

Table 2: Haplotype analysis for *Beauveria bassiana* isolates

Table 3. Number of haplotypes and isolates contained in each haplotypes for *B. bassiana* species


MALAWI MAP
N
W
E
S
Chitipa
Karonga
Tanzania
Rumphi
Zambia
Nkhata Bay
Mzimba
Chizumulu
Likoma
Lake Nyasa (Lake Malawi)
Nkhotakota
Kasungu
Ntchisi
Dowa
Mchinji
Salima
Lilongwe
Dedza
Mangochi
Malombe Lake
Ntcheu
Machinga
Balaka
Mozambique
Chilwa Lake
Neno
Zomba
Blantyre
Mwanza
Chiradzulu
Phalombe
Thyolo
Mulanje
Chikwawa
Nsanje
Chikwawa District Map
Kalambo EPA
Majete GR
Majete GR
Mitole EPA
Chikwawa Boma
Mitole
Kasinthula
Maseya
Phata
Lengwe NP
Mbewe EPA
Nchalo
Thyolomwani FR
Livunzu EPA
Alumenda
Mikalango EPA
Ngabu Township
Dolo EPA
MAP KEY
Country boundary
Disatrict boundary
River
Lake
Traditional fields
Outgrower fields
Commercial estates
0
50 Miles
0
50
100 Kilometers
Copyright © 2018 www.mapsofworld.com


A
Soil
Roots
Stem
Leaf
Total
Number of Beauveria spp. isolates
60
50
40
30
20
10
0
Alumenda
Nchalo
Kasinthula
Phata
Maseya
Mitole
Location


B
Soil
Roots
Stem
Leaf
Total
Number of Metarhizium spp. isolates
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8
6
4
2
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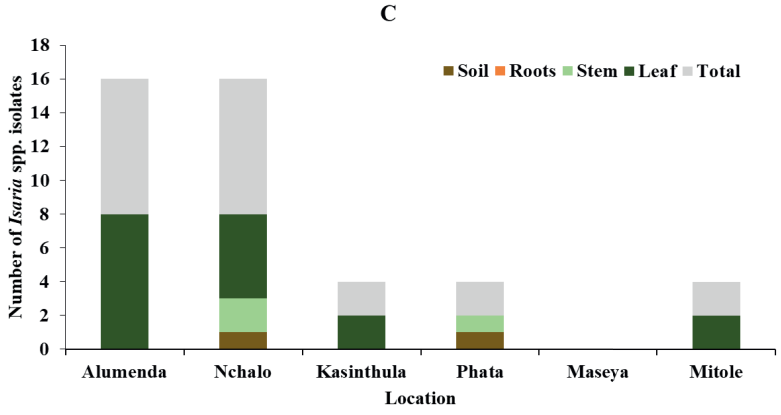
Alumenda
Nchalo
Kasinthula
Phata
Maseya
Mitole
Location


C
Soil
Roots
Stem
Leaf
Total
Number of Isaria spp. isolates
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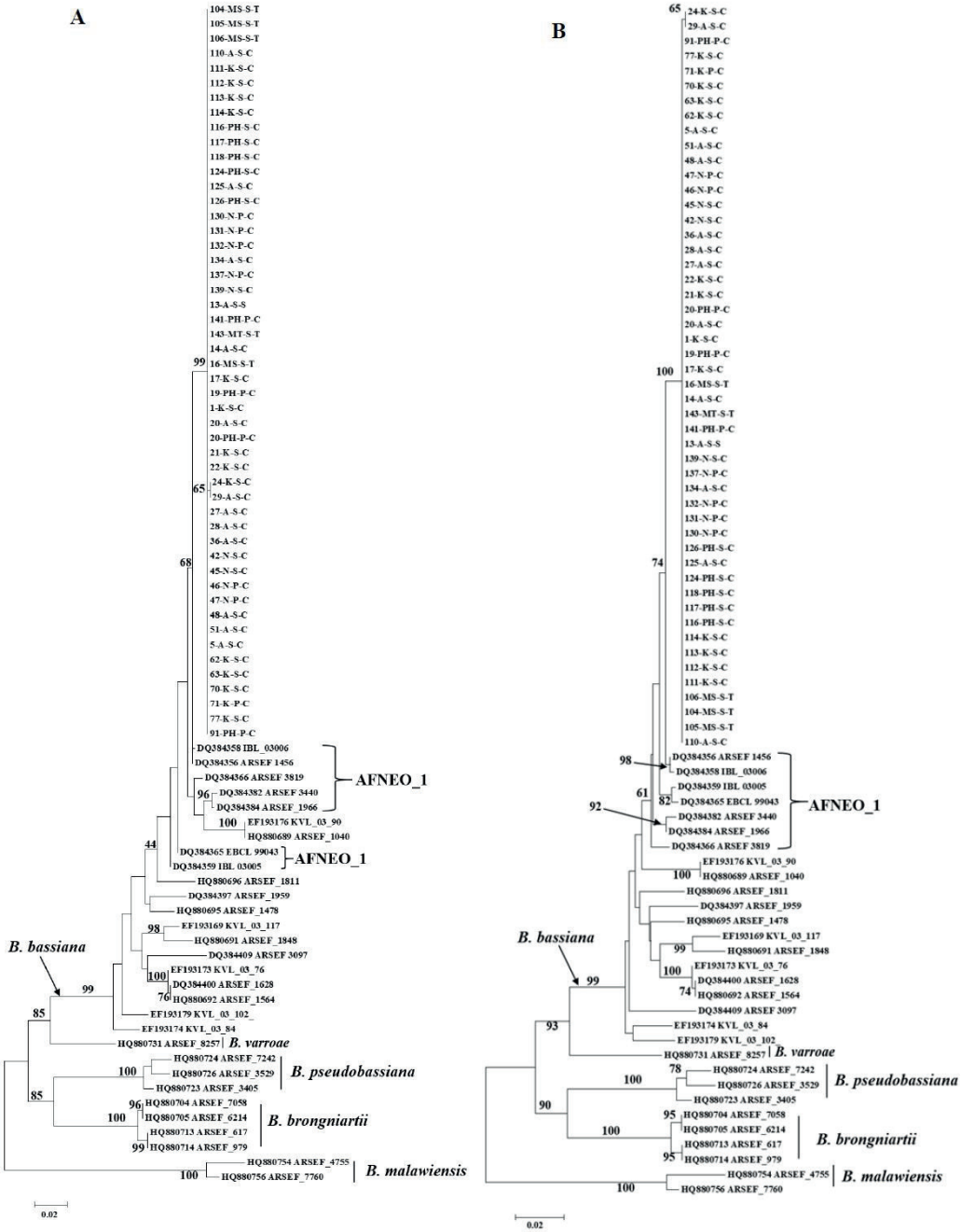
16
14
12
10
8
6
4
2
0
Alumenda
Nchalo
Kasinthula
Phata
Maseya
Mitole
Location


A
104-MS-S-T
105-MS-S-T
106-MS-S-T
110-A-S-C
111-K-S-C
112-K-S-C
113-K-S-C
114-K-S-C
116-PH-S-C
117-PH-S-C
118-PH-S-C
124-PH-S-C
125-A-S-C
126-PH-S-C
130-N-P-C
131-N-P-C
132-N-P-C
134-A-S-C
137-N-P-C
139-N-S-C
13-A-S-S
141-PH-P-C
143-MT-S-T
14-A-S-C
16-MS-S-T
17-K-S-C
19-PH-P-C
1-K-S-C
20-A-S-C
20-PH-P-C
21-K-S-C
22-K-S-C
24-K-S-C
29-A-S-C
27-A-S-C
28-A-S-C
36-A-S-C
42-N-S-C
45-N-S-C
46-N-P-C
47-N-P-C
48-A-S-C
51-A-S-C
5-A-S-C
62-K-S-C
63-K-S-C
70-K-S-C
71-K-P-C
77-K-S-C
91-PH-P-C
DQ384358 IBL_03006
DQ384356 ARSEF 1456
DQ384366 ARSEF 3819
DQ384382 ARSEF 3440
DQ384384 ARSEF_1966
EF193176 KVL_03_90
HQ880689 ARSEF_1040
DQ384365 EBCL 99043
DQ384359 IBL 03005
AFNEO_1
HQ880696 ARSEF_1811
DQ384397 ARSEF_1959
HQ880695 ARSEF_1478
EF193169 KVL_03_117
HQ880691 ARSEF_1848
DQ384409 ARSEF 3097
EF193173 KVL_03_76
DQ384400 ARSEF_1628
HQ880692 ARSEF_1564
EF193179 KVL_03_102_
EF193174 KVL_03_84
HQ880731 ARSEF_8257
B. varroae
HQ880724 ARSEF_7242
HQ880726 ARSEF_3529
HQ880723 ARSEF_3405
B. pseudobassiana
HQ880704 ARSEF_7058
HQ880705 ARSEF_6214
HQ880713 ARSEF_617
HQ880714 ARSEF_979
B. brongniartii
HQ880754 ARSEF_4755
HQ880756 ARSEF_7760
B. malawiensis
B. bassiana
0.02
B
24-K-S-C
29-A-S-C
91-PH-P-C
77-K-S-C
71-K-P-C
70-K-S-C
63-K-S-C
62-K-S-C
5-A-S-C
51-A-S-C
48-A-S-C
47-N-P-C
46-N-P-C
45-N-S-C
42-N-S-C
36-A-S-C
28-A-S-C
27-A-S-C
22-K-S-C
21-K-S-C
20-PH-P-C
20-A-S-C
1-K-S-C
19-PH-P-C
17-K-S-C
16-MS-S-T
14-A-S-C
143-MT-S-T
141-PH-P-C
13-A-S-S
139-N-S-C
137-N-P-C
134-A-S-C
132-N-P-C
131-N-P-C
130-N-P-C
126-PH-S-C
125-A-S-C
124-PH-S-C
118-PH-S-C
117-PH-S-C
116-PH-S-C
114-K-S-C
113-K-S-C
112-K-S-C
111-K-S-C
106-MS-S-T
104-MS-S-T
105-MS-S-T
110-A-S-C
DQ384356 ARSEF 1456
DQ384358 IBL_03006
DQ384359 IBL 03005
DQ384365 EBCL 99043
DQ384382 ARSEF 3440
DQ384384 ARSEF_1966
DQ384366 ARSEF 3819
AFNEO_1
EF193176 KVL_03_90
HQ880689 ARSEF_1040
HQ880696 ARSEF_1811
DQ384397 ARSEF_1959
HQ880695 ARSEF_1478
EF193169 KVL_03_117
HQ880691 ARSEF_1848
EF193173 KVL_03_76
DQ384400 ARSEF_1628
HQ880692 ARSEF_1564
DQ384409 ARSEF 3097
EF193174 KVL_03_84
EF193179 KVL_03_102
HQ880731 ARSEF_8257
B. varroae
HQ880724 ARSEF_7242
HQ880726 ARSEF_3529
HQ880723 ARSEF_3405
B. pseudobassiana
HQ880704 ARSEF_7058
HQ880705 ARSEF_6214
HQ880713 ARSEF_617
HQ880714 ARSEF_979
B. brongniartii
HQ880754 ARSEF_4755
HQ880756 ARSEF_7760
B. malawiensis
B. bassiana
0.02

Location	Field type	Crops grown	Cultivar grown	Pesticides used	Tillage system
Alumenda	Commercial	sugarcane	MN1	Acetamiprid, (acetamiprid 200g/L)	Convectional using tractor ploughs
Nchalo	Commercial	Sugarcane	MN1	Acetamiprid, (acetamiprid 200g/L)	Convectional using tractor ploughs
Kasinthula	Outgrower	Sugarcane	MN1, N32	Cypermethrin 200 EC (200g/L cypermethrin) Acetamiprid, (acetamiprid 200g/L)	Convectional using tractor ploughs
Phata	Outgrower	Sugarcane	N32	No information	Convectional using tractor ploughs
Maseya	Traditional	Sugarcane, maize, onions, tomato.	Mixture of local cultivars such as Msenjere, Chiutsa and Mkono wa Mwana	Dimethiote 40EC (400g/L dimethoate) Cypermethrin 200 EC(200g/L cypermethrin) Aceta 20 SL (200g/L acetamiprid) Marshal 250 EC (25% v/v carbosulfan)	Convectional using hoes
Mitole	Traditional	Sugarcane, maize, onions, tomato	Mixture of local cultivars such as Msenjere, Chiutsa and Mkono wa Mwana	Dimethiote 40EC (400g/L dimethoate) Aceta 20 SL (200g/L acetamiprid) Cypermethrin 200 EC(200g/L cypermethrin) Marshal 250 EC (25% v/v carbosulfan)	Convectional using hoes

Species	[1]Malawian *B. bassiana*	[2]AFNEO_1 *B. bassiana*	[3]Non-AFNEO_1 *B. bassiana*	*B. brongniartii*	*B. varroae*	*B. pseudobassiana*	*B. malawiensis*
Malawian *B. bassiana*							
AFNEO_1 *B. bassiana*	0.016						
Non-AFNEO_1 *B. bassiana*	0.045	0.043					
B. *brongniartii*	0.121	0.120	0.123				
B. varroae	0.085	0.081	0.088	0.109			
B. pseudobassiana	0.132	0.128	0.133	0.105	0.109		
B. malawiensis	0.171	0.166	0.170	0.175	0.176	0.169	

[1] All sequenced Malawian *B. bassiana* isolates collected from sugarcane fields in Chikwawa District, Malawi.

[2] *B. bassiana* s.l. isolated isolates collected from the coffee berry borer *Hypothenemus hampei* (Coleoptera: Curculionidae) from Africa (Cameroon, Côte d'Ivoire, Kenya, Togo) and the Neotropics (Brazil, Colombia, Costa Rica, Mexico, Nicaragua; Rehner *et al*., 2006).

[3] *B. bassiana* s.l. isolated isolates collected from insects in several orders and from countries not listed in the AFNEO_1 group (Rehner *et al*., 2006).

Species	No. of individuals (n)	No. of haplotypes (h)	Haplotype diversity (H_d)	No. of polymorphic sites (S)	Parsimony informative sites	Nucleotide diversity (π)	Intraspecific diversity
[1]Malawian *B. bassiana*	50	3	0.117	2	1	0.000	0.000
[2]AFNEO_1 *B. bassiana*	7	7	1.000	26	15	0.014	0.014
[3]Non-AFNEO_1 *B. bassiana*	14	13	0.989	111	65	0.042	0.043

[1] All sequenced Malawian *B. bassiana* isolates collected from sugarcane fields in Chikwawa District, Malawi.

[2] *B. bassiana* s.l. isolated isolates collected from the coffee berry borer *Hypothenemus hampei* (Coleoptera: Curculionidae) *from* Africa (Cameroon, Côte d'Ivoire, Kenya, Togo) and the Neotropics (Brazil, Colombia, Costa Rica, Mexico, Nicaragua; Rehner *et al*., 2006).

[3] *B. bassiana* s.l. isolated isolates collected from insects in several orders and from countries not listed in the AFNEO_1 group (Rehner *et al*., 2006).

Species	Haplotype	No. (n)	isolates
[1]Malawian *B. bassiana*	H-1	47	104-MS-S-T, 105-MS-S-T, 106-MS-S-T, 111-K-S-C, 112-K-S-C, 113-K-S-C, 114-K-S-C, 116-PH-S-C, 117-PH-S-C, 118-PH-S-C, 124-PH-S-C, 125-A-S-C, 126-PH-S-C, 130-N-P-C, 131-N-P-C, 132-N-P-C, 134-A-S-C, 137-N-P-C, 139-N-S-C, 13-A-S-S, 141-PH-P-C, 143-MT-S-T, 14-A-S-C, 16-MS-S-T, 17-K-S-C, 19-PH-P-C, 1-K-S-C, 20-A-S-C, 20-PH-P-C, 21-K-S-C, 22-K-S-C, 27-A-S-C, 28-A-S-C, 36-A-S-C, 42-N-S-C, 45-N-S-C, 46-N-P-C, 47-N-P-C, 48-A-S-C, 51-A-S-C, 5-A-S-C, 62-K-S-C, 63-K-S-C, 70-K-S-C, 71-K-P-C, 77-K-S-C and 91-PH-P-C
	H-2	1	110-A-S-C
	H-3	2	24-K-S-C, 29-A-S-C]
[2]AFNEO_1 *B. bassiana*	H-1	1	DQ384382
	H-2	1	DQ384384
	H-3	1	DQ384358
	H-4	1	DQ384359
	H-5	1	DQ384365
	H-6	1	DQ384356
	H-7	1	DQ384366
[3]Non-AFNEO_1 *B. bassiana*	H-1	1	DQ384397
	H-2	2	DQ384400, HQ880692
	H-3	1	DQ384409
	H-4	1	EF193169
	H-5	1	EF193173
	H-6	1	DQ384358
	H-7	1	EF193174
	H-8	1	EF193176
	H-9	1	EF193179
	H-10	1	HQ880689
	H-11	1	HQ880691
	H-12	1	HQ880695
	H-13	1	HQ880696

[1] All sequenced Malawian *B. bassiana* isolates collected from sugarcane fields in Chikwawa District, Malawi.

[2] *B. bassiana* s.l. isolated isolates collected from the coffee berry borer *Hypothenemus hampei* (Coleoptera: Curculionidae) *from* Africa (Cameroon, Côte d'Ivoire, Kenya, Togo) and the Neotropics (Brazil, Colombia, Costa Rica, Mexico, Nicaragua; Rehner *et al*., 2006).

[3] *B. bassiana* s.l. isolated isolates collected from insects in several orders and from countries not listed in the AFNEO_1 group (Rehner *et al*., 2006).

Establishment of the fungal entomopathogen *Beauveria bassiana* as an endophyte in sugarcane, *Saccharum officinarum*

Trust Kasambala Donga[a,b], Fernando E. Vega[c] and Ingeborg Klingen[d,*]

[a]Department of Plant Sciences, Nowergian University of Life Sciences (NMBU), Campus ÅS, Universitetstunet 3, 1433 Ås, Norway.

[b]Lilongwe University of Agriculture and Natural Resources (LUANAR), P.O. Box 219, Lilongwe, Malawi.

[c]Sustainable Perennial Crops Laboratory, United States Department of Agriculture (USDA), Agricultural Research Service, Beltsville, MD 20705, USA

[d]Division for Biotechnology and Plant Health, Norwegian Institute of Bioeconomy Research (NIBIO), Høgskoleveien 7, 1431 Ås, Norway.

* Corresponding author.

E-mail address: ingeborg.klingen@nibio.no

ABSTRACT

We investigated the ability of the fungal entomopathogen *Beauveria bassiana* strain GHA to endophytically colonize sugarcane (*Saccharum officinarum*) and its impact on plant growth. We used foliar spray, stem injection, and soil drench inoculation methods. All the three inoculation methods resulted in *B. bassiana* colonizing sugarcane tissues. Extent of fungal colonization differed significantly with inoculation method ($\chi^2 = 20.112$, d.f. = 2, $p = 0.000$), and stem injection showed the highest colonization level followed by foliar spray and root drench. Extent of fungal colonization differed significantly with plant part ($\chi^2 = 33.072$, d.f. = 5, $p = 0.000$); stem injection resulted in *B. bassiana* colonization of the stem and to some extent leaves; foliar spray resulted in colonization of leaves and to some extent, the stem; and soil drench resulted in colonization of roots and to some extent the stem. Irrespective of inoculation method, *B. bassiana* colonization was 2.8 times lower at 14-16 d post inoculation (DPI) than at 7-10 DPI ($p = 0.020$). Spraying leaves and drenching the soil with *B. bassiana* significantly ($p = 0.01$) enhanced numbers of sett roots. This study demonstrates for the first time that *B. bassiana* can endophytically colonize sugarcane plants and enhance the root sett and it provides a starting point for exploring the use of this fungus as an endophyte in management of sugarcane pests.

Key words: Biological control, endophytic fungus, entomopathogenic fungi, Hypocreales, *Beauveria bassiana*, phytobiome

1. Introduction

Sugarcane (*Saccharum officinarum*; Poaceae) is one of the world's most valuable crops. Although sugarcane originated in Polynesia, it is grown in approximately 120 tropical and subtropical countries with a global production of about 1.89 billion tonnes of crushed sugarcane in 2016 (FAOSTAT 2018). The sugarcane ecosystem (phytobiome) comprises numerous weeds, arthropods and more than 50 plant pathogens (Ferreira and Comstock 1993; Verma 2004; Leach et al. 2017). Arthropod pests associated with the crop worldwide include complexes of stalk feeders, sap sucking insects (e.g., aphids, thrips, mealybugs), root feeders (e.g., white grubs, stemborers), and spider mites (Dittrich *et al.* 2005; Barker et al. 2006; Leslie 2008, 2009; Goebel and Sallam 2011; Goble et al. 2014; SASRI 2014; Bharu 2015).

The main arthropod pests infesting sugarcane in Africa include stemborers (*Chilo* and *Sesamia* spp.), black maize beetles (*Heteronychus* spp.), thrips (*Fulmekiola serrata*), scale insects (*Aulacaspis tegalensis*), mealybugs (*Saccharicoccus sacchari*) and spider mites (*Tetranychus urticae*) (Smith-Meyer 1974; Nuessly 1994; Conlong 2001, 2008; SASRI 2014; Language 2015). The sugarcane yellow aphid (*Sipha flava*) was first recorded in southern Africa in 2013 (Conlong and Way 2014; Way et al. 2014). Management of all these pests currently relies on cultural methods, host plant resistance, chemical insecticide application, and biological control focusing on use of insect predators and parasitoids (Akbar et al. 2010; Goebel et al. 2010; Bowling et al. 2016). Chemical insecticides provide rapid and effective control of many pests and reduce labour cost associated with mechanical pest removal. However, health and environmental problems, the development of insecticide resistance, and cost, limits their use (WHO 2014; Kasambala Donga and Eklo 2018). Host plant resistance may contribute to reduced pesticide load in the environment, but it might not be long lasting or practical in instances of a

new virulent pest species (Humphries et al. 2010). Biological control agents are usually compatible with other pest control methods and are central in integrated pest management (IPM) programs of many crops.

Fungal entomopathogens belonging to the order Hypocreales (Ascomycota) or to the phylum Entomophthoramycota have been reported to protect plants from insect pests (Pell *et al.* 2009; Vega *et al*. 2012). Fungi in the Entomophthoromycota are generally associated with natural epizootics on foliar insect hosts and are mostly used in conservation biological control (Ekesi et al. 2005; Baverstock et al. 2008; Pell et al. 2009). The major disadvantage with Entomophthoromycota is that they are mainly biotrophic with a close association with their insect or mite host and many cannot be mass-produced on artificial media (Jaronski and Jackson 2012). On the other hand, hypocrealean fungi such as *Beauveria* and *Metarhizium* are hemibiotrophic, cosmopolitan and ubiquitous in the soil but do not commonly cause natural, large-scale epizootics on foliar insects in annual crops (Pell et al. 2009; Jaronski 2010). For instance, in a survey of natural enemies of *Chilo sacchariphagus* in sugarcane plantations in Moçambique, Conlong and Goebel (2002) found *B. bassiana* infesting only three cadavers of *C. sacchariphagus* larvae. Hypocrealean fungi are traditionally employed in both inundation and inoculation biological control (Maniania et al. 2001; Meyling and Eilenberg 2007; Remadevi et al. 2010; Klingen et al. 2014). Currently, large-scale inundation and inoculative biological control is being practiced in many countries including Austria, Brazil and South Africa (Lacey et al. 2015).

There is growing evidence that fungal entomopathogens occur naturally or can be established artificially as endophytes in various crop plants and that such establishment might adversely affect insect pests (Vega 2008; Quesada-Moraga et al. 2014a; Greenfield et al. 2016;

Vega 2018). *Beauveria bassiana* artificially introduced as an endophyte in cotton (*Gossypium hirsutum*) negatively affected cotton aphid reproduction (Castillo Lopez et al. 2014) and endophytic *B. bassiana* in maize (*Zea mays*) resulted in all-season suppression of the European corn borer, *Ostrinia nubilalis* (Bing and Lewis 1992a; 1992b). In banana (*Musa* spp.), endophytic *B. bassiana* significantly reduced damage caused by larvae of *Cosmopolites sordidus* by 42-87% depending on the plant tissue (Akello et al. 2007).

Several approaches have been used in establishing *B. bassiana* as an endophyte in target plants. Lewis and Bing (1991), Bing and Lewis (1992a; 1992b) and Wagner and Lewis (2000) successfully established *B. bassiana* as an endophyte in maize using foliar application at the two-leaf or whorl stage. *Beauveria bassiana* was also established as an endophyte in cocoa (*Theobroma cacao*; Posada and Vega 2005) and coffee (*Coffea arabica*; Posada and Vega 2006) by inoculating the main radicle of seedlings. Posada et al. (2007) also established *B. bassiana* in coffee seedlings using stem injections, foliar sprays, and soil drenches, with highest endophytic recovery obtained in plants whose stems had been injected with a *B. bassiana* spore suspension. Tefera and Vidal (2009) reported that *B. bassiana* could be established as an endophyte in different sorghum (*Sorghum bicolor*) tissues through seed dressing, foliar sprays, and soil inoculation, with foliar sprays being the best method. Brownbridge et al. (2012) introduced *B. bassiana* into pine seedlings (*Pinus radiata*) using seed coating and root dipping. Quesada-Moraga et al. (2014b) established *B. bassiana* as an endophyte in opium poppy (*Papaver somniferum*) tissue via seed soaking and found that *B. bassiana* was vertically transmitted via seeds from endophytically colonized maternal plants. Evaluating the potential of an entomopathogenic fungal species to establish as an endophyte in a given plant species is the first step in the process of determining whether this fungus might protect the plant from insect pests

or mites. The most common method for evaluating endophytic establishment is the fragment plating method (Torres et al. 2011). This method involves the elimination of epiphytes, by surface sterilizing plant tissue sections, and plating the sterilized sections on selective growth media (Vega, 2018). Post-inoculation time for performing this step varies. Ten days were enough to confirm that *B. bassiana* could establish endophytically in artichoke, *Cynara scolymus* (Guesmi-Jouini et al. 2014). Greenfield et al. (2016) evaluated *B. bassiana* endophytic colonization of cassava (*Manihot esculenta*) at 7-9 and 47-49 d. Renuka et al. (2016) traced post-inoculation persistence of *B. bassiana* in maize (*Zea mays*) for 90 d.

Information on the ability of *B. bassiana* to endophytically colonize sugarcane and effects of *B. bassiana* on sugarcane plant growth is not available. We report that *B. bassiana* can become established as an endophyte in sugarcane using foliar spray, stem injection and soil drench and that endophytism with *B. bassiana* resulted in enhanced sugarcane plant growth.

2. Materials and methods

2.1. Treatments, study location, and experimental design

The experiment was conducted in a greenhouse at the ILLOVO Malawi sugarcane quarantine facility at Bvumbwe Agricultural Research Station, Thyolo District, Malawi (15°55'27.1"S 35°04'12.5"E, 1174 m.a.s.l). The experiment was set up as a completely randomized design with subsampling, and treatments consisted of three different fungal inoculation methods (foliar spray, stem injection, soil drench) and the control. The experiment was repeated four times. Each replicate had 36 plants: 9 foliarly-sprayed plants, 9 stem-injected plants, 9 soil-drenched plants, and 9 control plants. Therefore, the experiment consisted of 144 plants. Destructive sampling of plant tissue (leaves, stems, roots) to evaluate endophytic colonization by *B. bassiana* was done 7

and 14 d post-inoculation (DPI). For method, see below. Evaluation of plant growth was done 16 DPI.

2.2. Plants

The sugarcane variety MN1 was used. This is a commonly grown variety in Malawi (Kasambala Donga and Eklo, 2018). Sugarcane stems free from pests and diseases were collected from 7-10-month-old irrigated seedcane growing at the ILLOVO Nchalo Sugar Estate (Chikwawa District, Malawi). The stems were cut into smaller sections approximately 13.5 cm long. Each of these sections had two buds. These stem cuttings are referred to as 2-bud cane-setts (Fig. 1A). To prevent ratoon stunting disease and other bacterial sugarcane pathogens, cane-setts are routinely dipped in 50 °C water for 2 h. This treatment could have negative effects on germination (McFarlane 2013); therefore, surface sterilization in alcohol and sodium hypochlorite was used as described below. Two-bud cane-setts were washed for 1 min in running tap water to remove any debris before surface sterilizing by immersing for 3 min in 1% sodium hypochlorite followed by 1 min in 70% ethanol (Parsa et al. 2013; McKinnon et al. 2016). The tissues were then rinsed in sterile distilled water three times. The sterilized plant tissues were dried on sterile filter paper for 30 min before plating. Effectiveness of the sterilization process was evaluated by plating 100 µl of the last rinse water on Sabouraud dextrose agar (SDA) and incubating the plate for 10 d at 25 °C. Imprints of sterilized plant tissue were also prepared to ensure that the sterilization was successful. This was done by momentarily placing and pressing a surface sterilized plant tissue on SDA and incubating the plate for 10 d at 25 °C.

Two surface sterilized two-bud cane-setts were horizontally planted in each 10 L plastic bucket (height 235 mm, upper diameter 265 mm, lower diameter 170 mm) containing a steam-sterilized mixture (2:1:1) of sandy loam soil, bagasse and sand from the ILLOVO Nchalo Sugar

Estate (Fig. 1B). The sterilization of the soil involved leading steam from a 210 L metal drum into a perforated hosepipe under a heavy-duty PVC black sheet secured at the edges by heavy stones. The temperature inside the PVC sheets was maintained at 92-95 °C for 5 h. The soil was cooled for 24 h before planting. Diammonium phosphate (25 g) was mixed with the soil mixture to provide phosphate in each 10 L plastic bucket. The soil was moistened using sterile distilled water 24 h before planting. After germination, buckets were thinned by discarding cane-setts with poorly growing shoots; therefore, each plastic bucket had only one two-bud sett with one or two healthy shoots. Plants were watered with sterile distilled water as required. The plastic buckets were kept in a greenhouse for 14 d after planting.

2.3. Fungal strain

A commercial strain of *B. bassiana* (GHA) formulated as BotaniGard® ES was used (Laverlam International Corporation, Butte, MT). The strain was chosen based on its registered use against aphids and sugarcane borers. To generate the stock inoculum, one inoculating loop of liquid emulsifiable suspension was suspended in 1 ml of a 0.1% sterile water solution of Tween 80 (Sigma-Aldrich, St. Louis, MO) and vigorously hand-shaken for 30 sec. From the suspension, 100 µl was plated on SDA and incubated for 24 h at 25±5 °C. A single germinating conidium was transferred to a 90 mm diameter Petri dish containing SDA mixed with a 0.1% stock solution of antibiotics to inhibit bacterial growth (Posada and Vega 2005). The antibiotic stock consisted of 0.2 g of each of three antibiotics (chloramphenicol, penicillin and tetracycline) dissolved in 10 mL sterile distilled water, followed by filter sterilization through a 0.2 mm filter. From this, 1 mL was added to each liter of medium. The fungus was grown in the dark at 25±5 °C until it covered the entire plate.

The fungus was then harvested by scraping it off the SDA using a sterile spatula and suspending it in 10 ml sterile 0.1% Tween 80 and vigorously hand-shaking for one min. The suspension was filtered through sterile cheesecloth to remove hyphae and to obtain the stock suspension. An improved Neubauer haemocytometer was used to estimate the spore concentration of the stock suspension. Sterile distilled water was used to adjust the stock concentration to a final concentration of $1x10^8$ conidia ml^{-1}. Conidial viability was assessed just after harvest and prior to inoculation of plants by plating 100 µl of 1.7 $x10^9$ conidia ml^{-1} on SDA and incubating at 25±5 °C for 24 h. Three random groups of 100 spores were examined using a stereoscope to estimate percent germination. A conidium was considered germinated when a visible germ tube longer than half the diameter of the conidium was observed. Conidial germination was > 90% and was considered acceptable for use in the experiments. The stock suspension was stored in sterile 300 ml glass bottles in darkness at 4°C for 24 h before use.

2.4. Plant inoculation

Plants were watered to saturation using sterile distilled water 24 h before inoculations. Seven days after the emergence of the primary shoot, the plants were inoculated with *B. bassiana*. Three different inoculation methods were used: foliar spray application; stem injection and soil drench. For inoculation by foliar spray, plants were sprayed in a separate room to prevent accidental inoculation of the other treatments via spray droplets. A manual atomizer was used to apply 100 ml inoculum ($1x10^8$ conidia ml^{-1}) onto the sugarcane leaves. The top of the plastic bucket was covered with aluminum foil to avoid conidial runoff to the soil. After spraying, the plants were covered with a plastic bag for 24 h to maintain a high level of humidity to facilitate fungal germination and plant colonization (Parsa *et al.* 2013) before being returned to the experimental blocks in the greenhouse. For inoculation by stem injection, a hole was made on

the primary shoot using a 5 ml sterile disposable insulin hypodermic needle to facilitate injection of 1 ml of conidial suspension (Akello *et al.* 2007; Posada *et al.* 2007). For inoculation by soil drench, 100 ml of inoculum was applied to the soil surface in close proximity with the root area. Control plants of all three treatments were treated with sterile water with 0.1% Tween 80.

2.5. Sampling for endophytic colonization

The first sampling was done 7 DPI. Due to problems with availability of a consistent power supply throughout the experiments, collection, surface sterilization and plating of plants samples onto Petri dishes was done on four consecutive days for the first sampling. The second sampling was done 14 DPI and took three consecutive days to process. At each sampling time, 3 foliarly-sprayed plants, 3 stem-injected plants, 3 soil-drenched plants, and 3 control plants were carefully uprooted (from each replicate) to avoid damage to roots using a sterilized garden spade and placed in plastic bags. The garden spade was dipped in 70% alcohol after each plant was uprooted. The plants were then transferred to the laboratory for examination of endophytic colonization by *B. bassiana*. The base of the plant was washed under running tap water to remove debris and soil while carefully avoiding destruction of root tissue. After washing, leaves were processed first followed by roots, and lastly the stems.

The endophytic colonization evaluation method outlined by Greenfield *et al*. (2016) was followed. Leaves (60 mm), stems and roots sections were surface sterilized (McKinnon et *al.* 2016) as described above. The outer edges of the tissues were dissected and discarded. Each trimmed sample was cut into six sections, averaging 6x6 mm for leaves and 6 mm long for stems and roots and plated on a 90 mm Petri dish with SDA supplemented with antibiotics (as described above). The Petri dish was sealed with parafilm and incubated in the dark at 25±5 °C. The last rinse water was changed after processing each block of a given treatment. Before

discarding the final rinsing water, a 100 µl sample was plated on SDA and incubated for 10 d at 25±5 °C to assess sterilization success. Imprints as described above were also done to assess sterilization success. The plates were inspected for fungal growth every 2-3 d for 20 d. If fungal growth was detected, the corresponding samples were discarded. No fungal growth on the medium used for the imprint indicated that sterilization was successful. Each plant yielded six plates, two per plant part divided into proximal and distal parts as described in Fig. 1C.

2.6. Growth of B. bassiana*-treated sugarcane plants*

The following sugarcane growth parameters were determined 16 DPI: number of healthy green leaves; sett roots and shoot roots; plant height; length of longest root (the distance in cm from plant base to the tip of the root); length of newly emerged leaves (the distance in cm from stem/leaf joint blade to the tip of the leaf); and wet and dry biomass. Plant height was measured from the soil surface to the tip of the stem. Dry weight was determined after oven-drying whole plant samples at 50°C for 72 h (Greenfield *et al.* 2016).

2.7. Data analysis

Colonization was considered as the number of tissue parts showing *B. bassiana* growth in each Petri dish. We modelled the number of tissue part inoculated using a negative binomial regression model. The model was chosen because overdispersion was observed under Poisson distribution (sample mean of the outcome = 0.71, variance = 2.07, variance/mean ratio = 2.92). Inoculation method, sampling time and plant part were the predictors in the model. We included an interaction term of treatment sampling time (time 1 = 7-10 DPI; time 2 = 14-16 DPI) and plant part (plant part 1 = leaf distal; plant part 2 = leaf proximal; plant part 3 = stem distal; plant part 4 = stem proximal; plant part 5 = root distal; plant part 6 = root proximal) inoculated was

used to test if colonization in the different plant tissues differed with time. All plant growth data was subjected to general linear model multivariate procedures. Prior to analyses number of bud roots data were subjected to $\log_{10+1}$ transformation because positive skewness was observed. Tukey HSD test ($p = 0.05$) was used to separate significant means. Model estimation and multivariate analysis were performed in SPSS software version 24 (IBM® Corp. 2016).

3. Results

3.1. Evaluation of endophytic colonization

All three inoculation methods resulted in *B. bassiana* becoming established as an endophyte in sugarcane tissues. Fungal colonization levels differed significantly with inoculation method ($\chi^2 = 20.112$, d.f. = 2, $p = 0.000$), sampling time $\chi^2 = 11.187$, d.f. = 1, $p = 0.001$) and plant part ($\chi^2 = 33.072$, d.f. = 5, $p = 0.000$). Foliar spray resulted in successful colonization of leaves and stems but not roots (Fig. 2). When using foliar sprays, the highest mean number of leaves colonized by *B. bassiana* was recorded at 7-10 DPI in distal leaves (2.6±0.05) and at 14-16 DPI in distal parts of the stem (2.44±0.97). These were significantly ($p = 0.000$) higher than that in proximal leaf and stem (Fig. 2). *Beauveria bassiana* colonization of leaf tissues significantly ($p = 0.000$) decreased between 7-10 and 14-16 DPI (Fig. 2).

Stem injections led to *B. bassiana* colonizing stems and leaves but not roots (Fig. 2), and colonization was significantly higher in proximal parts of stems at both 7-10 and 14-16 DPI (4.6±0.05) compared to distal stems (1.67±0.70) (Fig. 2). *Beauveria bassiana* also colonized leaves following stem injection but at significant ($p = 0.000$) lower levels than that in stems. *Beauveria bassiana* recovery in stems and leaves did not change over time (Fig. 2).

Soil drench inoculation resulted in successful colonization of roots, and there was no significant ($p = 0.05$) difference in the colonization of proximal and distal roots. The highest root colonization (1.6±0.05) was recorded 7-10 DPI and it was significantly ($p = 0.01$) higher than at 14-16 DPI. *Beauveria bassiana* was also detected in stems following soil drenches only at 7-10 DPI (Fig. 2).

Based on the negative linear regression analysis and irrespective of inoculation method, *B. bassiana* colonization was 2.8 times lower at 14-16 DPI than at 7-10 DPI ($p = 0.020$). Based on the same analysis, expectations of *B. bassiana* colonization level of sugarcane was higher than the observed *B. bassiana* colonization level for all factors tested (inoculation methods, plant parts, time).

Beauveria bassiana was never recovered from control plants. *Penicillium* and *Aspergillus* were the only other fungi isolated from plants receiving stem injections and foliar sprays.

3.2. Growth of B. bassiana*-treated sugarcane plants*

Plant growth data indicate that inoculation method affected plant height ($F = 3.985$; $df = 3$; $p = 0.013$), number of sett roots ($F = 6.762$; $df = 3$; $p = 0.01$) and fresh weight ($F = 6.430$; $df = 3$; $P = 0.011$). Plants in the foliar spray and soil drench treatments developed more sett roots than plants in the stem injection and control treatments (Table 1). The length of leaves and height of plants that had received stem injections or a soil drench were not significantly different from each other (Table 1). None of the plants showed any signs of disease.

4. Discussion

This study has demonstrated for the first time the ability of *B. bassiana* to endophytically colonize sugarcane roots, stems and leaves following foliar spray, stem injection and soil

drenching. Our results agree with Behie et al. (2015) who found that, unlike *Metarhizium* spp., *B. bassiana* does not display preferential tissue colonization. In addition, *B. bassiana* recovery was significantly higher in plants inoculated via foliar sprays and stems injections than soil drenching. Several papers have reported similar results (Quesada-Moraga et al. 2007; Tefera and Vidal 2009; Guesmi-Jouini et al. 2014; Russo et al. 2015; Jaber and Enkerli 2017). In a study involving coffee plants, soil drenching was a more effective way of introducing *B. bassiana* as an endophyte than foliar sprays (Posada *et al.* 2007). One possible explanation for this finding is that the leaf might be a poor route of entry for *B. bassiana* due to the absence of stomata on the adaxial (upper) surface and the presence of substances/structures on the leaf surface that may have negatively affected germination of conidia (Posada *et al.* 2007). In contrast to coffee plants, sugarcane has stomata on both sides of the leaf (Ferreira *et al.* 2007). Considering that spray droplets from foliar spray application may not totally cover the abaxial leaf surface, the adaxial stomata are probably an important route of entry for the *B. bassiana* germination tube in sugar cane. However, the germinating conidium has to overcome a cuticular wax layer that may completely cover the sugarcane plant stomata (Ferreira *et al*. 2007). Use of stem injection as an inoculation method bypasses these physical hurdles.

Drenching the soil with *B. bassiana* did result in root colonization. *Beauveria bassiana* persistence in root tissue did not result in systemic colonization of other sugarcane tissues, as has been reported for banana (Akello et al. 2007), sorghum (Tefera and Vidal, 2009), and red campion (*Silene dioica*; Yan et al. 2015). There was no statistical difference in *B. bassiana* establishment in distal and proximal part of the roots. This is in contrast with what Greenfield et al. (2016) reported for cassava roots. The following explanation could account for this difference. During the first weeks of sugarcane germination, the root system is comprised chiefly

of thin, hairy and highly branched sett roots arising from the root band and thick, fleshy, and less branched shoot roots (Smith et al. 2005). These roots are concentrated in the top 20 cm of soil (Blackburn, 1984). Using a pot experiment, Kim et al. (2010) found that within 18 d of soil inoculation, *B. bassiana* strain GHA growth was concentrated in the upper soil surface. In our study, both the proximal and distal portions of the roots were concentrated in the upper soil surface. In addition, we could not attribute the reason for the poor establishment of *B. bassiana* in roots following soil drenches to the presence of *B. bassiana* antagonists in the soil as suggested by Tefera and Vidal (2009), since the soil used in our experiment had been sterilized. Furthermore, no other endophytes were isolated from roots of plants inoculated by soil drenching. Lastly, *B. bassiana* has been reported to have lower soil persistence when applied as unformulated conidia using the soil drench method (Vänninen et al. 2000).

Overall, the incidence rate of *B. bassiana* colonization of sugarcane decreased over time and significantly differed among sugarcane tissues irrespective of inoculation method. This observation is similar to previous findings in other crops such as maize (Renuka et al. 2016), crested wheat grass (*Agropyron cristatum*) (Inglis et al. 1993) and iceberg lettuce (*Lactuca sativa* cv. Mirette) (Shrestha et al. 2015). Dilution of initial fungal inoculum due to rapid plant growth (Inyang et al. 1998) may account for the low persistence of *B. bassiana*. We would expect *B. bassiana* persistence to be very low as the plant ages. Therefore, multiple applications may be required to ensure persistence in the first 5 months when the plant is established.

Recovery of *B. bassiana* from the distal part of leaves, stems, and roots following foliar sprays, stem injections, and soil drenches indicate that *B. bassiana* was capable of some movement within the plant, as already reported for maize (Bing and Lewis 1991; 1992a, Wagner and Lewis 2000), coffee (Posada et al. 2007), tomato (*Solanum lycopersicum*) (Klieber and

Reineke 2015) and pine trees (*Pinus radiate*) (Lefort et al. 2016). Yan et al. (2015) found that fungal endophytes displayed very limited systematic growth within plants; the inoculated fungal endophyte remained localized in the plant part that had received the initial fungal treatment. This seems to be the case with sugarcane, where the level of *B. bassiana* recovered was significantly higher in the plant part that received the initial fungal inoculum. In maize, however, mycelial growth in xylem vessels was the main mechanisms in which the fungus applied on the leaves colonized the stem (Wagner and Lewis 2000; Cherry et al. 2004).

It is important to note that fungal entomopathogen endophytism might induce plant responses that might have an effect on the plant, insects and/or plant pathogens (Cory and Hoover 2006; Gomez-Vidal et al. 2006; Cory and Ericsson 2010; Yan *et al*. 2015). If compounds involved in host plant resistance are induced, systematic colonization over long periods by an entomopathogenic fungus in a given plant tissue may not be essential for detrimental effects on insect pests. For instance, terpenoids are an integral part of the plant chemical defense system (Singh and Sharma 2015). Shrivastava et al. (2015) found that *B. bassiana*-inoculated tomato leaves significantly altered the plants' terpenoid chemistry (α-phellandrenec, δ-2-carene, sabinene, and α-humulene) and a monoterpene (myrcene) was detected in *B. bassiana*-treated but not in control plants. Similarly, Gan et al. (2017) found that the concentration of carbon was significantly higher in roots of *B. bassiana*-treated tall fescue plants (*Festuca arundinacea*) compared to control plants. Therefore, even though the fungus might not be detected, induced plant responses might still be present. The effect of endophytic *B. bassiana* on sugarcane biochemistry and the possible interaction with other beneficial endophytes that colonize sugarcane (Rodrigues et al. 2016; Jaber and Ownley 2017) needs further investigation.

Inoculation method, inoculum concentration and host plant properties are important factors in evaluation of the effect of fungal endophytes as plant growth promoters (Jaber and Enkerli 2017). Several studies have reported enhanced plant growth following *B. bassiana* inoculation via foliar spray, soil drench, or seed immersion (Reddy et al. 2009; Gurulingappa et al. 2010; Lopez and Sword 2015; Jaber and Enkerli 2017). In our study, spraying the leaves and drenching the soil with *B. bassiana* did result in enhanced plant growth (number of sett roots). Sett roots play a significant role in the establishment of the sugarcane plant. In addition, growth of the primary shoot is significantly affected by the growth and functionality of the sett root system (Pankhurst et al. 2004; Blair and Stirling 2006). It will be worth investigating whether promotion of sett roots through foliar sprays and soil inoculation could confer inoculated plants an advantage to better withstand abiotic stresses such as drought during the germination phase especially in this era of changing climate and extreme weather variability. In terms of plant height, plants that had received stem injection were significantly shorter than control plants. Stem injection involved wounding of the stalk and this action could have affected plant health (Akello et al. 2007; Doccola and Wild 2012). However, according to Yan et al. (2015), an introduced fungal inoculum may interact with the host plant defense system. The results of this interaction could be beneficial or detrimental. For instance, *B. bassiana* inoculated into tall fescue negatively affected the ability of the plant to regrow after root herbivory infestation (Gan et al. 2017). In faba beans (*Vicia faba*), inoculating the plants with *B. bassiana* did not result in consistent growth promotion (Jaber and Enkerli 2017). In-depth studies aimed at elucidating the mechanism responsible for enhanced plant growth need to be conducted.

Foliar spray for endophytic establishment of *B. bassiana* could have a potential in sugarcane IPM programs since *B. bassiana* is already known to be effective against arthropod

pests that infest sugarcane (Cherry et al. 2004; Tefera and Pringle 2004; Goble et al. 2012; Wu et al. 2014). In addition, the *B. bassiana* strain used in this study is commercially available and can be sprayed using conventional farm equipment (Legaspi et al. 2000), which would facilitate its use in sugarcane plantations. Future studies will focus on determining *B. bassiana* endophytism effects on sugarcane insect pests, interaction with host-plant's endophytes and elucidating the mechanism responsible for enhanced plant growth.

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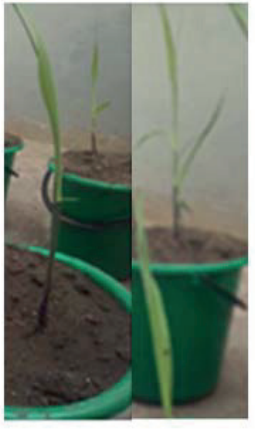
Figure legends

Fig. 1. Sugarcane stem cutting with two buds (two-bud cane-sett) used in propagating sugarcane in this study (A). Sugarcane plants growing in 10 L plastic buckets (B). Definition of proximal and distal in reference to sugarcane leaves, stems and roots used in this paper (C, D; photo credits: Blackburn 1984)

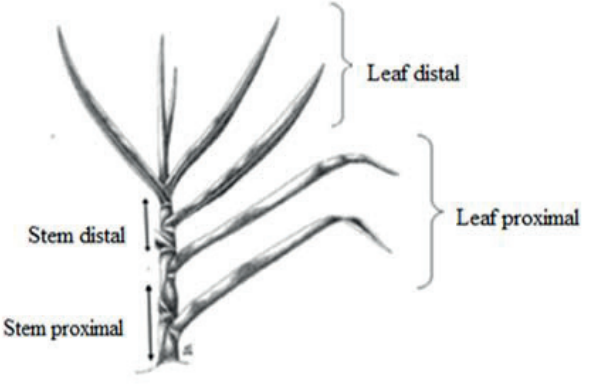
Fig. 2. Mean number (± SE) of plant part pieces with *B. bassiana* isolate GHA recovered from sugarcane leaves, stems, and roots 7 and 14 d post-inoculation (DPI) following foliar spray (A), stem injection (B), or soil drench (C). Different letters above columns indicates statistical difference using negative binomial regression ($p = 0.05$).

A

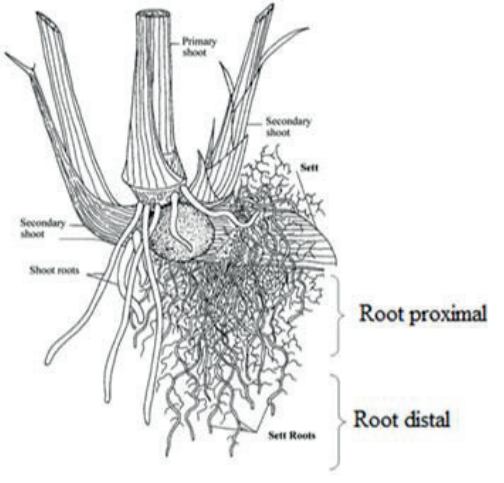
B



C


Leaf distal
Leaf proximal
Stem distal
Stem proximal

D


Primary shoot
Secondary shoot
Sett
Secondary shoot
Shoot roots
Root proximal
Root distal
Sett Roots

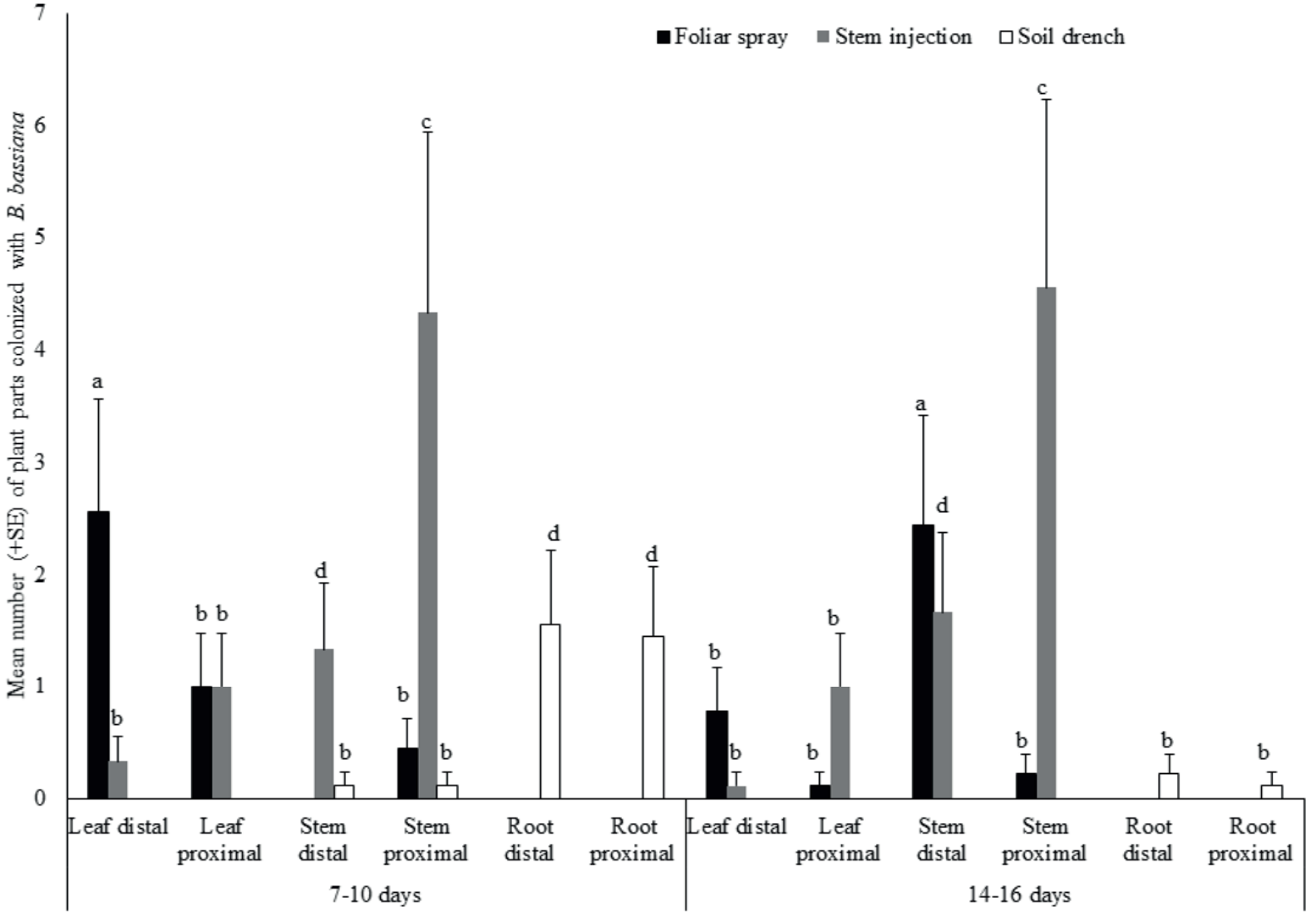

Foliar spray
Stem injection
Soil drench
Mean number (+SE) of plant parts colonized with B. bassiana
0
1
2
3
4
5
6
7
a
b
b b
d
b
b
c
b
d
d
b
b
b
b
a
d
b
c
b
b
Leaf distal
Leaf proximal
Stem distal
Stem proximal
Root distal
Root proximal
Leaf distal
Leaf proximal
Stem distal
Stem proximal
Root distal
Root proximal
7-10 days
14-16 days

Table 1. Effects of *B. bassiana* strain GHA inoculation method (foliar spray, stem injection and soil drench) on mean (±SE) plant height, leaf length, number and length of sett and shoot roots, and fresh and dry weight 16 days post-inoculation (DPI).

Inoculation method	Plant height (cm)	Leaf length (cm)	# sett roots	# shoot roots	Sett roots length (cm)	Shoot root length (cm)	Fresh weight (g)	Dry weight (cm)
Foliar spray	24.9±1.1b	94.3±5.5b	36.7±2.3b	2.2±0.6a	15.2±1.3a	6.0±1.3a	23.4±2.0b	2.8±0.2a
Stem injection	20.7±1.0a	66.3±5.5a	26.4±2.0a	1.1±0.6a	15.6±1.1a	1.7±1.1a	10.7±1.8a	2.0±0.2a
Soil drench	24.0±1.1ab	79.5±5.5ab	36.3±2.1b	0.7±0.6a	13.9±1.2a	2.3±1.2a	15.9±1.9ab	2.1±0.2a
Control	25.1±1.1b	82.5±5.5ab	28.1±2.0a	2.0±0.6a	14.0±1.1a	2.9±1.1a	16.0±1.8ab	2.2±0.2a

Different letters following means in the same column indicates statistical difference using Tukey HSD test ($p = 0.05$).

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Norwegian University
of Life Sciences

Postboks 5003
NO-1432 Ås, Norway
+47 67 23 00 00
www.nmbu.no