UNIVERSITY OF NAIROBI

INDUCTION OF SECONDARY METABOLITES BY ENDOPHYTIC FUNGI IN *PHASEOLUS VULGARIS* FOR *LIRIOMYZA* LEAFMINER AND FALL ARMYWORM MANAGEMENT

BY

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DECLARATION

I declare that this thesis is my original work and has not been submitted elsewhere for research, examination, award of a degree or publication. Where other people's work or my own work has been used, this has properly been acknowledged and referenced in accordance with the University of Nairobi's requirements.

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DEDICATION

This thesis is dedicated to my parents Mr. Kiprotich Ngeno and Mrs. Agnes Ngeno.

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ABSTRACT

The *Liriomyza* leafminer flies (LMF) are invasive pests that attack several horticultural crops one of which is the common bean *Phaseolus vulgaris*. Endophytic fungi have been shown to deter performance, oviposition and feeding of these *Liriomyza* flies in beans. However, the clear infection mechanism or pathways of inoculated fungal endophytes against LMF has not been well established. This study, therefore, investigated the induction of active compounds in the endophytically colonized *P. vulgaris* plants, as defense mechanism against herbivorous insects. Two fungal isolates *Beauveria bassiana* (G1LU3) and *Hypocrea lixii* (F3STI) were used for artificial seeds inoculation. Inoculation was done by soaking *P. vulgaris* seeds in these fungal conidial suspensions prior to planting. Colonization of the different plant parts was assessed to confirm the endophytic property of the inoculated fungi. Volatiles emitted from endophytically colonized *P. vulgaris* were collected into adsorbent Super-Q traps and evaluated using gas chromatography combined with mass spectrometry (GC-MS). Liquid extraction was conducted with methanol and dichloromethane solvents followed by chromatographic analysis. Methanol and dichloromethane extracts of endophytically colonized plants were screened for their efficacy against leafminer fly, *Liriomyza huidobrensis* Blanchard and Fall Armyworm (FAW), *Spodoptera frugiperda* larvae using leaf dipping method and topical application, respectively. The two isolates successfully colonized the entire host plant (roots, stems, and leaves) with significant variation ($F = 19.22$, $Df = 6$, $P = 0.01$) between fungal isolates and the controls. The results also showed qualitative differences in the volatile profiles between the control *P. vulgaris* plants, endophytically colonized plants and insect damaged plants. The volatile blend from the control *P. vulgaris* plants consisted mainly of the following compounds: *meta* cresol (**52**) (35%) and *para* cresol (**53**) (64%). The most abundant emissions from the insect damaged plants included terpinen-4-ol (**59**) (28%) and benzaldehyde dimethyl acetal (**61**) (21%). The *H. lixii* inoculated plants consistently released *m*-cresol (**52**) (72%) and *p*-cresol (**53**) (13%). The most abundant emissions from *H. lixii* colonized plants with insect damage included benzaldehyde dimethyl acetal (**61**) (6%), butylated hydroxytoluene (**63**) (4%) and methyl salicylate (**76**) (3%). *Beauveria bassiana* colonized plants released the highest number of volatiles with the most abundant including benzaldehyde dimethyl acetal (**61**) (6%), butylated hydroxytoluene (**63**) (5%), (*E*)- γ-bisabolene (**86**) (4%), methyl salicylate (**76**) (4%) and 4,8,12-trimethyl-1,3(*E*),7(*E*),11-tridecatetraene (**87**)

(4%). The most abundant volatiles detected from *B. bassiana* colonized plants with insect damage include benzaldehyde dimethyl acetal (**61**) (16%) and butylated hydroxytoluene (**63**) (4%). Qualitative and quantitative differences were also reported between solvent extracted compounds detected from control *P. vulgaris* plant extracts and fungal extracts. All extracts except the *B. bassiana* fungal extract contained the hexadecenoic acid methyl ester (**92**) (18%) and 9,12-octadecanoic acid methyl ester (**93**) (13%). Among the extracts' compounds, the most abundant included 9,12,15-octadecatrienoic acid methyl esters (**94**) (70%) and 1-hydroxy-4-methylanthraquinone (**102**) (58%). The bioassay results showed significant differences between the liquid crude extracts from endophytically colonized plants and the controls plants with regard to the effects on pupation of 2nd instar LMF larvae ($F = 4.33$, $Df = 6$, $P = 0.03$) and adult *Liriomyza* flies emergence of LMF pupae ($F = 5.4$, $Df = 6$, $P = 0.02$). The survival of the 1st instar FAW larvae dipped into the methanolic endophytically colonized plant extracts was also significantly reduced ($F = 3.7$, $Df = 8$, $P = 0.001$) as compared to the controls. The extracts of *B. bassiana* inoculated plants were the most lethal to FAW larvae with median lethal time (LT_{50}) of 4.42 days. Profiled compounds have previously been identified in plant extracts and volatiles with activities including insect repellence, predator attractant and insecticidal properties. Colonization of the host plant *P. vulgaris* therefore triggers production of compounds for defense against herbivorous insects including the leafminer and fall armyworm. The compounds deter feeding and oviposition of the pest through insect repellence and predator attraction. The toxic compounds also infect the pest through effects on its physiology. However, the identities of compounds were based on comparisons of mass spectra available from an MS library. Therefore, some of the identifications may be tentative especially for volatiles with *trans-cis* isomers. This study demonstrated the high potential of endophytic fungi *H. lixii* and *B. bassiana*, to induce mainly specific defense compounds in the common bean *P. vulgaris* that can be used against the *Liriomyza* leafminers and Fall Armyworm.

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LIST OF ABBREVIATIONS AND ACRONYMS

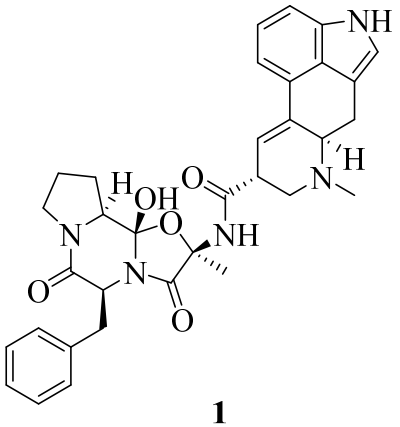
a.s.l	Above sea level
$CDCl_3$	Deuterated trichloro methane
EPF	Entomopathogenic Fungi
FSNWG	Food Security & Nutrition Working Group
FAW	Fall Armyworm
GDP	Gross Domestic Product
HPLC	High Pressure Liquid Chromatography
ICIPE	International Centre of Insect Physiology and Ecology
KNBS	Kenya National Bureau of Statistics
LMF	Leafminer flies
LT_{50}	Median lethal time
MS	Mass Spectroscopy
PTLC	Preparative Thin Layer Chromatography
UNEP	United Nations Environment Programme

CHAPTER ONE: INTRODUCTION

1.1 Background Information

Ornamental crops and vegetables production contribute significantly to the economic development in Africa, mostly as a result of high financial returns and employment creation (Ekesi *et al*., 2016). The agricultural sector in Kenya is the largest contributing up to 24% of the economy (UNEP, 2018). The horticultural division is among the rapidly growing sectors in Kenya with exports growing by 29.3% from $28.7 billion in 2017 to $37.1 billion in the 2018 Gross Domestic Product (GDP) review (KNBS, 2018). In the efforts to improve horticultural productions, it has been observed that the productivity of crop systems in Kenya is constrained by factors including outbreaks and high prevalence of pests and diseases (Mangeni *et al*., 2014). The *Liriomyza* leafminer flies (LMF) are polyphagous invasive pest species that attack common bean crops in Kenya. Leafminer larvae mines under the leaf surface creating winding trails on the foliage; and a heavy infestation causes degradation of plant tissues and eventual death (Gathage *et al*., 2016). Three key *Liriomyza* species, *L. sativae (*Blanchard)*, L. trifolii (*Burgess) and *L. huidobrensis (*Blanchard) have been identified in Kenya, with *L. huidobrensis* (Blanchard) being identified as the most devastating and most predominant. Crop losses due to LMF range between (20%-100%) depending on the affected crops or regions (Chabi-Olaye *et al*., 2013). The quarantine status of leafminers has also caused export restrictions to international markets and as a result, loss of revenue. To control leafminer attacks, farmers rely heavily on the application of chemical insecticides which are harmful not only to humans but also to the environment. Problems associated with chemical insecticides also include elimination of the natural enemies of the pest, and they also speed up the development of resistance to these chemical interventions (Johnson *et al*., 1980). In recent years, research has shifted to exploration of sustainable alternative methods

such as the usage of parasitoids and inundative use of entomopathogenic fungi (EPF) (Migiro *et al*., 2010). The use of fungal endophytes has also been demonstrated to be effective against LMF with *Phaseolus vulgaris* and *Vicia faba* as host plants (Akutse *et al*., 2013; Gathage *et al*,. 2016). Similar systemic endophytic properties were also reported on thrips (Muvea *et al*., 2014) and bean stem maggot (Mutune *et al*., 2016). However, the clear infection mechanism or pathways of inoculated fungal endophytes against insect pests has not been well established. Metabolic interactions of fungal endophytes and the host plants stimulate the synthesis of bioactive secondary compounds such as alkaloids and phenols that are toxic to the pest at larval stages (Zhao *et al*., 2012). Secondary metabolites that have been characterized from fungal extracts such as pyrrolizidine alkaloids, and indole derivatives have been associated with insecticidal activity on arthropods (Kumar *et al*., 2008). The alkaloid ergotamine (**1**) caused reduced larval weight and leaf area consumption of the Fall Armyworm (FAW) *Spodoptera frugiperda* (Lepidoptera: Noctuidae), a newly invasive pest in Africa and Asia (Wink et al., 2018, Akutse *et al*., 2019, 2020). An indole derivative, 6-isoprenyl indole-3-carboxylic acid (**2**) from the endophyte *Collectotrichum* species showed growth inhibitions against plant fungi (Kumar *et al.,* 2008). Endophytic fungi are therefore prospective inducers of defense compounds in host plants that can be exploited in maintaining healthy crops (Puniani *et al*., 2010). This study therefore investigated the induction of active defense compounds in endophytically colonized *P. vulgaris* plants and tested their toxicity against *L. huidobrensis (*Blanchard) and the newly invasive Fall Armyworm (FAW).



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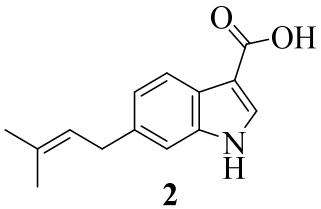

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1.2 Statement of the Problem

Horticultural crops production in Kenya is extremely strained by attacks of the invasive *Liriomyza* species and other arthropod pests. Up to 100% losses have been recorded on beans due to leafminer damage (Chabi-Olaye *et al*., 2013). The quarantine status of *Liriomyza* species has resulted in export restrictions and rejections at international inspections therefore causing further losses of lucrative markets and revenue. In addition to LMF, FAW is another notorious invasive pest that is affecting food security in Africa and Asia. Attempts to manage these insects via the usage of synthetic insecticides are heavily afflicted by elimination of natural enemies and development of pest resistance (Johnson *et al*., 1980). Campaigns on food security and safety, health issues and environmental protections have put pressure on agricultural producers to search for alternative pest control approaches. The use of biopesticides has been promoted and the application of entomopathogenic fungi and fungal endophytes as biocontrol management has been proven to be efficient on various pest species. However, there is still a need to understand and establish the systemic infection mechanism of the endophytically colonized host plant against these herbivorous pests. This study, thus, aimed to investigate the induction of defense compounds from endophytically colonized *P. vulgaris* plant and assess their *in vitro* efficacy on LMF and the newly emerging Fall Armyworm larvae.

1.3 Objectives

1.3.1 General Objective

To assess the induction of secondary metabolites by endophytic fungi in the common bean *Phaseolus vulgaris* for sustainable management of *Liriomyza* leafminer and Fall Armyworm.

1.3.2 Specific Objectives

i. To evaluate the colonization of *P. vulgaris* host plant by the endophytic fungi *H. lixii* F3STI and *B. bassiana* GILU3 through seeds inoculation

ii. To characterize the active secondary metabolite compounds identified from endophytically colonized *P. vulgaris* plant

iii. To assess the insecticidal effects of active extracts on LMF and FAW larvae *in vitro*.

1.4 Justification of the Study

The outcomes of this study will enable us not only to identify and characterize the secondary metabolites, but also to establish and understand the systemic infection mechanism of an endophytically colonized *P. vulgaris* plant against the pests. It will also improve the adoption and application techniques of fungal endophytes in pest management programs. Bioactive compounds from an endophytically colonized plant that were profiled could be used as potential pest control components against other key agricultural and livestock pests. This study would therefore allow/ facilitate adaptation of microbial insecticides that are environmentally friendly, while reducing the abusive use of synthetic pesticides with maximum residue limits and eventually open more lucrative markets to horticultural crops in the country.

2 CHAPTER TWO: LITERATURE REVIEW

2.1 Host Plant: Common Bean *Phaseolus vulgaris*

The common bean *Phaseolus vulgaris,* is a major green legume consumed worldwide. It is an herbaceous plant with edible seeds and it is important in Africa as a source of proteins, minerals, vitamins, carbohydrates and oil (Mitchell *et al.,* 2009). The common bean is one of the staple foods in Kenya rated second after maize, and is cultivated in all the regions, where Western, Rift Valley, Nyanza, Central and Eastern are considered as the major production areas (Mangeni *et al*., 2014). The world production area is over 28 million hectares yielding over 40 million tons. Common beans production in Kenya covers over 500,000 hectares with more than 150,000 tons yield per year (Mangeni *et al*., 2014). The common bean is also an important supply of income for low-income households where a significant share goes into exports to European markets (Chabi-Olaye *et al*., 2013). However, the production of the common bean has remained below world averages as a result of adverse effects of pre-harvest pests and diseases (Mangeni *et al*., 2014). Common beans production in Kenya is hampered greatly by the invasive *Liriomyza* species. Leafminers, accidentally introduced into East Africa, are among the most important pests of the common bean causing up to 100% losses (Chabi-Olaye *et al*., 2013).

2.2 Species Composition of *Liriomyza* Leafminers

The *Liriomyza* spp. (Diptera: Agromyzidae) commonly known as leafminer insects originated from the neotropics regions and was introduced from the New World to new regions in the mid-1970s (Murphy *et al*., 1999). The invasive species has since established new territories in Asia, Latin America, and Africa (EPPO, 2006). Over 300 species of *Liriomyza* have been recorded, among which 20 are considered serious pests of agricultural crops (Kang *et al*., 2008). Six polyphagous species of the leafminers have achieved significant economic status with most

attention on the *L. bryoniae (*Kaltenbach)*, L. huidobrensis (*Blanchard)*, L. strigata (*Meigen)*, L. longei, L. trifolii (*Burgess) and *L. sativae (*Blanchard) (Morgan *et al*., 2000). Leafminer insects repeatedly puncture the epidermis of the affected leaves but their main feeding behavior is mining of the leaves by the larvae. *Liriomyza huidobrensis (*Blanchard)*, Liriomyza trifolii (*Burgess) and *Liriomyza sativae (*Blanchard) are the most regularly reported and also the predominant invasive species in Kenya, attacking a variety of commercially valuable crops including beans and peas (Gitonga *et al*., 2010).

2.3 Ecology and Geographical Distribution of *Liriomyza* Species

*Liriomyza trifolii (*Burgess) has been regularly reported from various African countries including South Africa, Kenya, Mauritius, Tanzania, and Senegal. *Liriomyza* species was introduced accidentally in East Africa in the late 1970s via flower cuttings from Florida (Mujica *et al*., 2011). Since that period, *Liriomyza* species has been found throughout the country. The invasive *Liriomyza* species is widely distributed across the tropical African region since the early 1970s (Rauf *et al*., 2000). *Liriomyza huidobrensis (*Blanchard)*,* the most devastating LMF species (80%), is predominant in high altitudes (above 1000 m a.s.l) in Kenya, whereas *Liriomyza sativae (*Blanchard) and *Liriomyza trifolii (*Burgess) are well-known to adapt predominantly at warmer lowland areas (Mujica *et al*., 2011). The three species in Kenya mainly infest commercially valuable crops including various cut flowers, beans, peas, potatoes and tomatoes (Chabi-Olaye *et al*., 2013).

2.4 Biology of *Liriomyza* species

Liriomyza leafminers are polyphagous insects with a complete metamorphosis life cycle. Adults' length is about 2 mm long and the larvae sizes ranges from 2-3 mm (Murphy *et al*., 1999). LMF have a grayish color and some are black with yellow markings. Female adult flies are slightly

bigger than the adult males. Different *Liriomyza* species can be morphologically identified from the positions of the double vertical setae features on top of the head of the insect and the color of the frons, orbits and femur (Muchemi *et al*., 2018). The life cycle of LMF includes several developmental stages. The female adult lays eggs on the plant tissue by puncturing on the leaf surface. The eggs are white or translucent. They hatch into the larva in 2-3 days which is dependent on environmental circumstances. The larvae are small yellow maggots that feed on the internal tissues of the leaf creating winding tunnels (Chabi-Olaye *et al*., 2013). There are three larval stages and after 5-7 days, the larvae pupate in the top soil or on the face of the leaf. For the pupal stages, they reposition in the leaf axils or under flowers and fruits for 4-5 days. The pupa is oval with variable colors and cone-like appendages (Gitonga *et al*., 2010). (Figure 2.1).

Figure 2.1. *Liriomyza* leafminer fly on okra leaf (left), newly hatched larvae (middle), and pupa within tunnel of onion (right) (Source: https://infonet-biovision.org)

2.5 Economic Impact of Leafminers

The *Liriomyza* spp. causes damage to crops in several ways. Damage is first caused by the insect which punctures the leaf surface for feeding by sucking contents of the epidermal cells and for laying eggs. Punctures provide opening for entry of disease and pathogens. Characteristic larval mining of the leaves and discolorations, (Figure 2.2) reduce the aesthetic value of ornamental crops and reduces the photosynthetic capacity of the crops (Parrella *et al*., 2015). Intense mining damage on young plant tissues causes defoliation which leads to significant losses in crops and fruits. However, the main damage is exhibited in discolorations of flowers and fruits, distortion of leaves

and unappealing feeding scars (Johnson *et al*., 1980). Affected fruits and leaves of host plants reduce the marketability of the produce, photosynthetic capacity, growth, yield and vigor of the crop. Beyond production losses, the quarantine status of the leafminers results in restrictions at market inspections, resulting in losses of lucrative international markets and revenue for smallholder growers (Gathage *et al*., 2016).

Figure 2.2. Damage caused by leafminers (Source: https://infonet-biovision.org/)

2.6 Fall Armyworm

Fall armyworm (FAW) *Spodoptera frugiperda* (J.E Smith) (Lepidoptera:Noctuidae) is currently one of the highly critical pest species affecting maize production. Fall Armyworm is indigenous to sub-tropical and tropical America. FAW is an economically critical polyphagous insect that consumes maize and other horticultural crops in the Gramineae family (Wheeler *et al*., 1989). However, the pest's host range includes almost a 100 documented species of plants in about 27 families (de Oliveira *et al*., 2016). FAW is a periodic and a long-distance migratory pest. The adult moths are adept in flying over 100 km in a just a night (de Oliveira *et al*., 2016). The larvae of *S. frugiperda* feeds on immature greenery, whorls, leaves, ears and tufts bringing about considerable destruction to maize, intermittently causing a full yield loss (Sarmento *et al*., 2002). The pest account for annual losses of crops valued at approximately 500 million dollars (De Groote *et al*., 2020).

2.7 Management Strategies for Leafminers and Fall Armyworm

Management of LMF and FAW is hard because of the cryptic nature of the larval stage of the pests. LMF and FAW control is therefore generally focused on the utilization of Integrated Pest Management methodologies involving cultural practices, chemicals, and biological controls.

2.7.1 Cultural practices

Basic controls recommended for LMF and FAW management include row cover placements and removal of damaged plant materials. Cultural practices also involve the use of natural enemies and insecticide rotation. Ploughing to expose buried pupae has also been effective, and the exposed pupae are killed by natural enemies or solarization (Parrella *et al*., 2015). Inter-cropping helps to slow movement of the pest through crops. The use of reflective mulches and monitoring of nitrogen levels has been reported to significantly reduce leafminer population (Parrella *et al*., 2015).

2.7.2 Chemical control

Use of pesticides is the major strategy against the *Liriomyza* pest. Chemical control involves the use of carbamates, organophosphates and carbaryls. Chemical pesticides that have been employed effectively in the management of LMF include; abamectin, β-cyfluthrin, dimethoate, alphacypermethrin and imidacloprid (Gitonga *et al*., 2010). Some modes of actions have been reported with mechanisms such as insecticidal and suppression of reproduction (Guantai *et al*., 2015). Problems exhibited by chemical control results from the cryptic nature of larval miners which tend to be concealed in sheltered places on the plant such as under the epidermis of leaves and beneath the foliage. Pesticides also kill the natural enemies of the pest and frequent application also allows the development of resistance (Murphy *et al*., 1999). To prevent or delay resistance and other associated problems, insecticide rotations and use of alternative methods have been explored.

Control of FAW has been predominantly approached through traditional chemical management policies which are often unreliable and inadequate to manage the pest in maize fields (Jarrod *et al*., 2018). Use of conventional chemicals to manage the pest is complicated due to chronic poisoning to farmers owing to inaccurate usage (Tinoco and Halperin, 1998).

2.7.3 Biological control

Biological management involves the utilization of organisms to control crop pests, for example parasitoids, predators, and pathogens of the pest. Parasitoids of LMF including exotic natural enemies from Peru (*Phaedrotoma scabrientris, Halticoptera arduine* and *Chrysocharis flacilla*) have been established as efficient parasitoids against the pest in East Africa (Foba *et al*., 2015; Muchemi *et al*., 2018). Biological management also comprises of the application of entomopathogens including *Beauveria bassiana* and *Metarhizium anisopliae* (Seal & Kumar, 2010; Migiro *et al*., 2010). Isolates of entomopathogenic fungi that are virulent to LMF have been identified and tested to compliment the use of parasitoids in LMF management (Gathage *et al*., 2016). Entomopathogenic fungi is normally applied inundatively but the cost of such application method is high, therefore autodissemination strategy has also been employed through biopesticides combination with an insect attractant (Migiro *et al*., 2010). In addition, it has been established that several of entomopathogenic fungi were viable to colonize *Liriomyza* preferred host plants as endophytes, and induce systemic resistance against LMF (Akutse *et al*., 2013) . Presently, three biopesticides based on *M. anisopliae* strains researched by ICIPE have been commercialized by Real IPM (Thika, Kenya) and are used on 132,994 ha in sub-Saharan Africa, with registration of additional products against animal ticks and the fall armyworm *Spodoptera frugiperda* pending.

2.8 Fungal Endophytes in Pest Management

Endophytic fungi have been incorporated into Integrated Pest Management (IPM) packages as bio-control tools (Akutse *et al*., 2013) . Entomopathogenic fungi including *Hypocrea lixii*, *Metarhizium anisopliae* and the *B. bassiana* can successfully colonize a range of host plants (Table 2.1) and confer systemic resistance against arthropod herbivores that feed on them (Akutse *et al*., 2014) . They exist in the plant as endosymbionts benefitting nutritionally from the plant while protecting it from the primary herbivores, pests, and diseases. There are also reports of other roles associated with fungal endophytes including drought and abiotic stress tolerance enhancers, soil nutrient distribution, plant growth and yield promotion (Keppanan *et al*., 2018). Fungal endophytes are microbes that are mostly associated with plants, living part of their life cycle within them. Endophyte colonization does not result in degradation to the host plant such as discolorations, chlorosis or lesions (Puri *et al*., 2016). Some of the fungal endophytes have been found to be pathogenic to adult insects and their larvae. They have been shown to infect pests such as thrips, leafminers (Akutse *et al*., 2013), aphids (Muvea *et al*., 2014) and bean stem maggot (Mutune *et al*., 2016) which are deemed as great concerns in agriculture. Fungal endophytes are categorized depending on their functional roles, reproductive system, host range, nutritional sources, colonized part of the plant, mode of transmission and the capability to manifest symptoms on the host plant (Purahong *et al*., 2011). Successful colonization depends on the host plant and the fungal endophyte. Various works have been done in the adoption and application of fungal endophytes to manage crop pests and diseases. Use of fungal endophytes as a biopesticide to reduce pest attackes through inoculation with modified fungal strains which reduces toxicity and improves yields. Selection of endophytes for agricultural use considers the impact of infection on pests and

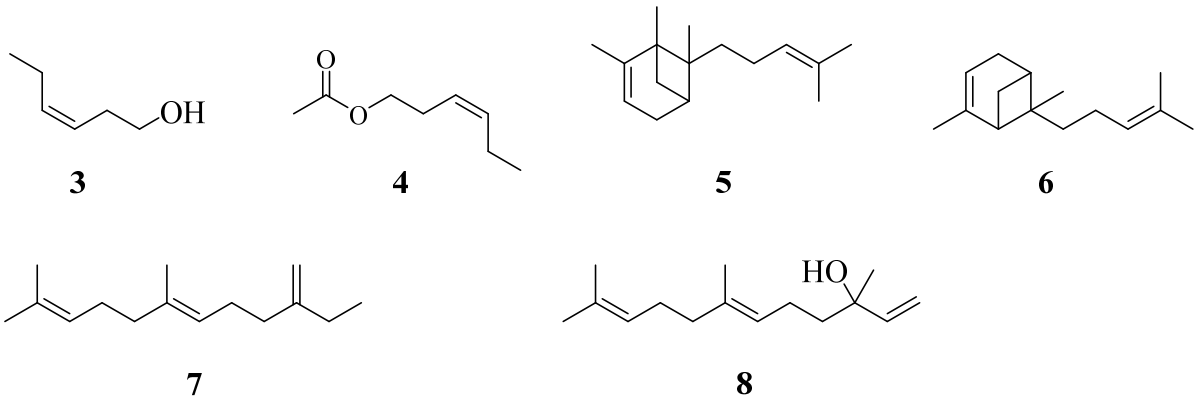
predators so as to increase crop yield without sacrificing environmental, livestock and human health.tal, livestock and human health.

Table 2.1. Colonization of various plants from artificial inoculation of entomopathogenic fungi

Plant	**Scientific name**	**Reference**
Common bean	*Phaseolus vulgaris*	Parsa *et al*., 2018, Akutse *et al*., 2013
Soybeans	*Glycine max*	Russo *et al*., 2015
Coffee	*Coffea sp.*	Posada *et al*., 2007
Maize	*Zea mays*	Wagner & Lewis, 2000
Breadseed poppy	*Papaver somniferum*	Quesada-Moraga *et al*., 2009
Tomato	*Lycopersicon esculentum*	Ownley *et al*., 2008
Cassava	*Manihot esculenta*	Greenfield *et al*., 2016
Sorghum	*Sorghum bicolor*	Tefera & Vidal, 2009
Cotton	*Gossypium hirsutum*	Ownley *et al*., 2008

The host plant is protected from infection of pests through several mechanisms including reproductive rate reduction (Akello *et al*., 2012), feeding deterrence (Vega, 2008), growth retardation (Lacey *et al*., 2006) and survival and oviposition reduction (Martinuz *et al*., 2011). *Beauveria bassiana* and *Hypocrea lixii* are endophytic fungi known to colonize the common bean plant, and have also shown infection properties against LMF pests (Akutse *et al*., 2013). However, the chemical defense mechanism of these endophytes in common bean plants was not established. This research aimed to investigate the infection process of the endophytically colonized plant on the herbivorous attacks with reference to LMF. Induced plant resistance to arthropod herbivores could be attributed to production of chemical compounds that affect the feeding, development and reproduction of the damaging pest negatively (Walling, 2000). The defense phytochemicals that are already well known include carboxylic acids, alkanes, alkenes, ethers and esters, ketones, aldehydes and alcohols. *cis*-3-Hexen-1-ol (**3**), *cis*-3-hexenyl acetate (**4**), and *cis*-α-bergamotene (**5**) have been reported to increase egg preying levels by a common predator and reduced lepidopterous insect oviposition counts (Kessler *et al*., 2001). Production of secondary metabolites

can depend on abiotic factors such as wounding, or biotic factors including plant hormones and microorganisms (JI et al., 2006). The produced metabolites could affect the physiology, biochemistry, anatomy and development of the attacking pest and thus can be used as an alternative tool in IPM programs (Turlings *et al*., 2015). Terpenoids released by caterpillar eaten corn seedlings with ability to attract parasitic wasps include *trans*-α-bergamotene (**6**), (*E*)-*β*-farnesene (**7**), and (*E*)-nerolidol (**8**) (Turlings *et al*., 2015).



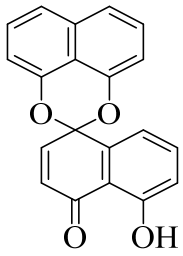
3 **4** **5** **6**

7 **8**

2.9 Active Compounds Isolated from Endophytic Fungi

The production of active defense metabolites by endophytic fungi is correlated to its ecological habitat. Synthesis of biologically active defense chemicals by the microorganisms is attributed to metabolic relations between the fungal endophytes and its host plant (Manasa *et al*., 2014). Fungal endophytes have exoenzymes necessary for colonization of the hosts and their metabolic interactions that include an increase in the plant's defense metabolites to balance their association (Schulz *et al*., 2002). Induced systemic resistance in infected plants is due to activation or production of mycotoxins. Palmarumycin (**9**) and benzopyroanone (**10**) are novel metabolites isolated from endophytic fungi with anti-bacterial activity (Schulz *et al*., 2002). Pharmaceutical and agricultural industries have also explored endophytic fungi as a source of novel compounds.

Fungal endophytes have the capacity to produce active secondary metabolites that can be used as therapeutic agents directly and indirectly (Kusari *et al*., 2012). Taxol (Paclitaxel) (**11**) a broadly utilized cancer drug has been discovered in *Taxomyces andeanae*, a fungal endophyte isolated from *T. brevifolia*, by (JI et al., 2006). The lignan podophyllotoxin (**12**) a forerunner to clinically effective anti-cancer drugs have been isolated from endophytic fungi *Phialocephala fortinii* previously isolated from roots of the host plant *Podophyllum peltatum* (Cragg *et al*., 2005). Infected plants have also been found to contain several toxic alkaloids that are insect repellants for example peramine (**13**) from *epichloe* fungus in grasses has been documented as a feeding deterrent for insects (Schardl *et al*., 2004). Colonized host plants were also found to release camptothecin (**14**) as defense against insect and pathogen attacks (Sirikantaramas *et al*., 2009). Endosymbiotic relationships of dicotyledonous plants and clavicipitaceous fungi have also been shown to induce the production of ergoline (**15**) an alkaloid for protection against herbivory (Leistner *et al*., 2009).



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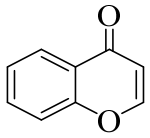

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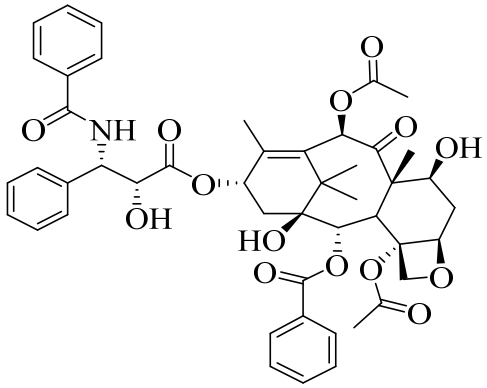

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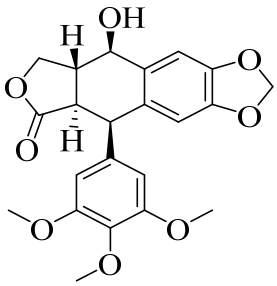

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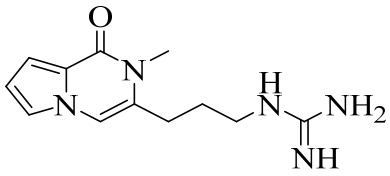

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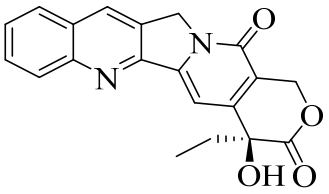

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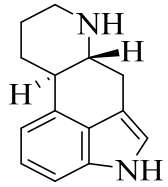

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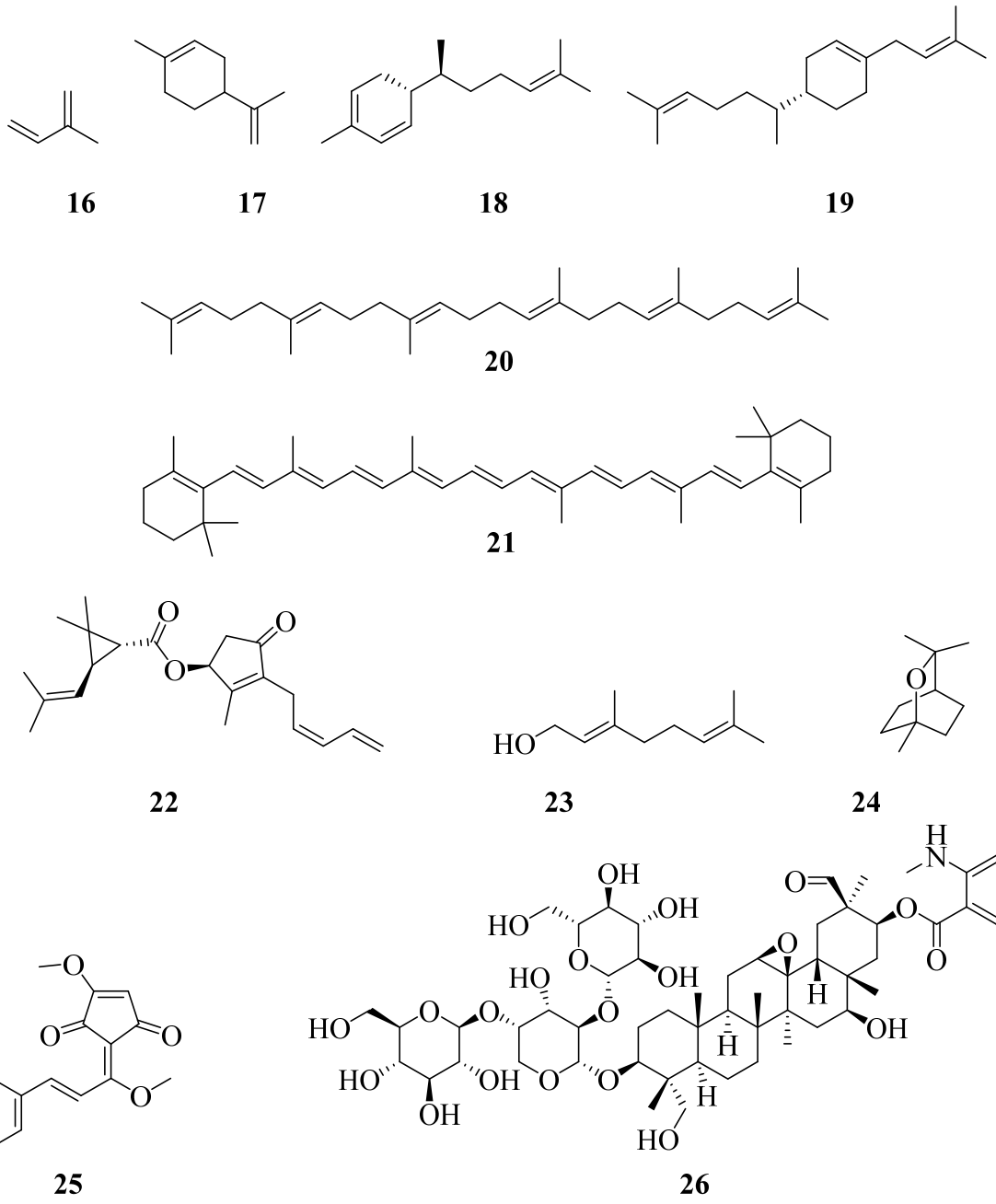
2.10 Chemical Plant Defense

Phytochemicals are categorized into primary and secondary metabolites. Sugars, nucleic acids, amino acids and proteins are primary plant chemicals produced for the purposes of development and growth or reproduction (Butnariu, 2018). In contrast, secondary plant metabolites are mainly

produced for plant defense against pathogens and herbivory. Chemical plant defense against arthropod pests is by production of terpenoids, phenolics and alkaloids (Freeman, 2008).

2.10.1 Terpenes for Plant Defense

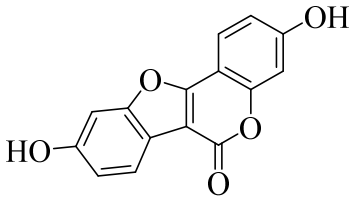
Terpenes are compounds constructed from isoprene units (C_5H_8) (**16**); a volatile gas produced by all plants. Terpenes are grouped according to the number of isoprene (C_5H_8) groups. Monoterpenes have 2 isoprene units for example limonene (**17**) a component of oils from citrus. Sesquiterpenes have 3 isoprene units for example zingiberene (**18**) a sesquiterpene abundant in ginger. Diterpenes have 4 isoprene units for example camphorene (**19**) detected in plant essential oils. Triterpenes have 6 isoprene units for example squalene (**20**) produced by all plants and animals. Tetraterpenes have 8 isoprene units for example carotene (**21**) an organic pigment produced by plants and algae (Kim *et al*., 2020). Monoterpenes and sesquiterpenes are volatile organic compounds produced by plants to repel against insects or attract insect predators for example pyrethrin I (**22**) from chrysanthemum (Butnariu, 2018). Monoterpenoids and sesquiterpenoids are derivatives of terpenes with an oxygen functionality. They are a major constituent of plant essential oils which function as insect toxins with anti-bacterial and anti-fungal activity (Yoshitomi *et al*., 2016). Geraniol (**23**) and eucalyptol (**24**) are monoterpenoids from geranium and eucalyptus essential oils with insect repellant activity (Sfara *et al.,* 2009) Monoterpenoids are mostly used as insecticides and are harmless to humans. Diterpenoids such as methyl lucidone (**25**) have shown to disrupt the development of the larvae and increase insect mortality (Shin, 2018). Saponins are glycosylated triterpenoids for example avenacin A-1 (**26**) from oats which degrade cell membranes of invasive fungal pathogens (JH, 2015). The presence of saponins and other terpenes have been found in common bean extracts (Guajardo-Flores *et al*., 2013). Sesquiterpenes have been isolated previously from fungi including *Beauveria bassiana* (Patočka, 2018).



2.10.2 Phenolics For Plant Defense

Phenolics are produced mainly through the shikimic acid and malonic acid pathways (Butnariu, 2018). Phenolics involved in plant defense include tannins, anthocyanins, flavonoids, furanocoumarins, and lignins. Antibiotic and antifungal polyphenols such as coumestrol (**27**)

produced by beans are phytoalexins that disrupt the cell structure and metabolism of fungal pathogens (Durango *et al*., 2013). Tannins are insect toxins that cause protein inactivation by binding to salivary proteins and digestive enzymes. Ingestion of high amounts of tannins results in growth retardation and eventual death of insect herbivores. Tannins are flavonoid polymers that are soluble in water (Carmona *et al*., 1991). Tannins including catechin (**28**), gallcatechin (**29**) and afzelechin (**30**) have been extracted from beans (Blair, 2010). Lignin is a heterogeneous polymer forming secondary cell walls for plants. Lignin is indigestible therefore providing a physical barrier against attack (Khoddami *et al*., 2013). Furanocoumarins are produced in defense against herbivore and pathogen attack. Most are highly toxic to vertebrates and invertebrate herbivores. Furanocoumarins contribute to faster cell death by integration into DNA (Aparicio-Fernández *et al*., 2008). Gossypol (**31**) is a phenol produced by cotton with strong anti-fungal and anti-bacterial activities. Phenolics extracted previously from beans are largely polyphenols including isoflavones and tannins for example quercetin (**32**) cyanidin (**33**) and delphinidin (**34**) (Rocha-Guzmán *et al*., 2007). Phenolics analyzed from *Beauveria bassiana* extract also included the flavonoid quercetin (**32**) (Azadi *et al*., 2016).



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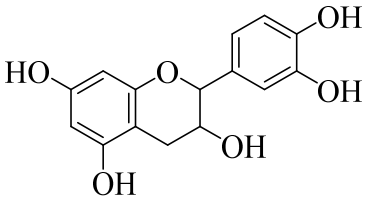

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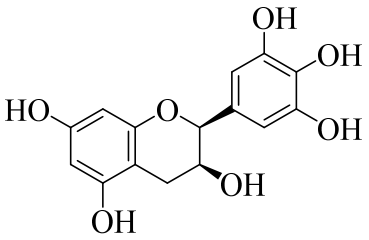

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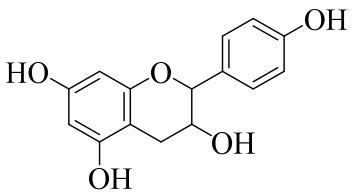

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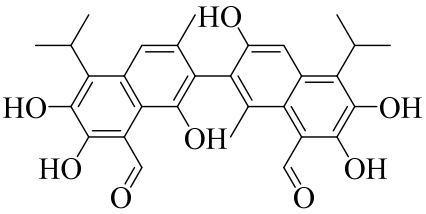

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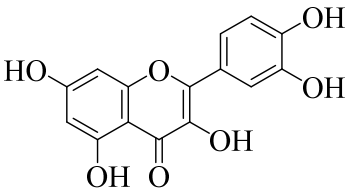

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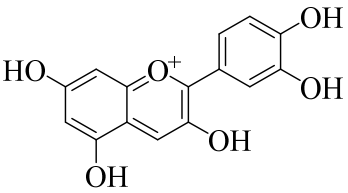

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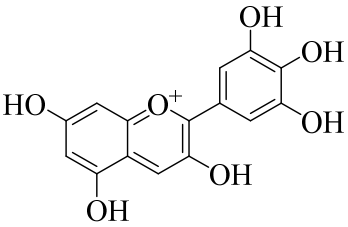

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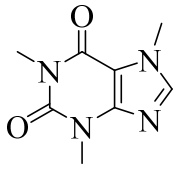
2.10.3 Alkaloids for Plant Defense

Alkaloids are nitrogenous compounds produced by vascular plants. Alkaloids are amino acid derivatives with powerful effects on insect physiology and fungi (Menjivar *et al*., 2012). Caffeine (**35**), cocaine (**36**), morphine (**37**) and nicotine (**38**) are plant alkaloids with toxic effects against insects and fungi (Doughari, 2015). Alkaloids from bean extracts include glycosides and isoquinolines (Atchibri *et al*., 2010). Alkaloids previously isolated from *B. bassiana* are derivatives of 2-pyridine for example tennelin (**39**), bassianin (**40**) and pyridovericin (**41**) (Patočka, 2018).



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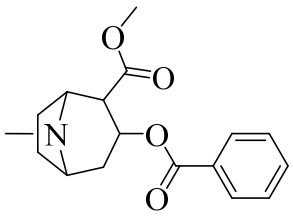

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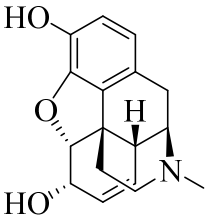

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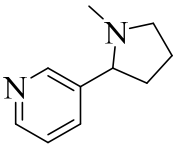

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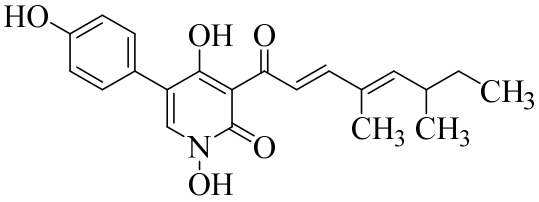

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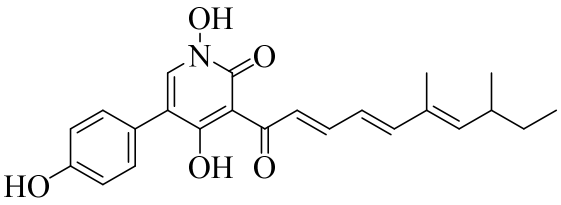

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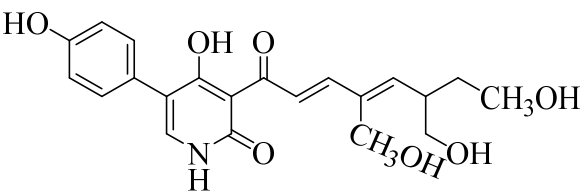

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2.11 Mechanism of Plant Defense Compounds

The mode of action of toxic secondary metabolites produced by non-pathogenic endophytic fungi in a host plant, against herbivorous pests, involves effects on the physiology of the insect. Toxic compounds that result in symptoms such as growth and performance deterrence for both the insect and its larvae (Freeman, 2008). Mode of action for defense compounds can also be direct or indirect for example nicotine (**38**) acts as insect repellants (Heldt, 1951).

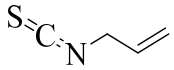
2.11.1 Production of Prussic Acid by Wounded Plants

Prussic acid (HCN) (**42**) is a poison that inhibits the final step in the respiratory chain, cytochrome oxidase. 10% of all plants is reported to utilize prussic acid in defense against herbivores. Prussic acid is produced in its non-toxic form cyanogenic glucoside. Plant wounding and the resulting hydrolysis of cyanogenic glycoside produces the toxic prussic acid (Wink *et al*., 2018).

42

2.11.2 Production of Volatile Mustard Oils

Mustard oil glycosides are glucosinalates found in plants including radish and cabbages. Wounding of the plant results in the hydrolysis of the glycoside producing a sulfate residue, mustard oil (isothiocyanate) (**43**) (Fatouros *et al*., 2014). Mustard oils with a pungent smell deter insect feeding. High concentrations are also deadly to insects, nematodes, fungi, and bacteria (Beran *et al*., 2014).



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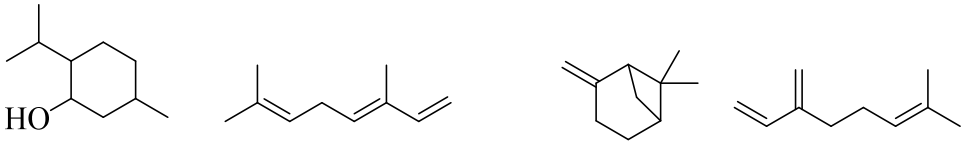
2.11.3 Production of Isoprenoids

Isoprenoids volatiles are emitted by plants to repel insects or attract predators. Linalool is a monoterpene defense substance that acts as a predator attractant (Heldt, 1951). Menthol (**44**) is a monoterpene that acts as an insect repellent (Wink *et al*., 2018). Compounds including ocimene (**45**), pinene (**46**) and myrcene (**47**) are toxic to the physiology of herbivores affecting their respiratory and digestive systems, they also attract natural enemies (Arimura *et al*., 2004). Saponins for example yamonin (**48**) are toxic in their ability to dissolve the plasma membranes of

fungi (Abdul Rasheed et al., 2019). Phyto ecdysones (**49**) with similar structures to ecdysones, insect hormones, cause disturbances in the pupation process resulting in larval death (Fürstenberg-Hägg *et al*., 2013).



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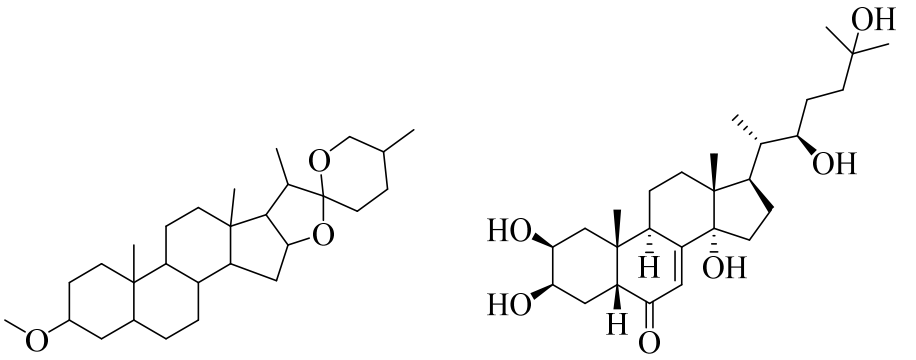

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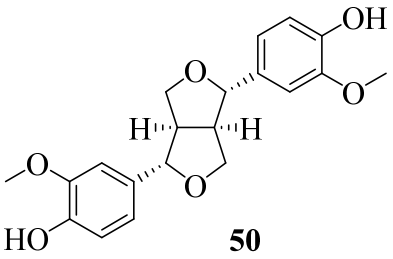
2.11.4 Production of Phenols

Lignan pinoresinol (**50**) is toxic to microorganisms because it inhibits the regulatory action of Camp phosphodiesterase, a messenger component (Heldt, 1951). Malognol (**51**) inhibits growth of bacteria and fungi (Wink *et al*., 2018). Lignans also provide mechanical strength and thus deters feeding (Wink *et al*., 2018). Phenolic groups of tannins react with enzymes of herbivores digestive tracts, binding with aggressive enzymes thereby deactivating them (War *et al*., 2019).



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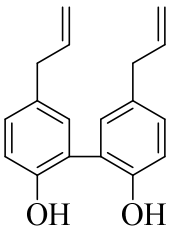

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3 MATERIALS AND METHODS

3.1 General

Analysis of volatiles was carried out using a Hewlett-Packard (HP) 5890 Series II GC-MS equipped with an HP-1 column (30 m length × 0.25 mm inner diameter × 0.25 µm film thickness) with nitrogen as the carrier gas at 1 mL/minute. For identification, the active compounds in the volatile extracts were analyzed by comparing their mass spectral data with those recorded in the Mass Spectral Library NIST/EPA/NIH 2005a. Thin layer chromatography of crude extracts was carried out on (Merck, 4 cm × 8 cm) plates. A Shimadzu QP 2010-SE GC-MS coupled to an auto sampler was used for the crude extract analysis. Ultrapure He was used as the carrier gas at a flow rate of 1 mL/minute. A BPX5 non-polar column, 30 m; 0.25 mm ID; 0.25 µm film thickness, was used for separation. RP-HPLC were performed on a HEWLETT PACKARD (HP) system using a LiChrospher®100 C18 reversed phase column (250 mm x 4.0 mm, 5µm), preceded by a LiChrospher® C18 reversed phase guard column (4.0 mm x 4.0 mm, 5µm). The HPLC system consisted of a HP 1050 pump unit, HP 1050 diode array detector and 1050 autosampler which were controlled by ChemStation for LC 3D systems.

3.2 Fungal Cultures and Suspensions Preparation

Two fungal isolates *Beauveria bassiana* G1LU3 and *Hypocrea lixii* F3ST1 were acquired from the International Centre of Insect Physiology and Ecology, ICIPE, Arthropod Germplasm Centre. The fungal isolates (*H. lixii* F3ST1 and *B. bassiana* G1LU3) were initially isolated from the above ground parts of maize plants. The isolates were cultured on potato dextrose agar (PDA), and then maintained at 25 ± 2 °C in complete darkness. Conidia were harvested by scraping the surface of the 2-3-week-old sporulating cultures with a sterile spatula as described by Akutse *et al*., (2014). The harvested conidia were mixed in 10 mL sterile distilled water containing 0.05% Triton X-100

and vortexed for 5 minutes to produce homogenous conidial suspensions. Conidial counts were made using a Neubauer Hemocytometer (Goettel & Inglis, 1997). The conidial suspension was adjusted to 1×10^8 conidia mL^{-1} through dilution prior to inoculation of *P. vulgaris* seeds. Spore viability was determined by plating 0.1 mL of 3×10^6 conidia mL^{-1} onto 9 cm petri dishes containing (PDA). Three sterile microscope cover slips (2 × 2 cm) were placed on the top of the agar in each plate. Plates were incubated in complete darkness at 25 ± 2 °C and examined after 16-20 hours. The percentage germination of conidia was determined from 100 randomly selected conidia on the surface area covered by each cover slip under the light microscope (400×) using the method described by Goettel & Inglis, (1997). Conidia were deemed to have germinated when the length of the germ tube was at least twice the diameter of the conidium. Four replicates were used for each fungal isolate.

3.3 Plant Inoculation and Endophytes Colonization Assessment

Inoculation was done by soaking *P. vulgaris* seeds (Brown Rose Coco) in conidial suspensions titrated at the concentration of 1 x 10^8 conidia mL^{-1} for 2 hours. Prior to inoculation, seeds were surface sterilized in 70% ethanol for 2 minutes followed by 1.5% sodium hypochlorite for 3 minutes and rinsed three times with sterile distilled water. For the controls, sterilized seeds were soaked in sterile distilled water for 2 hours as described by Gathage *et al*., (2016). The last rinse water was plated out to assess the effectiveness of the surface sterilization procedure (Schulz *et al*., 2002). Seeds were transferred into plastic pots (8 cm diameter × 7.5 cm high) containing the planting substrate (mixture of crop waste manure and soil 1:5 according to previous protocol described by (Akutse *et al*. 2013)). The planting substrate was sterilized in an autoclave for 2 hours at 121 °C and allowed to cool for 72 hours prior to planting. Five seeds were sowed per pot and maintained at room temperature (25 ± 3 °C) and 60 % relative humidity (RH). Pots were

transferred immediately after germination to a screen house (2.8 m length × 1.8 m width × 2.2 m height) at 25 ± 3 °C, for 2 weeks. Seedlings were thinned to three per pot after germination and watered twice per day (morning and afternoon). No additional fertilizer was added to the substratum.

To determine the colonization of inoculated fungal isolates in *P. vulgaris*, plants were carefully removed from the pots two weeks after inoculation and washed with running tap water. Colonization through seed inoculation has been shown previously to be optimal at two weeks (Akutse *et al*. 2013). Seedlings (*ca*. 30 cm in height) were cut into different sections (*ca*. 5 cm long): leaves, stems, and root sections. Five randomly selected leaf, stem and root sections from each plant were surface sterilized as described above. The different plant parts were aseptically cut under a laminar flow hood into 1 × 1 cm pieces before placing the pieces, 4 cm apart on PDA plates amended with a 0.05% solution of antibiotic (streptomycin sulfate salt) (Dingle *et al*., 2003). Plates were incubated at 25 ± 1 °C for 10 days, after which the presence of endophytes was determined. The last rinse water was also plated out to assess the effectiveness of the surface sterilization procedure as described above. The colonization of the different plant parts was recorded by counting the number of pieces of the different plant parts that showed the presence of inoculated fungal growth/mycelia according to Koch's postulates (Petrini *et al*., 1986). Only the presence of endophytes that were inoculated were scored. Slides were prepared from the mother plates and used for morphological identification. Treatments were randomized in complete block design (RCBD) and the experiment replicated four times over time.

3.4 Insect Rearing and Treatment

FAW and LMF were obtained from the *icipe*'s Animal Rearing and Quarantine Unit (ARQU). The initial colonies originating from adult LMF and FAW were collected from wild crucifers and maize

crops, respectively, at the *icipe* campus (01°13.3′S 36°53.8′E, 1600 m a.s.l) and reared on beans and maize leaves in plexiglass cages (50 cm × 50 cm × 45 cm) for 8 to 10 generations prior to experiments. LMF and FAW colonies were maintained at 27 ± 2 °C under a photoperiod of 12L: 12D and relative humidity of approximately 40%. Two-week seedlings from the above treatments were used for secondary metabolites assessment. Two-week old seedlings were randomly selected from each treatment and placed inside meshed cages (50 cm × 50 cm × 50 cm). These included: endophyte-free plants, and endophyte colonized plants. In addition, four plants (from endophyte-free and endophytically-colonized plants) were placed in each cage and exposed to two-day old, mated adult flies (30 individuals at sex ratio 1:2, male: female) for infestation, and later also used for volatiles and secondary metabolites assessment.

3.5 Collection and Analysis of Volatiles

Volatiles released from the intact aerial parts of *P. vulgaris* plants inoculated with fungus, controls and exposed to insects were collected by enclosing an intact plant in an air-tight plastic chamber and passing air through it (at a flow rate of 350 mL/minute) into adsorbent Super-Q traps. Talento timer based volatile collection system was employed in capturing volatiles released at night (19:00-06:59). The Super-Q traps were eluted with 200 µL GC/GC-MS-grade dichloromethane (CH_2Cl_2) and the eluate was stored at −80 °C until used. Volatiles were analyzed in the split less mode at an injector temperature of 280 °C and a split valve delay of 5 minutes. The oven temperature was held at 35 °C for 3 minutes, then programmed at 10 °C/minute to 280 °C and maintained at this temperature for 10 minutes (De Backer *et al*., 2015).

3.6 Solvent Extraction

To analyze active compounds produced in the bean plants of the controls, endophyte treated and infested plants, solvent extraction using dichloromethane (CH_2Cl_2) and methanol (MeOH) was

performed, to target the non-polar and moderately polar constituents. The above plant materials were collected and freeze dried in liquid nitrogen. Dry samples were crushed using a pestle and mortar. Extraction of target compounds were processed by soaking the powdered material in solvents; MeOH and CH_2Cl_2 (5 g/mL) for 48 hours with occasional stirring in the dark at laboratory temperature 25 ± 2 °C. Whatman N° 1 filter papers were used to filter the crude extract. The solvents were evaporated and the extract concentrated under a vacuum using a rotary evaporator at 40 °C. The resulting dry residue of each treatment were expressed in mg.

3.7 Chromatography

About 2 µL of concentrated extracts of the treatments were loaded on Silica gel 60 W TLC plates (Merck, 4 cm × 8 cm, 250 µm layer thickness) using a micropipette. The plates were developed in ethyl acetate in *n*-hexane (30:70) to show the profile in terms of retention factor of the constituent compounds of the extracts. The developed plate was air dried and observed under UV_{254} nm lamp and then exposed to iodine vapor for visualization. The TLC was also sprayed with 5% sulfuric acid and heated for 5 minutes at 100 °C to test the presence of various hydrocarbons including flavonoids and terpenes.

3.8 Gas Chromatography Screening of Plant Extracts

Samples, diluted in methanol, were filtered through 0.22 µm PTFE syringe filters, and transferred to 2 mL vials for GC-MS analysis. The GC was programmed as follows: 50 °C (1 minute); 10 °C/min to 180 °C (1 minute); 3 °C/minute to 250 °C (22 minutes) where the total runtime was 60 minutes. Only 1 µL of the sample was injected. Injection was done at 200 °C in split mode, with split ratio set to 10:1. The interface temperature was set at 250 °C, while the Electron Spray Ionization (ESI) ion source was set at 200 °C. Mass analysis was done in full scan mode, 50-700 amu (to accommodate high molecular-weight-compounds), with an initial solvent-delay time of 3

minutes. Raw mass spectra were matched against the NIST 2014 library of mass spectra for possible identification of compounds.

3.9 High Pressure Liquid Chromatography Screening of Plant Extracts

To analyze the active compounds produced in the bean plant extracts, reversed phase high liquid chromatography (RP-HPLC) analysis was performed. Sample extracts at 1mL each were transferred into Eppendorf tubes and centrifuged at 14,000 rotations per minute at 25 °C for 5 minutes. From each sample the upper 600 µL were filter sterilized (0.45 µm) and transferred to HPLC vials. Before samples are injected, the column was equilibrated with 90% water, supplemented with 0.1% (v/v) MeOH (solvent A) and 10% acetonitrile (solvent B). After injection the samples were eluted at a flow rate of 1 mL per min using an isocratic flow of 90% solvent A and 10% solvent B for 2-minute, a linear gradient to 10% solvent A and 90% solvent B for 28 min, followed by a isocratic flow for 5 min with 10 % solvent A and 90% solvent B. Before the next sample was injected, the column was re-equilibrated by a 1 min linear gradient to 90% solvent A and 10% solvent B, followed by a 4-minute isocratic flow of 90% solvent A and 10% solvent B. The resultant chromatogram and mass spectra were recorded for interpretations and profile comparisons. Retention time and intensity of the recorded compounds were compared for the different treatments and the recorded mass were used for identification.

3.10 Plant Extracts Bioassays

The effects of plant extracts from the different treatments; the positive controls consisted of the extract of the non-inoculated and non-infested plants and the non-inoculated and infested plants; the negative controls consisted of the solvents; with the treatments being the extract of inoculated and non-infested plants, inoculated and infested plants; the standards that were used in this experiment consisted of the fungal extracts. These extracts were tested against the 2nd instar larvae

of leafminers under laboratory conditions. The crude extracts were dissolved in a proportion of 1g:10 mL of aqueous and 1% Tween 80 solution. The leaf-dip bioassay described by Cahill *et al*., (1996) was used to determine potent activities against the pest. The common bean plant leaves with 5 intact mines for each leaf of 2nd instar leaf miner larvae with 4 leaves in each treatment, were dipped for 20 seconds in the extract solution. The leaves were left to dry for 5 minutes under a hood at ambient temperature and then placed within sealed petri dishes for each individual treatment. For the controls, the leaves were dipped in 1% Tween 80 solution. The petri dishes were maintained in a chamber at 25 ± 1 °C with 70% RH and 14 hours Light:10 hours darkness photoperiod for 48 hours. Mortality was recorded for 7 days after treatment. Pupation, adult emergence and survival of the emerged adults were also recorded. The experiment was carried out in a randomized design with three replicates per treatment each composed of 10 mines. Similar dipping bioassay approach were used with 1st instar FAW larvae, where the extracts activity was assessed. The same number (10 1st instar larvae) were used per treatment and under the same experimental conditions as described above.

3.11 Statistical Analyses

Mortality, number of pupae, emergence and survival data were analyzed using both analysis of variance (ANOVA) and the use of Abbott's formula for mortality analysis. Fungal colonization percentages were square root-transformed before applying ANOVA analysis. Student Newman Keuls (SNK) Test was used to perform multiple comparisons of means.

The success rate (%) of fungal colonization of host plants parts were calculated as follows:

$$\% \text{ Colonization} = \frac{\text{Number of pieces exhibiting fungal outgrowth}}{\text{Total number of pieces plated out}} \times 100$$

All the analyses were performed using R (3.6.1) statistical software (R Development Core Team 2019).

4 RESULTS AND DISCUSSION

4.1 Colonization Assessment of *Phaseolus vulgaris* Plants Inoculated with Fungi

Viability tests for the harvested conidia from the fungal cultures exhibited germination of more than 90% for both isolates. The two isolates, *H. lixii* F3STI and *B. bassiana* G1LU3 successfully colonized the host plant *P. vulgaris* (Figure 4.1). The endophytic colonization of the common bean in the leaves, stems and roots of the plant varied significantly between fungal isolates and the controls (F = 19.22, Df = 5, P = 0.01) (Figure 4.1). *Hypocrea lixii* F3STI exhibited the highest percentage of colonization at (93.3%) in the roots and the leaves, while *B. bassiana* G1LU3 had the lowest percentage of colonization at (55.0%) in the leaves.

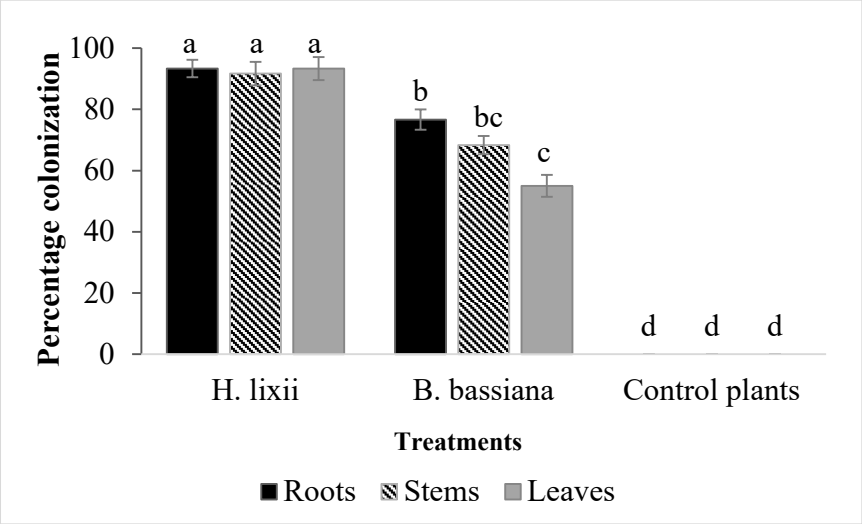


Figure 4.1. Percentage colonization of leaf, stem and root parts of *Phaseolus vulgaris* plants by endophytic isolates of *Hypocrea lixii* F3ST1, and *Beauveria bassiana* G1LU3. Means with the same letter are not significantly different.

Similar findings were described by Akutse *et al*. (2013) with *H. lixii* and *B. bassiana*. Both fungal isolates were capable of endophytically colonizing the entire *P. vulgaris* host plants (roots, stems

and leaves). Akello & Sikora (2012) likewise stated that *B. bassiana* (one of the isolates used in the current research), colonized maize plants. In the present study, there was no considerable differences in the colonization of the different parts of the plants, which was similar with the conclusions of Akutse *et al*. (2013). *Vicia faba* as host plant indicated substantial variations in the colonization of the different parts of the plants while *P. vulgaris,* plant used in present study showed no major differences in the colonization of the different parts of the plants (Akutse *et al*., 2013). The colonization of the various plant pieces reveals that the fungus travels inside the plant system. This implies that the degree of colonization of the separate plant sections also depends on the host plant. There were also differences in level of colonization of different parts of the plants among the fungal isolates. These results are consistent with results reported by Akello & Sikora (2012), and Gathage *et al*. (2016). The cause of higher levels of colonization in the stems and leaves is not apparent but could indicate disparities in physiological and microbial environments in the distinct plant parts. Petrini and Fisher (1987) reported that endophytic fungi showed tissue specificity because they are modified to certain special environments present in the allotted plant parts.

4.2 Secondary Metabolites Characterized from *Phaseolus vulgaris* Plants

In this study the inoculation of fungal endophytes in the common bean plants was assessed in their ability to induce alterations in the profiles of secondary metabolites of the *P. vulgaris* host plant. The emitted volatiles detected from control *P. vulgaris* plants, insect (LMF)-damaged plants, fungi-inoculated plants, and inoculated damaged plants, showed considerable variations in the number of compounds produced.

4.2.1 Control Phaseolus vulgaris Plants Volatiles Analysis

Only two volatiles were identified in control emissions *m*-cresol (**52**) and *p*-cresol (**53**) (Table 4.1, Figure 4.2).

Table 4.1. Volatiles identified from Control *Phaseolus vulgaris* plants.

Retention Time	Area Percentage	Library/ID	References
13.2954	35.6324	*m*-Cresol (**52**)	Naznin *et al*., 2014
13.6313	64.3676	*p*-Cresol (**53**)	Naznin *et al*., 2014

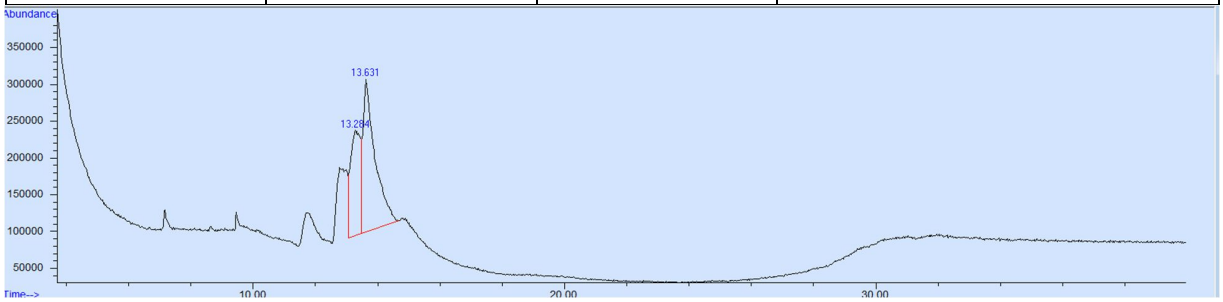
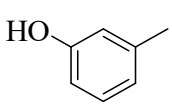
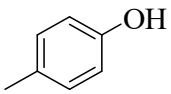


Figure 4.2. Control *Phaseolus vulgaris* plants chromatogram



52



53

The compounds *m*-cresol (**52**) and *p*-cresol (**53**) were identified in both controls and endophytically colonized plants. This implies that cresol compounds are produced by the common bean plants in both endophyte colonized and endophyte-free plants. Cresol compounds have previously been discovered as the main active volatile compounds from growth promoting fungi, *Ampetomyces* and *Cladosporium* species and consequently proven to elicit induced systemic resistance against disease pathogens (Naznin *et al*., 2014). Cresol compounds have also been identified in volatile blends of several plants including vanilla and coffee beans. This shows that plants produce these compounds for defence. Emission of cresol compounds were also considerably higher in control plants compared to endophytically treated plants. This points to reduction in the production of

cresol compounds in inoculated plants. Reduction may be due to changes in metabolism of the plant that caused limited production of specific compounds alongside induction of other compounds as a response to the treatment. Fungi such as the necrotrophic pathogen *Botrytis cinerea* has been previously demonstrated to suppress defense-related oxidative burst in the leaf tissues of the bean (Unger *et al.,* 2005).

4.2.2 Leafminer-Damaged Phaseolus vulgaris Plants Volatiles Analysis

The GC-MS chromatogram (Figure 4.3) revealed fourteen (14) peak signals of which, ten (10) of them were identified as volatiles consistently released by leafminer-damaged bean plants (Table 4.2). These compounds are largely terpenes including β-phellandrene (**54**), α-terpinene (**55**), *cis*-sabinene hydrate (**56**), *trans*-sabinene hydrate (**57**), camphor (**58**), terpinen-4-ol (**59**) and (*E*)-caryophyllene (**60**). The other compounds identified include benzaldehyde dimethyl acetal (**61**), heneicosane (**62**) and butylated hydroxytoluene (**63**).

Table 4.2. Volatiles identified from Leafminer-damaged *Phaseolus vulgaris* plants

Retention Time	Area Percentage	Library/ID	References
11.8618	1.2192	β-Phellandrene (**54**)	Ji hong *et al*., 2017
12.3545	1.4147	α-Terpinene (**55**)	Yoshitomi *et al*., 201
12.5113	4.7784	*cis*-Sabinene hydrate (**56**)	Al-Kalaldeh *et al*., 2010
13.0488	4.663	*trans*-Sabinene hydrate (**57**)	Bertoli *et al*., 2004
13.2504	21.8358	Benzaldehyde, dimethyl acetal (**61**)	Watanabe *et al*., 2009
13.8103	1.5602	Camphor (**58**)	Pragadheesh *et al*., 2013
14.3478	28.6898	Terpinen-4-ol (**59**)	Hulshof *et al*., 200
15.8709	2.0209	Heneicosane (**62**)	Saïdana *et al*., 2008
17.8418	2.288	(*E*)-Caryophyllene (**60**)	Sabulal *et al*., 2006
18.9393	6.3855	Butylated hydroxytoluene (**63**)	Zouari *et al*., 2013

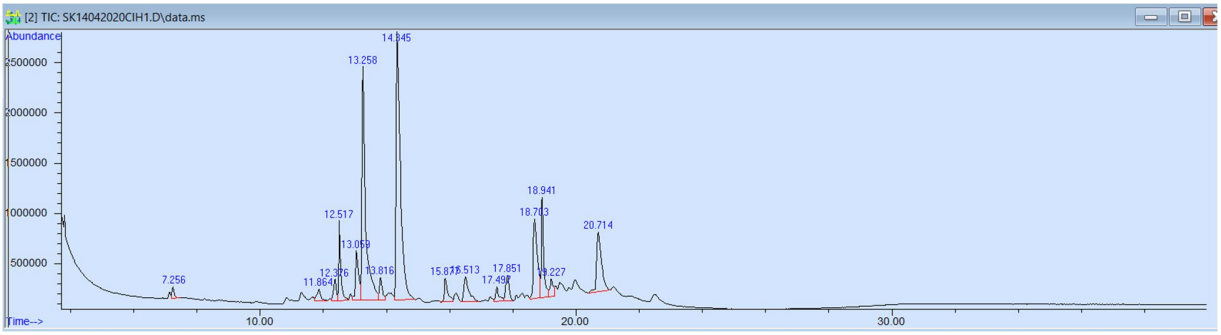
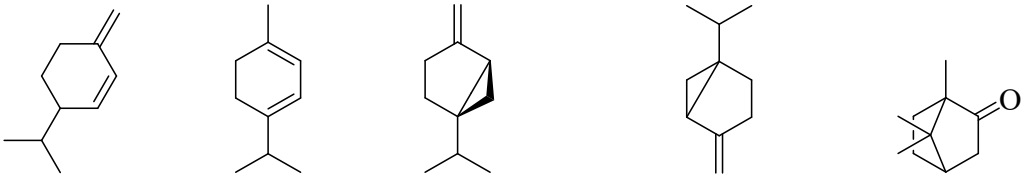
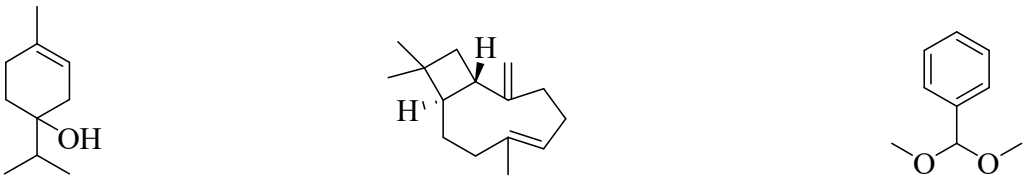


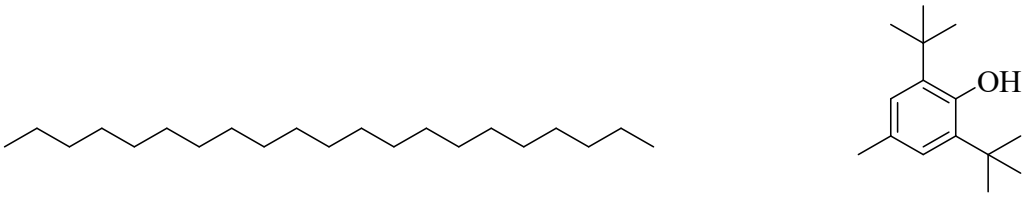
Figure 4.3. Insect damaged *Phaseolus vulgaris* plants chromatogram



54 **55** **56** **57** **58**



59 **60** **61**



62 **63**

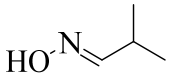
There are qualitative variations between volatile emissions from control bean plants and leafminer damaged plants. The difference in volatile emissions is related to cascade defence responses triggered by leafminer larval feeding. A puncture caused by leafminer larvae results in a large mine development that extend towards the base of the leaf. Plant wounding has previously been shown to result in reactions such as the hydrolysis of cyanogenic glycoside to produce insect-toxic prussic acid (Wink *et al*., 2018). Leafminer larvae consumes mesophyll in spongy and palisade tissue (Wei

et al., 2000). Wei *et al*. (2006) has shown production of defence volatiles when the common bean plants are damaged by pests or by artificial methods (Wei *et al*., 2006). Wei *et al*., (2006) detected terpenes and oximes in volatiles emitted by bean plants as a response to attack by agromyzid flies. They reported release of (*anti*)-2-methylpropanal oxime (**64**), (*syn*)-2-methylbutanal oxime (**65**), linalool (**66**) and (*E, E*)-α-farnesene (**67**) from insect-damaged plants. Activation of plant defence can, therefore, be linked to larval wounding/damage. The compound β-phellandrene (**54**) found in volatiles from the insect damaged plants and inoculated plants, is a cyclic monoterpene reported to have anti-microbial activity against various fungal and bacterial pathogens (Ji hong *et al*., 2017). It has also been documented to have attractive ability for the general insect predator *Macrolophus pygmaeus* (De Backer *et al*., 2015). The compound α-terpinene (**55**) produced by insect damaged and inoculated plants are volatile isomeric monoterpenes with reported anti-bacterial activity against rice pathogens (Yoshitomi *et al*., 2016). The compound α-terpinene, as a volatile constituent has also been reported to have anti-fungal effects (Sekine *et al*., 2007). The compounds *cis*-sabinene hydrate (**56**) and *trans*-sabinene hydrate (**57**) found in both insect damaged plant emissions and inoculated plant emissions are natural bicyclic monoterpenes. These compounds have been identified in several plant volatiles and oil compositions (Al-Kalaldeh *et al*., 2010; Bertoli *et al*., 2004). They have also been described to have anti-microbial activity by Deans *et al*., (1990) and Van Vuuren *et al*. (2010). The compound camphor (**58**) emitted by fungal inoculated plants is a terpene used in creams, lotions and ointments to reduce pain, irritation and itching, it has been identified in volatile oils from *Cinnanomum camphora* and ocimum plants. It has been proven to have anti-fungal property (Pragadheesh *et al*., 2013), insect repellence (Chokechaijaroenporn *et al*., 1994), and insect attractant abilities (Li *et al*., 2010). The compound terpinen-4-ol (**59**) was the major constituent of insect damaged plant at 28% (Table 4.2). It was

also emitted by inoculated insect-damaged plants. It is a terpineol isomer, a naturally occurring monoterpene. Plant volatiles containing terpinen-4-ol have been reported to affect feeding and oviposition of *Thrips tabaci* Lindeman (Thysanoptera: Thripidae) (Hulshof *et al*., 2002). It has also been tested for its anti-microbial activity (Njume *et al*., 2011) and anti-fungal property (Taylor *et al*., 2013). The compound (*E*)-caryophyllene (**60**) detected in all the treatments except in controls and F3ST1 inoculated plants is a naturally occurring sesquiterpene, a constituent of many essential oils. Volatiles constituting (*E*)-caryophyllene has been documented for its anti-microbial activity (Sabulal *et al*., 2006). The compound benzaldehyde, dimethyl acetal (**61**) was also a major constituent of insect damaged plants at 21% (Table 4.2). It is a dimethoxy toluene found in green vegetables and used in perfumes and food flavours. It is a major volatile constituent in the scent of flowers (Watanabe *et al*., 2009). It has shown anti-microbial and insect repellent activities (Demirci *et al*., 2013). The compound heneicosane (**62**) produced by inoculated plants is acyclic alkane that has been identified in volatile constituents from several plants (Saïdana *et al*., 2008). It has also been described to possess anti-microbial activity (Fitsiou *et al*., 2007) and as a kairomone (Renou *et al*., 1992). The compound butylated hydroxytoluene (**63**) was also identified in all the extracts except controls and *H. lixii* inoculated plants and, it is a lipophilic derivative of phenol, useful for its anti-oxidant properties (Zouari *et al*., 2013).

The major volatiles induced by leafminer larval wounding/damaged in previous studies include green leaf volatiles (esters, aldehydes and alcohols), nitrogen containing compounds and terpenoids (Wei *et al*., 2006). Related compounds have also been emitted by plants wounded by leaf-eating spider mites, sucking insects and caterpillars (Turlings *et al.,* 1998). The compounds identified in this study included the generally common volatile compounds identified from several plant species induced by various treatments and feeding herbivores. Production of these volatile

defence compounds by plants involve three biosynthetic pathways: the shikimic acid pathway for methyl salicylate, the isoprenoid pathway for terpenoids and the fatty acid/lipoxygenase pathway for green leaf volatiles (Tumlinson *et al.,* 1997).



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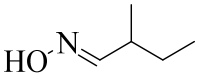

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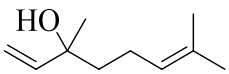

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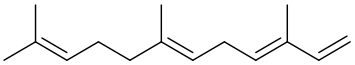


67

4.2.3 Hypocrea lixii Inoculated Phaseolus vulgaris Plants Volatiles Analysis

There were also qualitative variations in volatile emissions between control bean plants and fungal-inoculated plants. The GC-MS chromatogram (Figure 4.4) showed six (6) peak signals, five (5) of which were consistently detected from *H. Lixii* inoculated plants (Table 4.3), two (2) were previously identified in control emissions *m*-cresol (**56**) and *p*-cresol (**57**), three (3) more identified volatiles were released by *H. lixii* inoculated bean plants *cis*-1,1,3,5-tetramethyl cyclohexane (**68**), phenol (**69**), and benzyl alcohol (**70**) representing over 90% of the total blend (Table 4.3).

Table 4.3. Volatiles identified from *Hypocrea lixii* inoculated *Phaseolus vulgaris* plants

Retention Time	Area Percentage	Library/ID	References
9.4655	0.8916	*cis*-1,1,3,5-Tetramethyl cyclohexane (**68**)	Zhang *et al*., 201
11.0333	1.4779	Phenol (**69**)	Durango *et al*., 2013
11.9739	6.8814	Benzyl alcohol (**70**)	Aragüez *et al*., 201
12.6011	72.9283	*m*-Cresol (**56**)	Naznin *et al*., 2014
13.2282	13.2862	*p*-Cresol (**57**)	Naznin *et al*., 2014

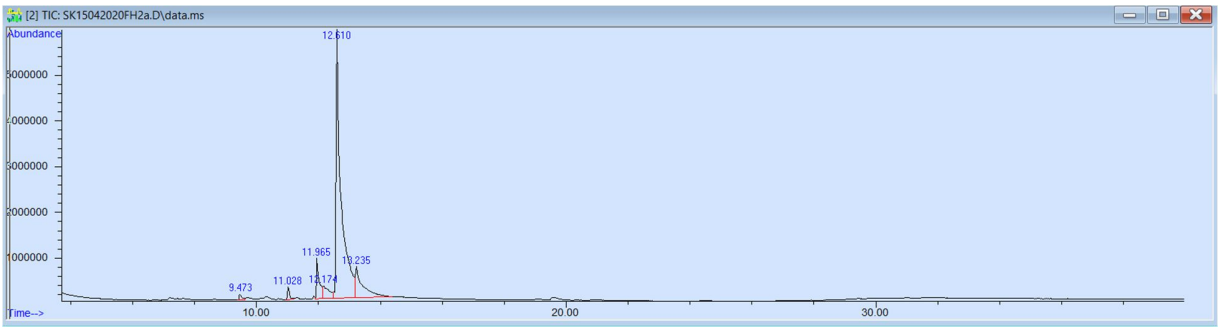
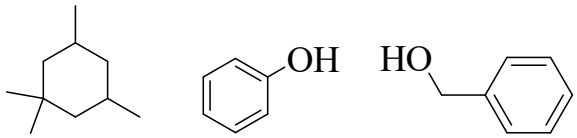


Figure 4.4. *Hypocrea lixii* inoculated *Phaseolus vulgaris* plants chromatogram



68 **69** **70**

Qualitative differences are also evidenced among bean plants inoculated with *H. lixii.* Volatiles absent in control plants were detected in this treatment. Production of compounds by *H. lixii* inoculated plants that were not present in control plants suggest induction as a result of *H. lixii* fungal colonization. The compounds identified have anti-fungal and insect attractive abilities that can be used for defence against both pathogens and herbivores. The activation of above-mentioned defences is therefore linked to endophytic inoculation. One of the compounds identified from *H. lixii* inoculated plants was a polymethylated cycloalkane known as *cis*-1,1,3,5-tetramethyl cyclohexane (**68**). It has been reported among anti-fungal volatiles produced by non-pathogenic fungi *Fusarium oxysporum* (Zhang *et al*., 2015). The compound phenol (**69**) produced by dicotyledonous plants are phytoalexins that disrupt the cell structure and metabolism of fungal pathogens (Durango *et al*., 2013). The compound benzyl alcohol (**70**) which was identified from *H. lixii* inoculated plants is an aromatic alcohol previously isolated from tomatoes, fruits and tea. These aroma components in fruits are responsible for attracting pollinators and seed dispersers and

also strengthening plant defence responses (Aragüez *et al*., 2013). The compounds *m*-cresol (**56**) and *p*-cresol (**57**) identified in both controls and fungi inoculated plants were the most abundant in *H. lixii* inoculated plant volatiles at 72% and 13% respectively (Table 4.3). The percentage abundance of cresol compounds is lower in inoculated plants compared to controls. This result implies a reduction in production of these compounds due to fungal inoculation while other defence compounds were induced. Plant-pathogen interactions depend on the presence of signal molecules, bearing information for the plant to commence appropriate defense reactions, or for the pathogen to conquer plant defense. Related results have been described by Unger *et al.,* (2005) where the necrotrophic pathogen *Botrytis cinerea* has been previously proven to supress defense-related oxidative burst in the leaf tissue of the bean.

4.2.4 Hypocrea lixii Inoculated Phaseolus vulgaris and Leafminer-Damaged Plants Volatiles Analysis

The GC-MS chromatogram (Figure 4.5) revealed more than twenty (20) peak signals of which, nineteen (19) of them were identified as volatiles consistently released by *H. lixii* inoculated and leafminer-damaged plants (Table 4.4). One (1) *p*-cresol (**57**) was previously identified in control emissions and *H. lixii* inoculated plant emissions. Four (4) were also previously identified in leafminer damaged control plants including (*E*)-caryophyllene (**60**), benzaldehyde dimethyl acetal (**61**), heneicosane (**62**) and butylated hydroxytoluene (**63**). Fourteen (14) more identified volatiles were consistently released by insect damaged *H. lixii* inoculated bean plants (Table 4.4). These compounds included 4-methyl octane (**71**), 3-methylanisole (**72**), (*Z*)-β-ocimene (**73**), (*E*)-β-ocimene (**74**), naphthalene (**75**), methyl salicylate (**76**), heptadecane (**77**), 6-propyl tridecane (**78**), propyl butanoate (**79**), tridecane (**80**), α-cedrene (**81**), octadecane (**82**), tetradecane (**83**) and dibutyl phthalate (**84**).

Table 4.4. Volatiles identified from *Hypocrea lixii* inoculated and Leafminer-damaged *Phaseolus vulgaris* plants

Retention Time (min)	Area Percentage	Library/ID	References
8.2336	0.0932	4-Methyloctane (**71**)	Ji hong *et al*., 2017
11.6604	0.4169	3-Methylanisole (**72**)	Azeem *et al*., 2013
11.9291	0.4492	(*Z*)-β-Ocimene (**73**)	Arimura *et al*., 2004
12.1307	1.5662	(*E*)-β-Ocimene (**74**)	Arimura *et al*., 2004
12.7802	1.9679	*p*-Cresol (**57**)	Naznin *et al*., 2014
13.2058	6.5047	Benzaldehyde dimethyl acetal (**61**)	Watanabe *et al*., 2009
14.4824	0.4742	Naphthalene (**75**)	Daisy *et al*., 2002
14.684	3.1435	Methyl salicylate (**76**)	Seskar *et al*., 1998
15.8934	0.2724	Heptadecane (**77**)	Hsouna *et al*., 2011
16.0726	0.4423	6-Propyl-tridecane (**78**)	Buchbauer et al., 1992
16.6773	0.3395	Heneicosane (**62**)	Saïdana *et al*., 2008
17.1477	1.4939	Propyl butanoate (**79**)	Buchbauer et al., 199
17.394	1.0589	Tridecane (**80**)	Buchbauer et al., 1992
17.73	0.5572	α-Cedrene (**81**)	Mahmud *et al*., 2009
17.8196	1.6471	(*E*)-Caryophyllene (**60**)	Sabulal *et al*., 2006
18.1779	1.2324	Octadecane (**82**)	Buchbauer et al., 1992
18.8946	4.8627	Butylated hydroxytoluene (**63**)	Zouari *et al*., 201
19.8577	3.3249	Tetradecane (**83**)	Zouari *et al*., 2013
23.9564	1.1746	Dibutyl phthalate (**84**)	Blažević *et al*., 2010

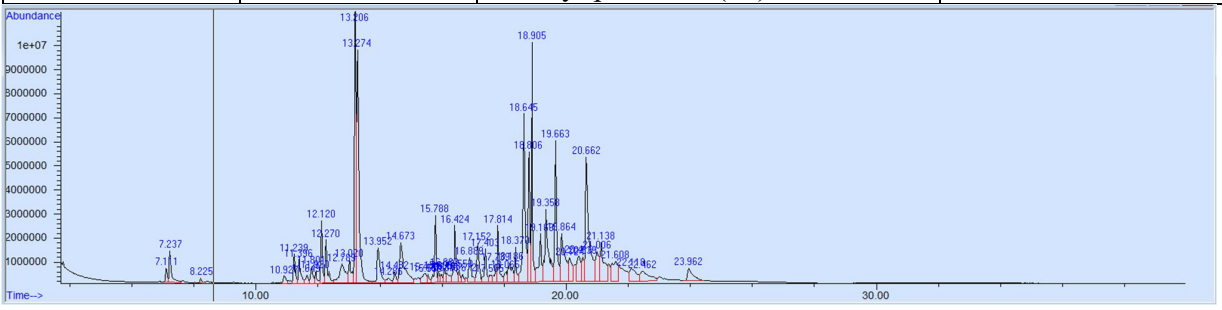
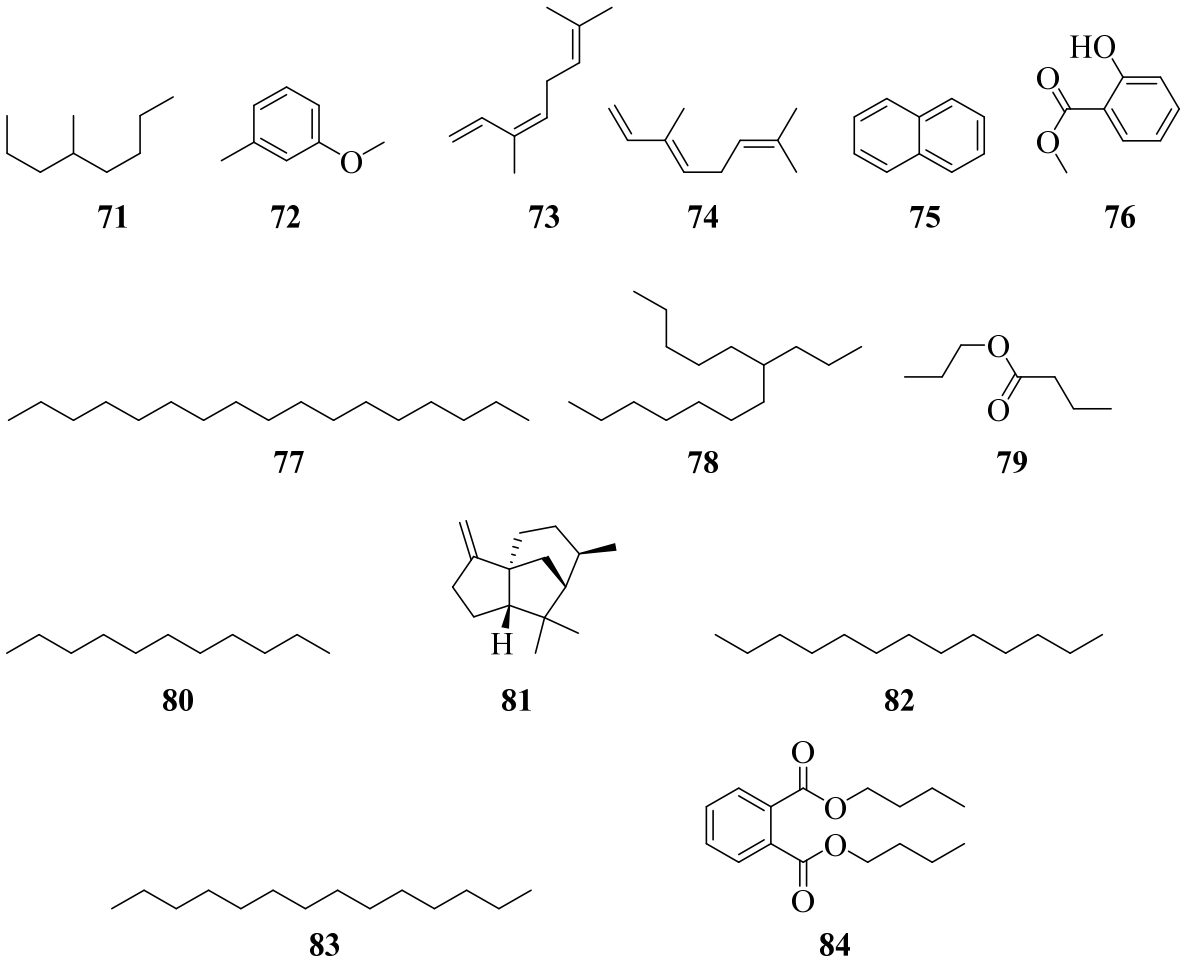


Figure 4.5. Leafminer-damaged *Hypocrea lixii* inoculated *Phaseolus vulgaris* plants chromatogram



Qualitative differences are also evidenced among *H. lixii* inoculated and leafminer-damaged plants. A combination of both inoculated and insect damage allowed for more changes in plant metabolism. Compounds previously linked to insect damage were also produced in combination with compounds linked to inoculation. More changes were also evident in the production of compounds not previously linked to either induction. The compound 4-methyloctane (**71**) emitted by *H. lixii* inoculated and leafminer damaged plants has been previously identified in headspace volatile samples of various plants including the grape fruit with reports of involvement in fruit ripening and resistance to fungus *Botrytis cinerea* (Ji hong *et al*., 2017). The compound 3-

methylanisole (**72**) released by both *H. lixii* and *B. bassiana* inoculated plants is a methoxy toluene, a microbial volatile that has been previously extracted from both fungi and bacteria. It has been shown to reduce pine weevil attraction to host plant (Azeem *et al*., 2013). It has also been described to have potential for plant growth and productivity (Kanchiswamy *et al*., 2015). The compounds (*Z*)-β-ocimene (**73**) and (*E*)-β-ocimene (**74**) in inoculated plants are monoterpenes found in various plants and fruits. Spider mites have been shown to induce emission of (*E*)-β-ocimene from the *Lotus japonicus* for defence (Arimura *et al*., 2004). Ocimene compounds have also been shown to activate genes and induce defence against *Botrytis cinera* (Kishimoto *et al*., 2005). The compound naphthalene (**75**) was also in inoculated and insect damaged plants and is the simplest polycyclic aromatic hydrocarbon. Naphthalene is an insect repellent that has been shown to be produced by fungal endophytes (Daisy *et al*., 2002). Volatiles containing naphthalene have also been shown to have anti-microbial properties (Vahedi *et al*., 2011). The compound methyl salicylate (**76**) is often used as an anti-inflammatory. Methyl salicylate has been shown to be an insect induced volatile (Ament *et al*., 2004). It has also been found in colonized plants (Seskar *et al*., 1998). Volatiles with methyl salicylate have shown anti-microbial activity (Papandreou *et al*., 2002). The compound heptadecane (**77**) is a straight chain alkane and a component of essential oils from plants with various metabolic functions. Volatile constituents with heptadecane have been shown to have anti-microbial and anti-oxidant properties (Hsouna *et al*., 2011). The compounds 6-propyl tridecane (**78**), tridecane (**80**) and tetradecane (**83**) have been found as a constituent of essential oils from the walnut plants responsible for their aromas (Buchbauer et al., 1992). The compound propyl butanoate (**79**) is a butyrate ester with roles as both human and plant metabolite and as an insect attractant. The compound α-cedrene (**81**) is a sesquiterpene metabolite that has been identified in several plant metabolites including cedar and citrus (Mahmud *et al*., 2009). Volatiles constituents

from endophytic fungi constituting cedrene has been tested for anti-fungal and anti-bacterial activities (Yan *et al*., 2018). The compound dibutyl phthalate (**84**) is a colourless oil that has been isolated from volatiles of plants and fungi with anti-microbial activity (Blažević *et al*., 2010; Radonić *et al*., 2011).

Production of compounds by *H. lixii* inoculated and damaged leafminer plants that were not present in control plants suggest induction as a result of both insect damage and fungal colonization. The compounds identified have anti-fungal and predator attractive abilities that can be used for defence against both pathogens and herbivores. The activation of above-mentioned defences is therefore linked to insect attack and endophytic inoculation. Benzaldehyde dimethyl acetal (**65**) produced by both insect damaged plants and *H. lixii* inoculated insect damaged plants, was the most abundant at 6% (Table 4.5). This implies that the compound is produced by beans during insect damage in the presence or absence of fungi. Demirci *et al*., (2013) has shown benzaldehyde dimethyl acetal (**65**) to have insect repellent activity.

4.2.5 Beauveria bassiana Inoculated Phaseolus vulgaris Plants Volatiles Analysis

The GC-MS chromatogram (Figure 4.6) revealed more than thirty (30) peak signals of which, sixteen (16) of them were identified as volatiles consistently released by *B. bassiana* inoculated plants (Table 4.5). Each experiment was repeated 3 times and *B. bassiana* inoculated plants emitted the highest number of compounds (Figure 4.6). One (1) compound *p*-cresol (**57**) was previously identified in control emissions, four (4) were also previously identified in leafminer damaged control plants including (*E*)-caryophyllene (**60**), heneicosane (**62**), benzaldehyde dimethyl acetal (**61**) and butylated hydroxytoluene (**63**). Six (6) compounds were also previously identified in insect damaged *H. lixii* inoculated bean plants including (*Z*)-β-ocimene (**73**) and (*E*)-β-ocimene (**74**), naphthalene (**75**), methyl salicylate (**76**), α-cedrene (**81**), tetradecane (**83**) and dibutyl

phthalate (**84**). Four (4) more identified volatiles were consistently released by *B. bassiana* inoculated bean plants (Table 4.5). These compounds include 1-methoxy-3-methylbenzene (**85**), (*E*)-γ-bisabolene (**86**), 4,8,12-trimethyl-1,3(*E*),7(*E*),11-tridecatetraene (**87**), sulfurous acid, pentyl undecyl ester (**88**) representing over 30% of the total blend.

Table 4.5. Volatiles identified from *Beauveria bassiana* inoculated *Phaseolus vulgaris* plants

Retention Time (min)	Area Percentage	Library/ID	References
11.6603	0.4001	1-Methoxy-3-methylbenzene (**85**)	Schnurer et al., 1999
11.9515	0.3093	(*Z*)-β-Ocimene (**73**)	Arimura *et al*., 2004
12.1307	1.4058	(*E*)-β Ocimene (**74**)	Arimura *et al*., 2004
12.8474	0.7414	*p*-Cresol (**57**)	Naznin *et al*., 2014
13.2058	11.8867	Benzaldehyde, dimethyl acetal (**61**)	Watanabe *et al*., 2009
14.5048	0.3716	Naphthalene (**75**)	Daisy *et al*., 2002
14.6839	4.4711	Methyl salicylate (**76**)	Seskar *et al*., 1998
17.4164	0.7988	Tetradecane (**83**)	Zouari *et al*., 2013
17.7524	0.5667	α-Cedrene (**81**)	Mahmud *et al*., 2009
17.8196	2.0802	(*E*)-Caryophyllene (**60**)	Sabulal *et al*., 2006
18.805	4.037	(*E*)-γ-Bisabolene (**86**)	Kurade *et al*., 2010
18.8946	5.3821	Butylated hydroxytoluene (**63**)	Zouari *et al*., 2013
19.6785	4.3292	4,8,12-Trimethyl-1,3(*E*),7(*E*),11-tridecatetraene (**87**)	Ishiwari et al., 2007
19.8577	3.3725	Sulfurous acid, pentyl undecyl ester (**88**)	Yan et al., 2010
21.0223	1.305	Heneicosane (**62**)	Saïdana *et al*., 2008
24.0236	1.3259	Dibutyl phthalate (**84**)	Blažević *et al*., 2010

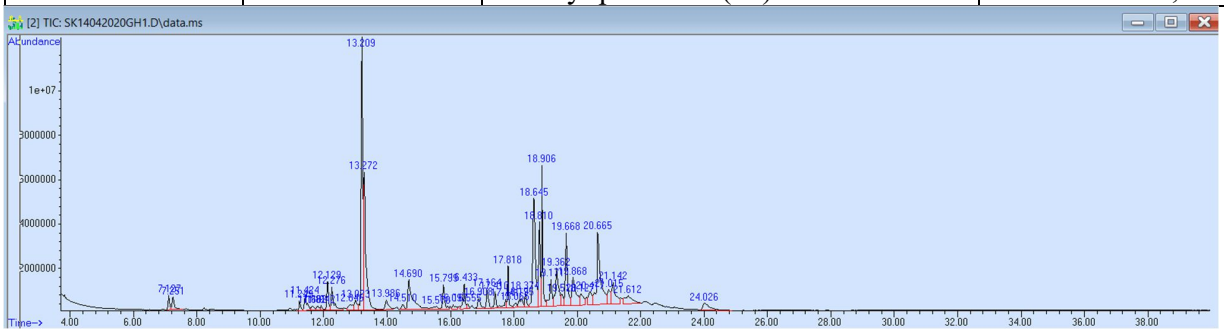
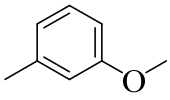


Figure 4.6. *Beauveria bassiana* inoculated *Phaseolus vulgaris* plants chromatogram



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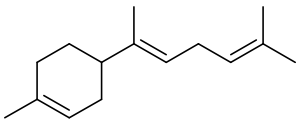

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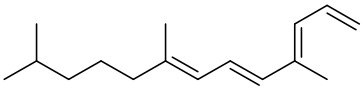

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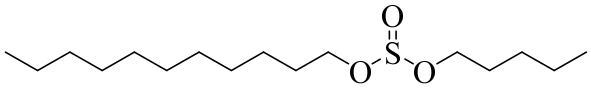


88

Although *H. lixii* inoculated plants consistently released volatiles absent in control plant emissions, their quantities were considerably lower compared to volatiles emitted by plants inoculated with *B. bassiana. Beauveria bassiana* inoculated plants produced the highest number of detected emissions. Synthesis of biologically active compounds through induction by the microorganisms is attributed to metabolic interactions between the fungal endophytes and their host plant (Manasa *et al*., 2014). Fungal endophytes have exoenzymes necessary for colonization of the hosts and their metabolic interactions that include an increase in the plant's defense metabolites to balance their association (Schulz *et al*., 2002). Induced systemic resistance in endophytically colonized plants is due to activation or production of mycotoxins. Plant responses to one fungus is therefore not similar to another fungus. *Hypocrea lixii* which exhibited higher colonization percentages produced fewer volatiles compared to *B. bassiana* inoculated plants. This shows that the interaction between the two fungi and the *P. vulgaris* host plant, and the consequent metabolism differed. This maybe because the fungal isolates are from different genera and compounds previously isolated from them have also been shown to be different (Patočka *et al.*, 2018). Major compounds produced from the two fungi are from different classes and thus interact differently with the host plant. The compound 1-methoxy-3-methylbenzene (**85**) produced by *B. bassiana* plants is reported to be produced in substantial quantities by the malodorous fungi. The compound

is included in fungal volatiles which are indicators for food and feed spoilage (Schnurer et al., 1999). The compound (*E*)-γ-bisabolene (**86**) is a sesquiterpene previously identified in several plant volatiles constituents which were shown to have anti-cancer, anti-bacteria and anti-fungal activities (Kurade *et al*., 2010). The compound 4,8,12-ttrimethyl-1,3(*E*),7(*E*),11-tridecatetraene (**87**) has been identified in essential compounds in plant volatiles induced by herbivores that attract the predatory mite *Neoseiulus womersleyi* (Ishiwari *et al*., 2007). The compound sulfurous acid, pentyl undecyl ester (**88**) has been previously characterized from essential oils of lemon and evaluated for anti-oxidant and anti-fungal properties (Yan *et al*., 2010). This therefore shows that induction of defense compounds by *B. bassiana* was successful. Compounds produced have all shown previous activities as anti-fungal, insect repellent or predator attractor. The compound benzaldehyde dimethyl acetal (**61**) produced by both insect damaged plants and *H. lixii* inoculated insect damaged plants, was also the most abundant at 11% (Table 4.5) for *B. bassiana* inoculated. This implies that the compound is produced by beans during insect damage in the absence or presence of fungi. Demirci *et al*. (2013) have shown benzaldehyde dimethyl acetal (**61**) to have insect repellent activity.

4.2.6 Leafminer-Damaged Beauveria bassiana Inoculated Phaseolus vulgaris Plants Volatiles Analysis

Leafminer-damaged *B. bassiana* inoculated plants emitted the most compounds (Figure 4.7). The GC-MS chromatogram (Figure 4.7) revealed more than thirty (30) peak signals of which, eleven (11) of them were identified as volatiles consistently released by *B. bassiana* inoculated plants (Table 4.6). Eight (8) of these compounds were also previously identified in leafminer damaged plants and *H. lixii* inoculated plants, α-Terpinene (**55**), *cis*-sabinene hydrate (**56**), camphor (**58**), terpinene-4-ol (**59**), benzaldehyde dimethyl acetal (**60**), heneicosane (**62)** butylated hydroxy

toluene (**63**) and α-cedrene (**81**). Three (3) more identified volatiles were consistently released by leafminer-damaged *B. bassiana* inoculated bean plants (Table 4.6). These compounds included benzaldehyde (**89**), 5,7-dimethyl undecane (**90**) and propanoic acid 2-methyl-2-ethyl 3-hydroxyhexyl (**91**).

Table 4.6. Volatiles identified from Leafminer-damaged *Beauveria bassiana* inoculated *Phaseolus vulgaris* plants

Retention Time (min)	Area Percentage	Library/ID	References
10.6972	0.6136	Benzaldehyde (**89**)	Velásquez et al., 2020
12.3322	0.9408	α-Terpinene (**55**)	Yoshitomi *et al*., 2016
13.0265	3.7344	*cis*-Sabinene hydrate (**56**)	Al-Kalaldeh *et al*., 2010
13.2281	16.511	Benzaldehyde, dimethyl acetal (**61**)	Watanabe *et al*., 2009
13.8104	0.9903	Camphor (**58**)	Pragadheesh *et al*., 2013
14.3256	16.685	Terpinen-4-ol (**59**)	Hulshof *et al*., 2002
15.8486	1.4502	5,7-Dimethyl undecane (**90**)	Journal *et al*., 2015
17.2372	0.7705	2-Methyl-2-ethyl-3-hydroxyhexyl propanoate (**91**)	Forti et al., 2018
17.7747	1.5039	α-Cedrene (**81**)	Mahmud *et al*., 2009
18.917	4.6411	Butylated hydroxytoluene (**63**)	Zouari *et al*., 2013
19.9025	4.5304	Heneicosane (**62**)	Saïdana *et al*., 2008

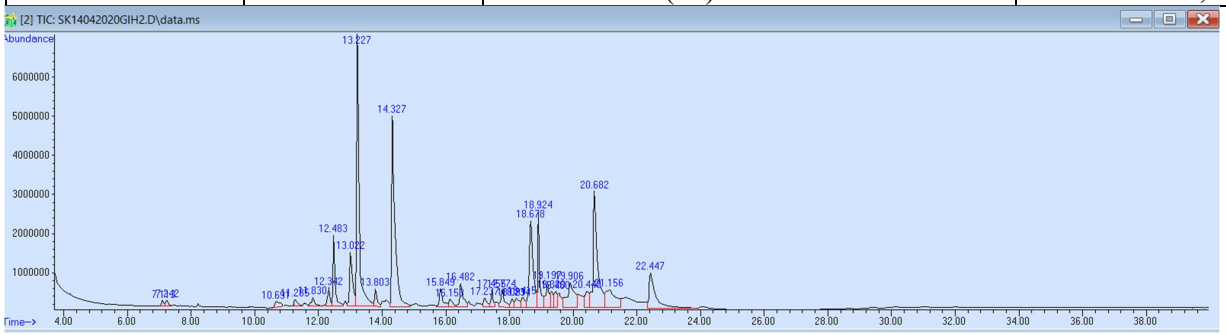
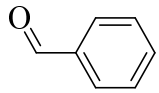
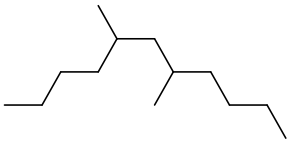


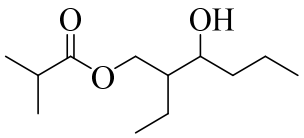
Figure 4.7. Leafminer-damaged *Beauveria bassiana* inoculated *Phaseolus vulgaris* plants chromatogram



89



90



91

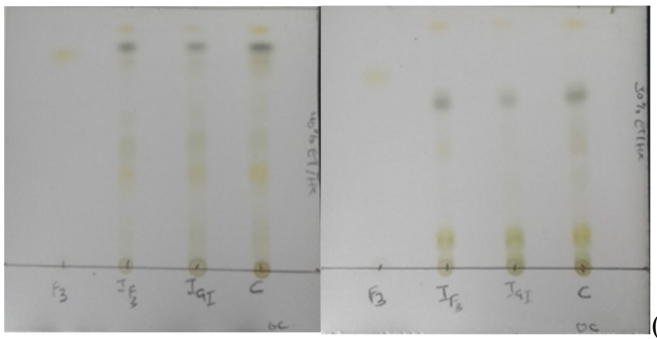
Changes in metabolism due to both insect damage and inoculation was also evident with *B. bassiana* inoculated plants. As previously discussed, compounds not related to either induction were also produced. The compound benzaldehyde (**89**) was reported in volatile organic compounds in *Vitis vinifera* as an outcome of induced metabolic changes in the host plant by the arbuscular mycorrhizal fungus *Funneliformis mosseae* (Velásquez et al., 2020). The alkane, dimethyl undecane (**90**) has been previously found in fungal volatile constituents of the *Monilinia* species (Journa *et al*., 2015). The compound 2-methyl-2-ethyl-3-hydroxyhexyl propanoate (**91**) is among flavor compounds produced by yeast (Forti et al., 2018). An insect repellent, produced by all plant treatments except control plants and *H. lixii* inoculated plants, benzaldehyde dimethyl acetal (**61**) was the most abundant at 16% (Table 4.5) for insect damaged *B. bassiana* inoculated plants. Another major constituent of the blend being terpinen-4-ol (**59**) at 16%. It has previously been identified in blends with insect predator attractive abilities.

Natural enemies including insect predators such as *Phaedrotoma scabriventris* (*Hymenoptera: Braconidae*) a leafminer parasitoid, attack a broad variety of herbivorous insects and are drawn by these chemical cues. The particular volatile blend produced from inoculated plants performs a vital role for natural enemies to detect hosts. Walling, (2000) has also shown that a specialist parasitoid could differentiate its host caterpillar from a non-host caterpillar based on specific volatile blends of host plants induced by caterpillars. The compounds identified in the study include volatiles previously identified as predator attractants. Volatiles induced by leafminer damaged bean plants

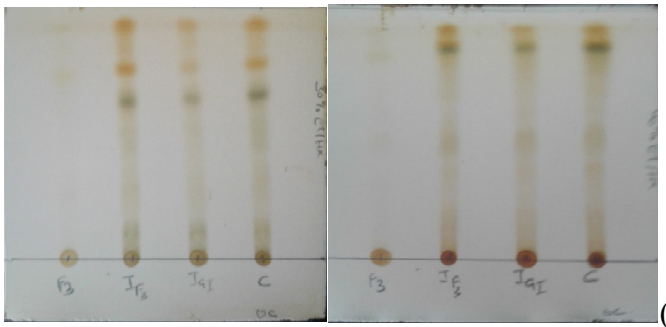
and *H. lixii* and *B. bassiana* fungi inoculated plants have potential to attract parasitoids as defence against herbivore attack. Two species of parasitic wasp, *Diglyphus isaea* and *Opius dissitus* parasitize *Liriomyza* leafminer larvae (Zhao et al., 2012). (Zhao et al., 2012) noted that adults of *Diglyphus isaea* orientate in the direction of plant smells correlated with *Liriomyza sativae* Blanchard-infected plants. Specific compounds from complex herbivore-induced volatile blends are proven to play a part in the in the discriminatory foraging behaviour of their natural enemies (Menjivar, 2010). Further studies on behavioural bioassays and electrophysiological tests of parasitoids in response to individual volatile compounds and different volatile blends have to be conducted to be able to understand the underlying mechanism. However, the identities of compounds were only based on comparisons of mass spectra available from an MS library. Therefore, some of the identifications may be tentative especially for volatiles with *trans-cis* isomers.

4.3 Thin Layer Chromatography

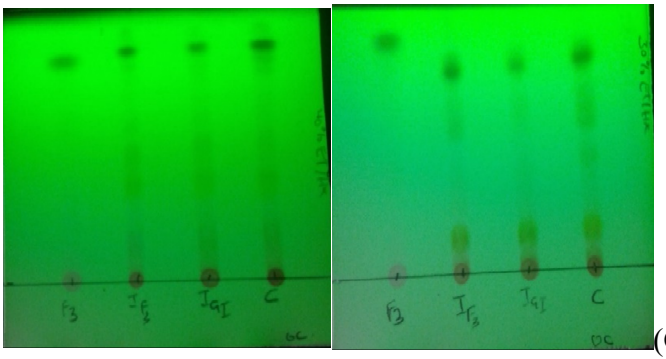
Preliminary screening of the plant extract using TLC plates in 30% ethyl acetate in hexane showed the presence of non-polar, mid-polar and polar compounds in the extracts. Coloured bands on the plate viewed under the UV lamp exhibited the presence of organic compounds chromophores. This compounds have also been isolated previously from the common bean by Rocha-Guzmán *et al*., (2007). The screening with sulfuric acid revealed the presence of compounds of interest, hydrocarbons. There was also a distinct variation in the number of compounds from the fungal extracts and the plant extracts. This was consistent with previous work from Panage *et al*. (1988).



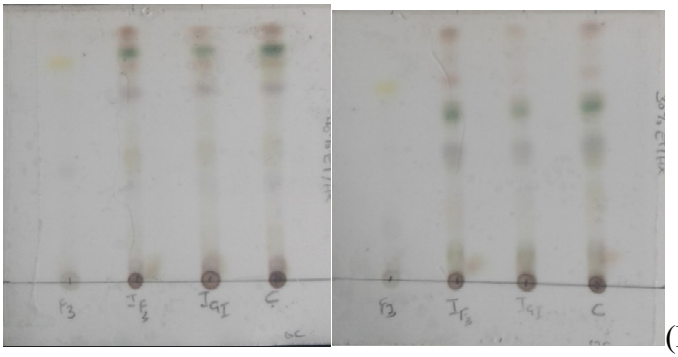
(A)



(B)



(C)



(D)

Figure 4.8. Comparison TLC plates for solvent system 30% ethyl acetate in *n*-hexane. Plates observed with the naked eye (A), plates exposed to visualization agent iodine (B) and plates observed under UV light (C) and plates screened with sulfuric acid (D). C – control plant extracts,

IG1 – *Beauveria bassiana* G1LU3 inoculated plant extracts, IF3 – *Hypocrea lixii* F3ST1 inoculated plant extracts, Fs – Fungal extract (*Beauveria bassiana*), F3 – Fungal extract (*Hypocrea lixii*).

4.4 Metabolic Screening with Gas Chromatography-Flame Ionization Detector

Qualitative and quantitative differences in detected compounds were expected from extract screening of the treatments by GC-FID from the results exhibited in prior screening by TLC.

4.4.1 Controls Plant Extracts Compared to insect (leafminer)-damaged Plant Extracts

Metabolic induction in the common bean plants by inoculation and exposure to insects was determined by GC-FID analysis of the dichloromethane extracts of freeze-dried material. More than eighty (80) peaks for every treatment were detected by the experiment. Nineteen (19) of these metabolite peaks revealed concentrations (areas measured in milli Absorbance units) different when compared to treatments exposed to leaf miner adult insects (Figure 4.9). These peaks had the retention times of (10.0 (a1), 13.6 (a2), 15.4 (a3), 16.2 (a4), 18.1 (a5), 18.6 (a6), 19.1 (a7), 19.5 (a8), 23.0 (a9), 23.4 (a10), 24.2 (a11), 25.3 (a12), 25.8 (a13), 26.0 (a14), 28.8 (a15), 29.5 (a16), 30.3 (a17), 31.2 (a18), 32.3 (a19)).

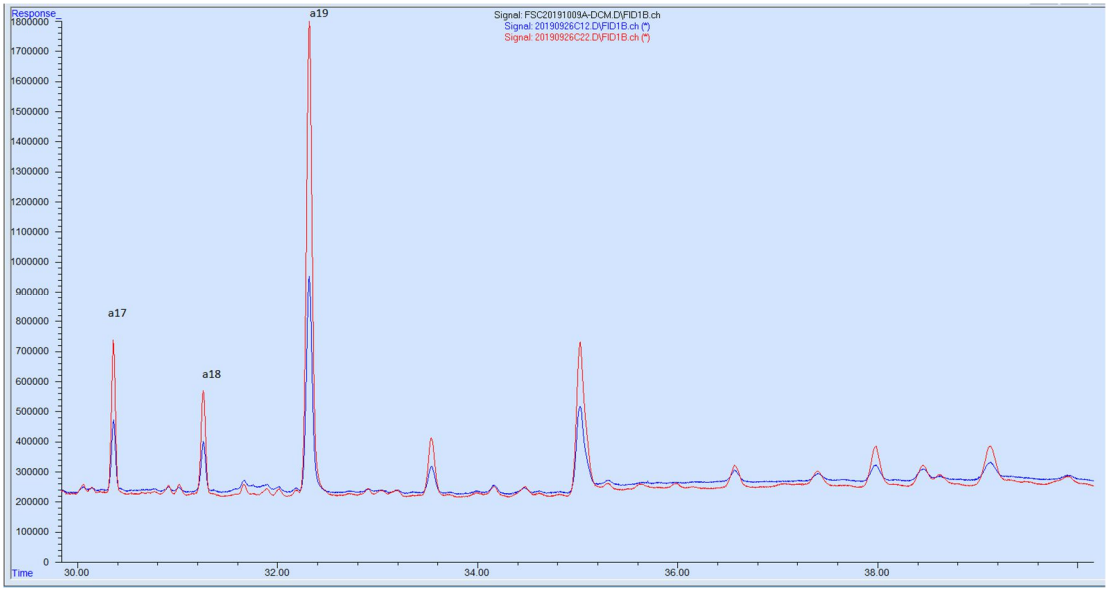


Figure 4.9. Metabolic accumulation in leafminer-damaged plants relative to control Phaseolus vulgaris plants as per observed peak areas measured in mAUs. Common bean plant controls extracts (blue) and plants exposed to adult leaf miners (red) at 30-40 min retention time (A)

(A)

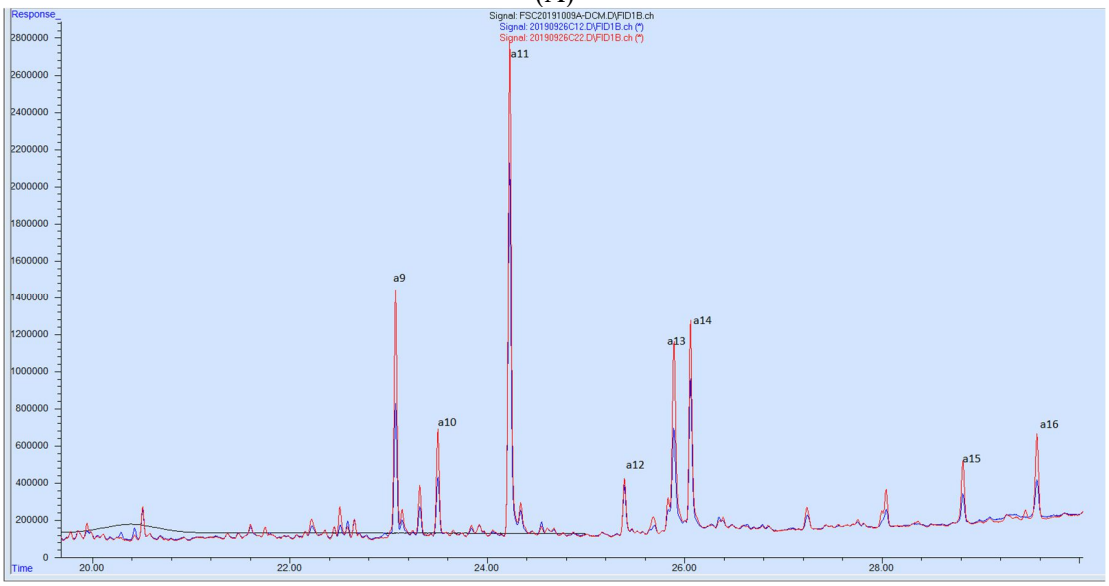


Figure 4.10. Metabolic accumulation in leafminer-damaged plants relative to control Phaseolus vulgaris plants as per observed peak areas measured in mAUs. Common bean plant controls extracts (blue) and plants exposed to adult leaf miners (red) at 20-30 min retention time (B)

(B)

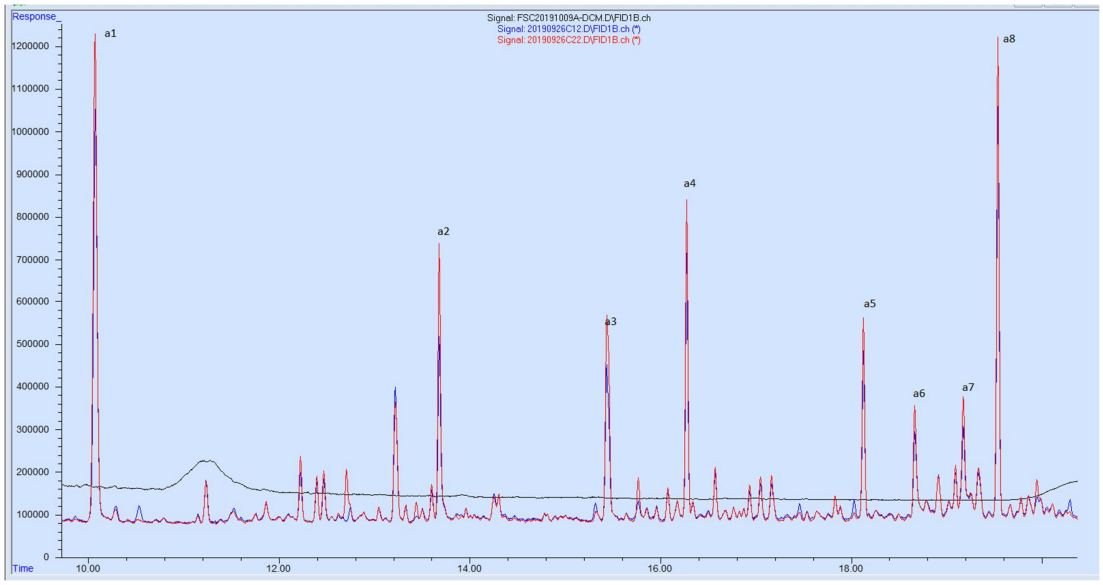
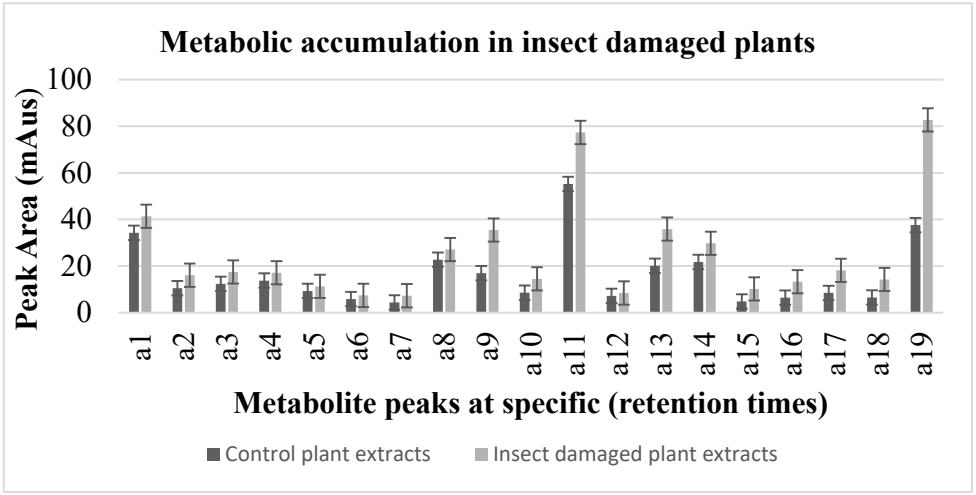


Figure 4.11. Metabolic accumulation in leafminer-damaged plants relative to control Phaseolus vulgaris plants as per observed peak areas measured in mAUs. Common bean plant controls extracts (blue) and plants exposed to adult leaf miners (red) at 10-20 min retention time (C)

(C)

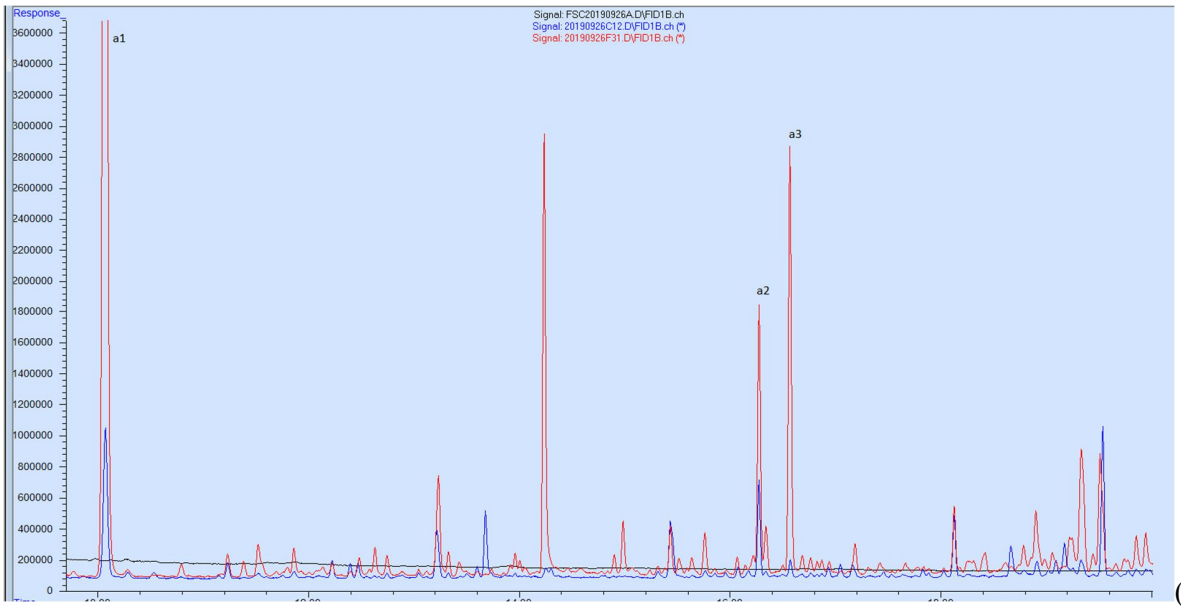


(D)

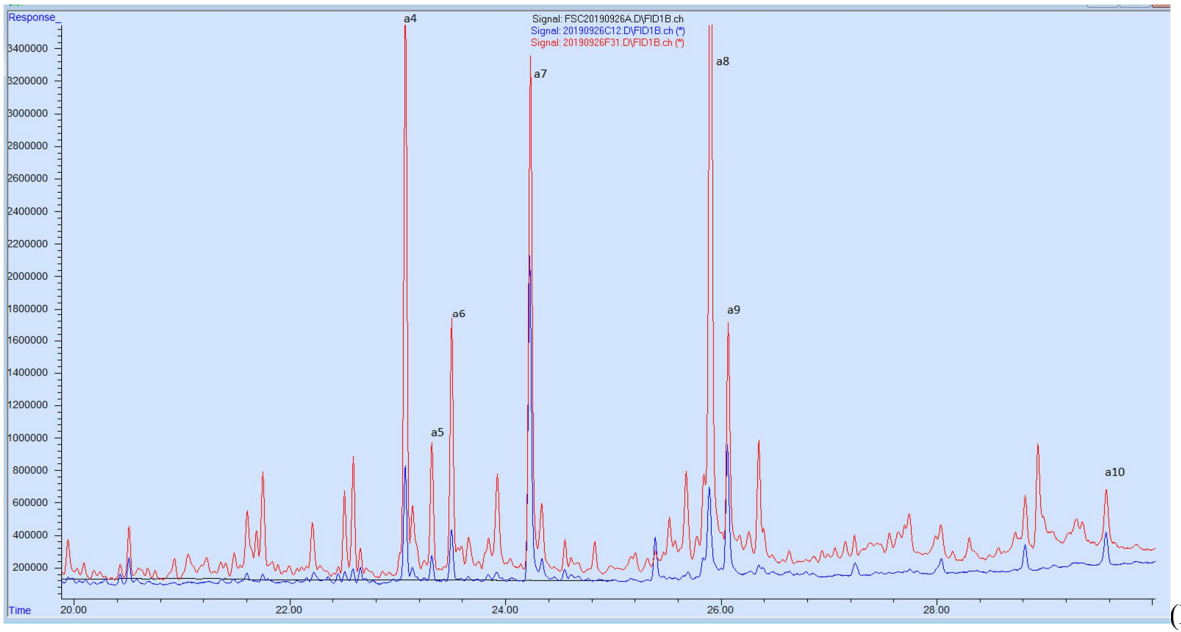
Figure 4.12. Metabolic accumulation in leafminer-damaged plants relative to control *Phaseolus vulgaris* plants as per observed peak areas measured in mAUs.) Peak area comparisons. Labelled peaks depict areas with significant differences.

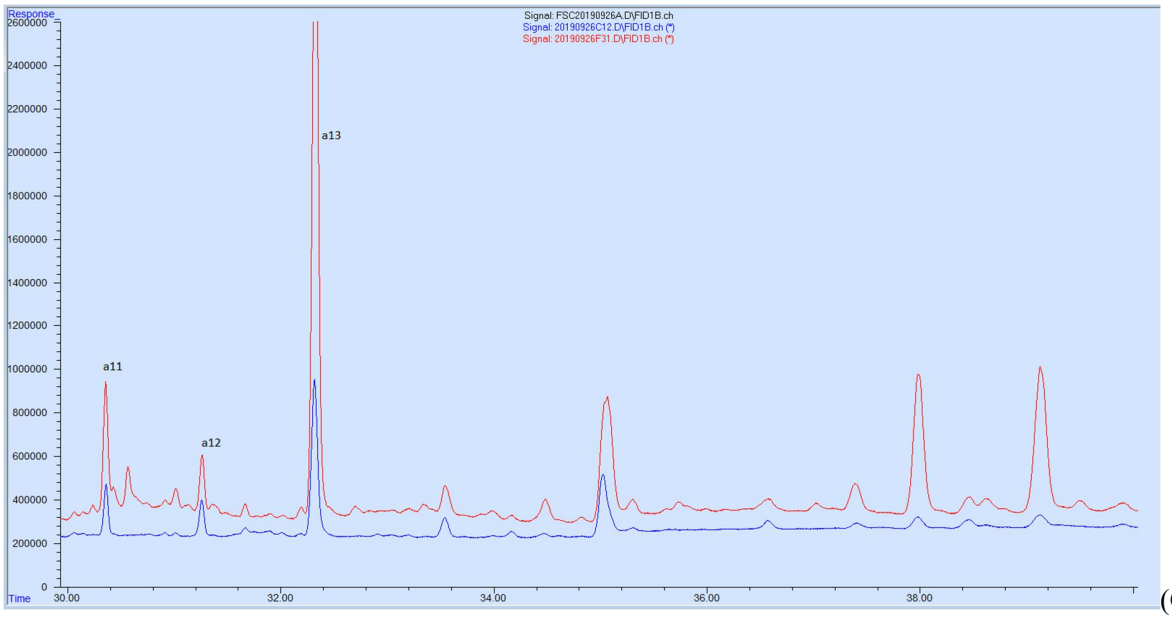
4.4.2 Controls Plant Extracts Compared to Hypocrea lixii F3ST1 Inoculated Plant Extracts

Metabolic induction in the common bean plants by inoculation with endophytic fungi was determined by GC-FID analysis of dichloromethane extracts of freeze-dried material. Thirty-three (33) peaks were detected in control plant extracts compared to Eighty-three (83) peaks detected in inoculated plant extracts. Several peaks were only detected in inoculated extracts including peaks at retention time, 5.8, 13.8 and 14.2. Thirteen (13) of these metabolite peaks showed concentrations (areas measured in milli Absorbance units) different when compared to treatments inoculated with F3ST1 (Figure 4.10). These peaks had the retention times of (10.0 (a1), 16.2 (a2), 16.5 (a3), 23.0 (a4), 23.3 (a5), 23.4 (a6), 24.2 (a7), 25.9 (a8), 26.0 (a9), 29.5 (a10), 30.3 (a11), 31.2 (a12), 32.4 (a13).



(A)


Response
Signal: FSC20190926A.D\FID1B.ch
Signal: 20190926C12.D\FID1B.ch (*)
Signal: 20190926F31.D\FID1B.ch (*)
a4
a7
a8
a6
a9
a5
a10
Time
20.00
22.00
24.00
26.00
28.00
(B)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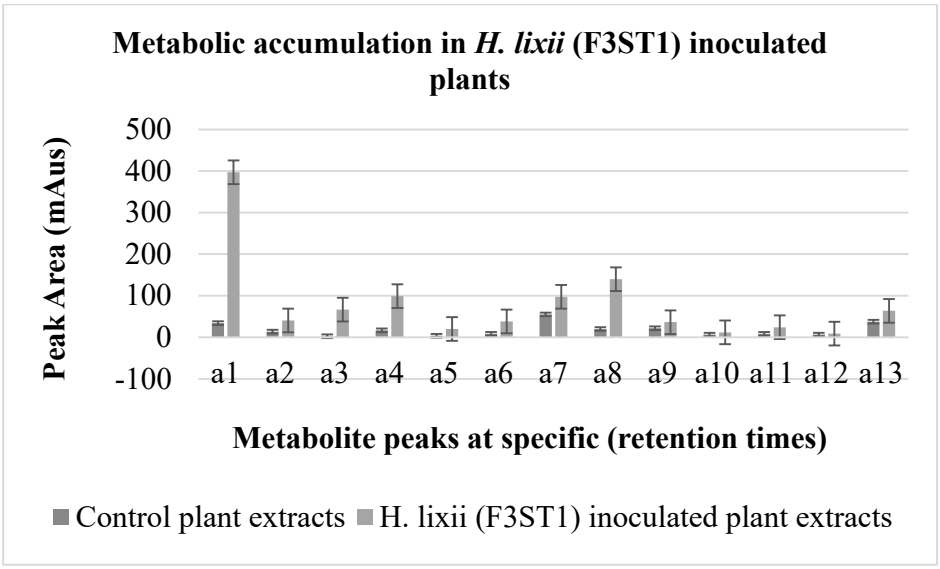
Signal: 20190926C12.D\FID1B.ch (*)
Signal: 20190926F31.D\FID1B.ch (*)
a13
a11
a12
Time
30.00
32.00
34.00
36.00
38.00
(C)

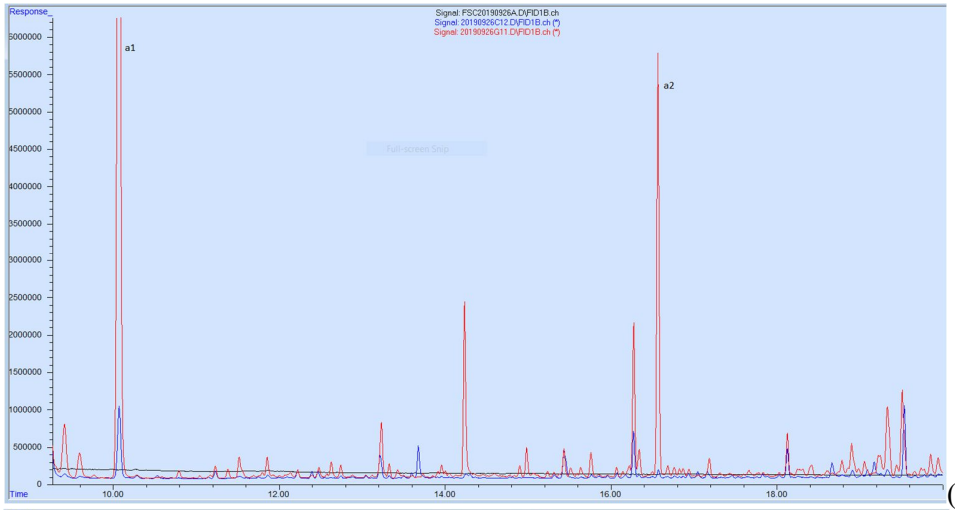


(D)

Figure 4.10. Metabolic accumulation in *Hypocrea lixii* inoculated plants relative to control *Phaseolus vulgaris* plants as per observed peak areas measured in mAUs. Common bean plant controls (blue) and plants inoculated with *Hypocrea lixii* F3ST1 (red) at 10-20 min retention time (A) and 20-30 min retention time (B) and 30-40 min retention time (C). (D) Peak area comparisons. Labelled peaks depict areas with significant differences.

4.4.3 Controls Plant Extracts Compared to Beauveria bassiana G1LU3 Inoculated Plant Extracts

Metabolic induction in the common bean plants by inoculation with endophytic fungi was determined by GC-FID analysis of dichloromethane extracts of freeze-dried material. Thirty-three (33) peaks were detected in control plant extracts compared to ninety-two (92) peaks detected in G1LU3 inoculated plant extracts. Several peaks were only detected in inoculated extracts including peaks at retention time, 14.2, 16.5, 23.5 and 36.4. Ten (10) of these metabolite peaks showed concentrations (areas measured in milli Absorbance units) different when compared to treatments inoculated with fungi G1LU3 (Figure 4.11). These peaks had the retention times of (10.0 (a1), 16.2 (a2), 23.0 (a3), 23.3 (a4), 23.5 (a5), 24.2 (a6), 25.8 (a7), 26.0 (a8), 30.3 (a9), 32.3 (a10).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Signal: 20190926G11.D\FID1B.ch (*)
a1
a2
Time
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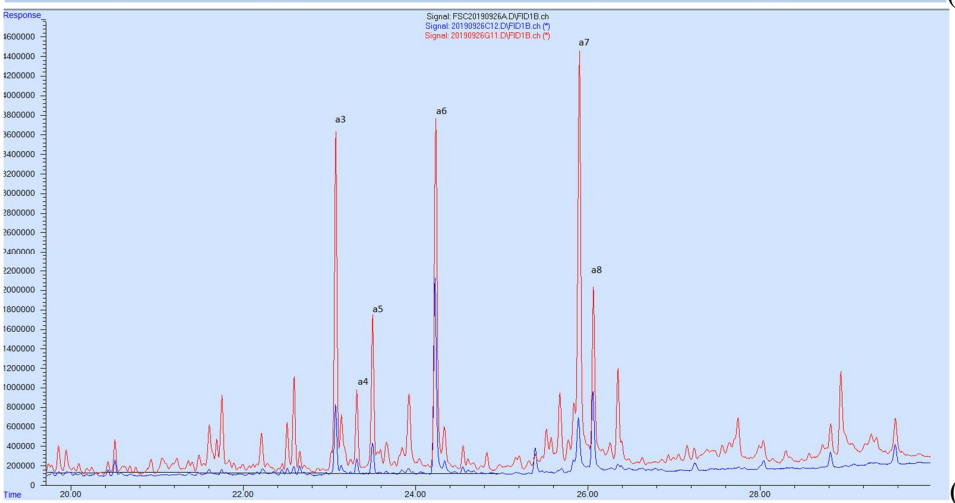
12.00
14.00
16.00
18.00

(A)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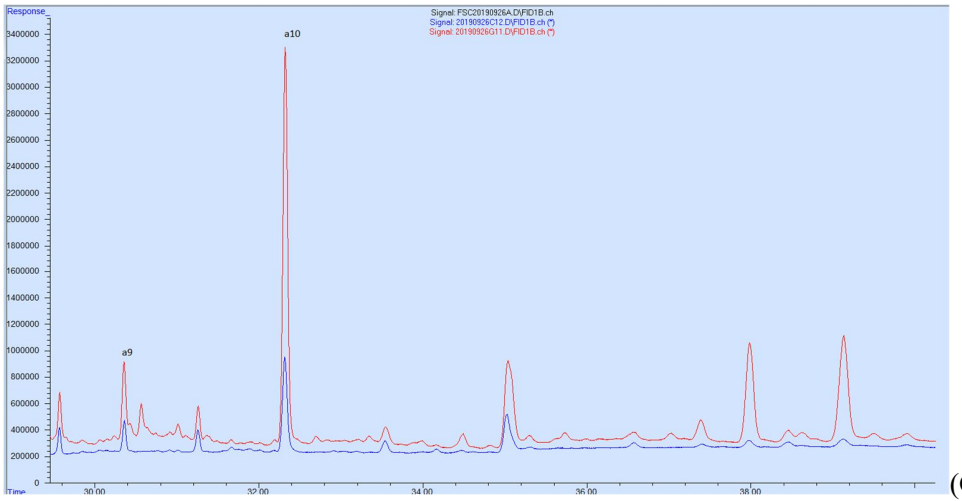
Signal: 20190926G11.D\FID1B.ch (*)
a3
a4
a5
a6
a7
a8
Time
20.00
22.00
24.00
26.00
28.00

(B)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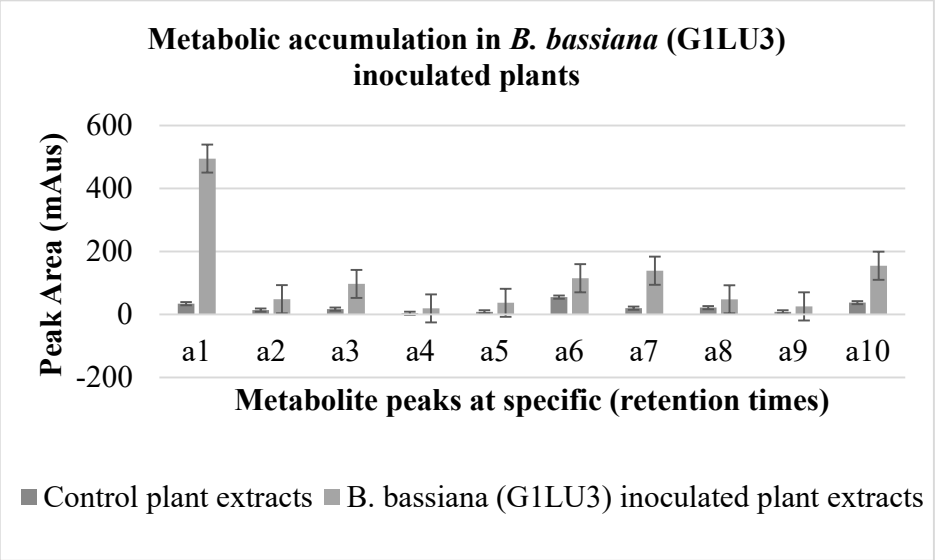
a9
a10
Time
30.00
32.00
34.00
36.00
38.00

(C)



(D)

Figure 4.11. Metabolic accumulation in *Beauveria bassiana* inoculated plants relative to control *Phaseolus vulgaris* plants as per observed peak areas measured in mAUs. Common bean plant controls (blue) and plants inoculated with *Beauveria bassiana* G1LU3 (red) at 10-20 min retention time (A) and 20-30 min retention time (B) and 30-40 min retention time (C). (D) Peak area comparisons. Labelled peaks depict areas with concentration differences.

Menjivar *et al*. (2012) also reported variation in quality and quantity of solvent extracts between control tomato plants and tomato plants with *Trichoderma atroviride* strain MT-20, *T. atroviride* strain S-2 and *Fusarium oxysporum* strain 162 implying changes in metabolism as a result of fungal colonization. Menjivar *et al*. (2012) reported metabolic accumulation in tomato leaves due to endophytic inoculation.

4.5 Metabolic Screening of Liquid Extracts with Gas Chromatography-Mass Spectrometer

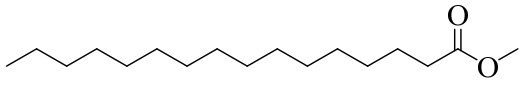
Similarities in the compounds produced between the controls and the treatments were observed (Table 4.11). All extracts except the *B. bassiana* fungal extract contained the hexadecenoic acid methyl ester (**92**), 9,12-octadecanoic (**93**) and 9,12,15-octadecatrienoic acid methyl esters (**94**).

Both control plants extracts contained 2-ethoxy-2-methylbutane (**95**) but was not present in other treatments. Compounds including 3-ethoxypentane (**96**), 2-pentanol, 2-methyl-2-pentanol (**97**) and 2-hexanol, 2-methyl-2-hexanol (**98**) were only present in inoculated plant extracts but not in the controls. 2-Hydroxy-2-methyl-8,8-diphenyl-octa-5,7-dien-3-one (**99**) was present in all plant extracts but not in fungal extracts. Control plants also exhibited the Acetic acid, 13-hydroxy-4,4,6a,6b,8a,11,11,14b-octamethyldocosahydropicen-3-yl ester (**100**) a plant growth regulator. *H. lixii* fungal extracts showed three potential defense compounds, methyl stearate (**101**), 1-Hydroxy-4-methylanthraquinone (**102**) and 1,8-dihydroxy-3-methyl-9,10-anthracenedione (**103**) which have previously been associated with critical bioactivity in plants.

Table 4.9. Compounds identified from liquid crude *Phaseolus vulgaris* plant extracts.

Compounds	**Retention Time**								**References**
Treatment	**C**	**C1**	**F**	**F1**	**G**	**G1**	**FF**	**GF**	
2-Ethoxy-2-methylbutane (**95**)	6.3	6.3		-	-	-	-	6.3	Xu *et al*., 2011
Hexadecenoic acid, methyl ester (**92**)	23.4	23.4	23.3	23.4	23.3	23.3	23.3	-	Abubacker *et al*., 2013
9,12-Octadecadienoic acid, methyl ester (**93**)	27.8	27.8	27.8	27.8	27.8	27.8	27.8	-	Antonious *et al*., 2007).
9,12,15-Octadecatrienoic acid, methyl ester (**94**)	28.0	28.0	28.0	28.0	28.0	28.0	-	-	Kumar *et al*., 2010
2-Hydroxy-2-methyl-8,8-diphenyl-octa-5,7-dien-3-one (**99**)	39.0	6.3	6.3	6.3	4.7	6.3	-	-	Toci et al., 2008
Acetic acid, 13-hydroxy-4,4,6a,6b,8a,11,11,14b-octamethyldocosahydropicen-3-yl ester (**100**)	39.8	-	-	-	-	-	-	-	Eberle *et al*., 1986
3-ethoxypentane (**96**)	-	-	6.0	-	-	-	-	-	Zvidzai *et al*., 2013
2-Pentanol, 2-methyl-2-pentanol (**97**)	-	-	6.3	6.3	-	6.3	-	-	Merk *et al*., 1997
2-Hexanol, 2-methyl-2-hexanol (**98**)	-	-	-	-	4.7	-	4.6	-	Merk *et al*., 1997
Methyl stearate (**101**)	-	-	-	-	-	-	28.6	-	Nonami, 2000
1-Hydroxy-4-methylanthraquinone (**102**)	-	-	-	-	-	-	32.3	-	Ee *et al*., 2009
1,8-dihydroxy-3-methyl-9,10-anthracenedione (**103**)	-	-	-	-	-	-	35.6	-	Ee *et al*., 2009

C – control plant extracts, C1 – control plant extracts exposed to leafminers, F – *H. lixii* F3ST1 inoculated plant extracts, F1 - *H. lixii* F3ST1 inoculated, exposed to leafminer plant extracts, G – *B. bassiana* G1LU3 inoculated plant extracts, G1 - *B. bassiana* G1LU3 inoculated, exposed to leafminer plant extracts, FF - *H. lixii* (F3ST1) fungal extracts, GF - *B. bassiana* G1LU3 fungal extracts.



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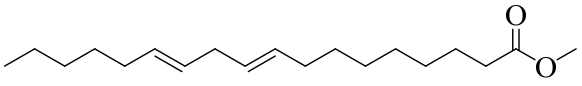

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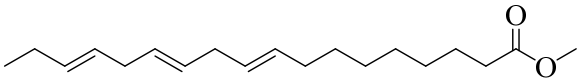

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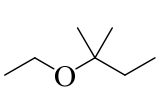

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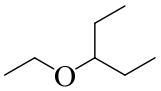

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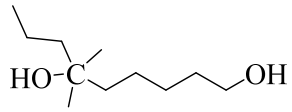

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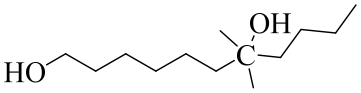

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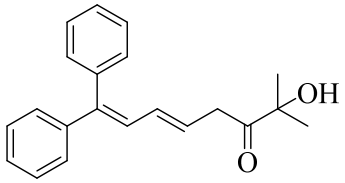

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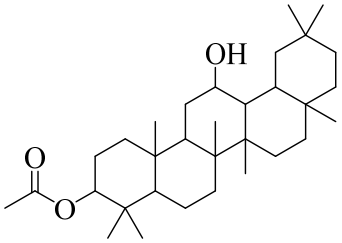

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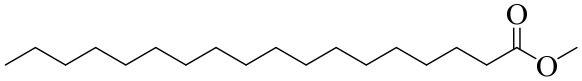

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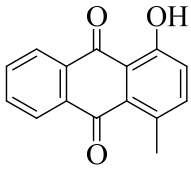

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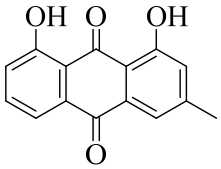


103

The study identified compounds in the methanolic crude plant extracts that have previously been isolated in other plants. Results showing metabolic accumulation as a result of endophyte inoculation are similar to reports by Menjivar *et al*. (2012) with *Fusarium oxysporum* strains in

tomato plants. Reported compounds have previously shown various activities associated with defense against herbivorous insects. The methyl ester of hexadecenoic acid (**92**) has been previously isolated from *Annona muricate* leaves and found to have highly effective anti-fungal properties (Abubacker *et al*., 2013). The methyl ester has also been identified in methanolic extracts of the calyx of green hibiscus and is associated with the anti-microbial activities reported for the plant (GA, 2015). The compound has been identified in several plants showing varying bioactivity including medicinal properties (Othman *et al*., 2015). The compound 9,12-octadecanoic acid, methyl ester (**93**) has been identified as a major component of fruits extracts from hot pepper, the extracts were observed to have insecticidal and acaricidal performance. The test showed that the acid was likely related to the mortality of cabbage looper and the repellency of spider mite (Antonious *et al*., 2007). The methyl ester has also been isolated from *Jatropha curcas* plant roots and shown to have anti-inflammatory activity (Othman *et al*., 2015). The compound has been isolated from several edible plants and various activities assessed (Tahir *et al*., 2018). The compound 9,12,15-octadecatrienoic acid, methyl ester (**94**) is a fatty acid that has been previously isolated from wallich leaves and shown to have anti-microbial activities (Kumar *et al*., 2010). The compound has also been isolated from jiringan seeds (Azizi *et al*., 2006). The linolenic acid has been identified in various phytochemical extracts assessed for bioactivities including analgesic properties (Aboutabl *et al*., 2002; Rammal *et al*., 2014). The compound 2-ethoxy-2-methylbutane (**95**) is an organic compound associated with leaf senescent progression (Xu *et al*., 2011), it has also been characterized as a biogenic emission due to volatilization of compounds contained in plant extracts (Liu *et al*., 2014; Statheropoulos *et al*., 2006). The compound 3-ethoxy pentane, (**96**) is an ether that has been detected in the analysis of insect extracts *Eniosternum delegorguei* (Zvidzai *et al*., 2013). The compound is included in the *denovo*

biosynthesis of chemical defense compounds via shikimic and isoprenoid pathways of sequestering plants as an adaptation to insect attack. It is also an alkaloid intermediate. The compounds 2-pentanol, 2-methyl-2-pentanol (**97**) and 2-hexanol, 2-methyl-2-hexanol (**98**) are aliphatic alkanols which presence with related compounds on plant surfaces contribute to biological processes such as emission of plant volatiles, interference with cuticular permeability and the chemical communications with arthropods (Merk *et al*., 1997). Hexanol compounds have been identified in VOCs of endophytic fungi *Fusarium* sp. (Katoch *et al*., 2017). Pentanol compounds have also been extracted from *P. indicus leaves* and tested as insect attractants (Zheng *et al*., 2014). Components of the odor of plums and apples have also shown the presence of 2-hexanol compounds. The compound 2-hydroxy-2-methyl-8,8-diphenyl-octa-5,7-dien-3-one (**99**) and 2-hydroxy-2methyl compounds have been identified previously in coffee beans as potential defective markers (Toci et al., 2008). The compound acetic acid, 13-hydroxy-4,4,6a,6b,8a,11,11,14b-octamethyldocosahydropicen-3-yl ester (**100**) is associated with metabolic pathways including plant growth regulators (Eberle *et al*., 1986). The methyl ester has been previously isolated from immature green peas leaves (Engvild *et al*., 1978). The compound methyl stearate (**101**) is a hydrophobic fatty acid found in vegetable oils. It has a role as an extracellular metabolite protecting cells from osmotic and oxidative stress for pathogen adaptation. It has been identified as a constituent for insect attractants (Nonami, 2002) and in the chemical composition of mediating ovipositional host plants (Phelan *et al*., 1991). The compounds 1-hydroxy-4-methylanthraquinone (**102**) and 1,8-dihydroxy-3-methyl-9,10-anthracenedione (**103**) are anthraquinones and anthracene derivatives that have been identified previously in plant extracts and marine organisms (Ee *et al*., 2009). Anthraquinones have been assessed for their anti-fungal

and anti-oxidant properties (Ng *et al*., 2003; Rath *et al*., 1995). These compounds have also been tested as potential inhibitors of platelet aggregation (Pmdurtl *et al*., 1989).

4.6 High Pressure Liquid Chromatogram Profile Comparisons

Over 80 peaks were detected in plant and fungal extracts (Figure. 4.16). Several peaks were common in all treatments including peaks with retention times (3.4, 3.5, 4.8, 9.0, 9.9, 10.0, 11.0, 14.4, 16.2, 16.6). Peaks of retention times (3.1, 5.9, 11.4, 13.6, 13.8, 14.0, 14.2, 14.3, 15.0, 15.1, 16.0) were detected in all the plant extracts but were not present in the fungi. Peaks of retention times (9.2, 9.3, 16.7) were detected in both controls only. Peaks of retention times (4.0, 6.8, 7.6, 12.6, 13.3, 13.7, 14.1, 17.2) were detected in the controls before exposure to leafminer insects and (8.3, 8.5, 10.4, 11.6, 15.3) with the insect introduction. All inoculated plants exhibited peaks of retention times (10.5, 12.8, 15.7 and 16.8) after exposure to insects. Plants inoculated with *Hypocrea lixii* produced peaks specific to it at retention times (4.3, 11.8, 15.4, 6.3, 7.7). Plants inoculated with *B. bassiana* produced peaks specific to it at retention times (5.0, 7.5, 8.8, 12.0). Both fungal extracts shared peaks at (2.7, 2.9, 11.1, 11.9). Common peaks found in both plant and fungal extracts showed significant differences in concentration (areas measured in mAUs) between different treatments.

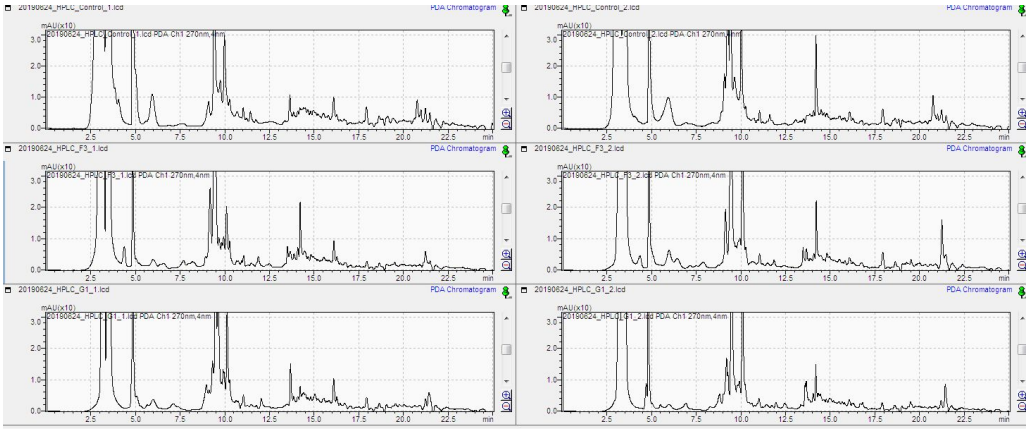


Figure 4.12. Chromatogram overlay data collected from extract run in a Shimadzu HPLC

Metabolite induction was observed at 270 nm in form of chromatograms depicting compound peak differences between the controls and the different treatments. Variations in the peaks and absorbances of the crude extracts profiles is suggested to be due to successful induction of defense compounds against insects as a result of fungal inoculation (Vega, 2008). Metabolite accumulation of certain compounds is also exhibited which corroborates with the findings of (Menjivar *et al*., 2012). In some instances, the concentrations of the compounds were higher in the controls compared to the treatments and this can be explained as a suppression of production of certain compounds by the endophytic fungi in the host plant.

4.7 Effects of Plant Extracts on Pupation of 2nd Instar Leafminer Larvae

Fewer leafminer pupae were produced in larvae dipped in plant extracts compared to the controls (Figure 4.9). There were significant differences in the pupation from 2nd instar leafminer larvae dipped into methanol extracts (F = 4.33, Df = 6, P = 0.03). For example, the percentage pupation of larvae dipped in *H. Lixii* inoculated plant methanol extracts (F31M) were 49.4% while the percentage pupation of larvae dipped in *B. Bassiana* inoculated plant methanol extract (G11M) was 52%. However, dichloromethane extracts did not show significant differences in the pupation of 2nd instar leaf miner dipped in dichloromethane extracts. The percentage pupation of larvae dipped in fungal extracts also did not show much variation from the controls. Larvae dipped in *B. Bassiana* inoculated exposed plant methanol extract (G12M) produced the least number of pupae (35%). Methanol and dichloromethane extracts did not show significant differences in their effects in pupation of the larvae (F = 0.613, Df = 8, P = 0.7523). There was also no significant interaction between the treatments and the solvents (F = 0.613, Df = 8, P = 0.7523). Exposure of the plants to *Liriomyza* adult flies before extraction did not show variation in the extract's effects to pupation of the larvae (F = 0.331, Df = 6, P = 0.9077).

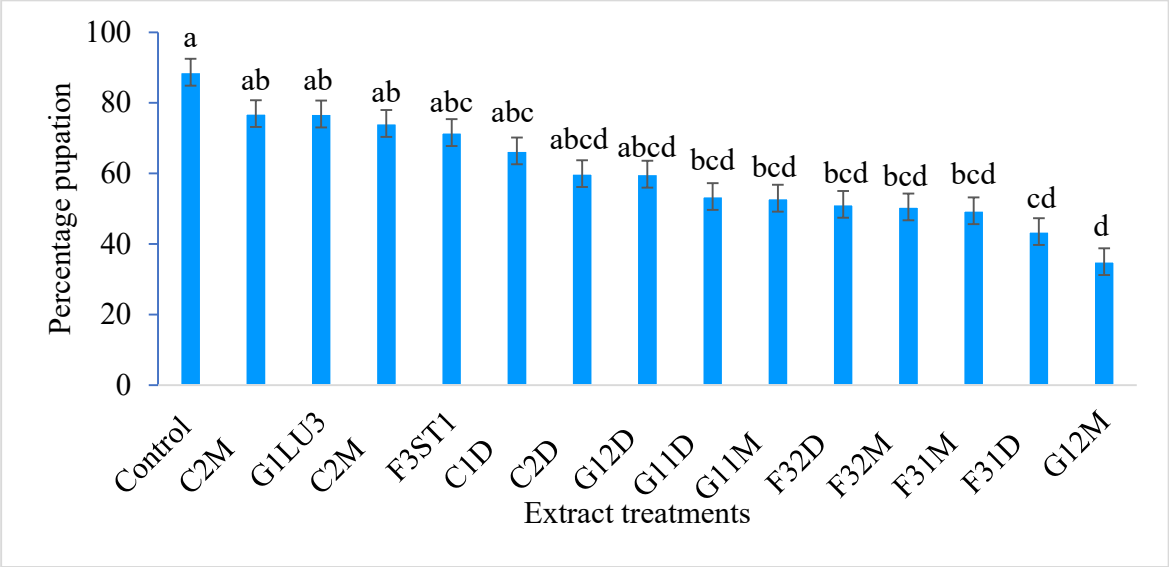


Figure 4.13. Effect of *Phaseolus vulgaris* methanol and dichloromethane plant extracts on the percentage pupation of 2nd instar LMF larvae. Mean pupation of 2nd instar leafminer larvae dipped in *Phaseolus vulgaris* plant extracts colonized with fungal isolates *Hypocrea lixii* F3ST1 and *Beauveria bassiana* G1LU3.

Bars denote means ± one standard error at 95 % CI (p = 0.05). Means with the same letter are not significantly different BY LSD test at 95% CI (p = 0,05). Control, Tween 80 solution (Control) (G1LU3) *B. bassiana* fungal extract, (F3ST1) *H. lixii* fungal extract, Control plants methanol extract (C1M), Control plants exposed to insects methanol extract (C2M), Control plants dichloromethane extract (C1D), Control plants exposed to insects dichloromethane extract (C2D), F3ST1 inoculated plants methanol extract (F31M) F3ST1 inoculated plants exposed to insects methanol extracts (F32M), F3ST1 inoculated plants dichloromethane extract (F31D), F3ST1 inoculated plants exposed to insects dichloromethane extracts (F32D), G1LU3 inoculated plants methanol extract (G11M), G1LU3 inoculated plants exposed to insects methanol extracts (G12M), G1LU3 inoculated plants dichloromethane extract (G11D), G1LU3 inoculated plants exposed to insects dichloromethane extracts (G12D).

4.8 Effects of Plant Extracts on Emergence of Leaf miner Adult Flies

Higher numbers of adult flies emerged from the controls compared to the extract treatments. (Figure 4.10). There were significant differences in the adult emergence from larvae dipped into methanol extracts (F = 5.4, Df = 6, P = 0.02). For example, the number of adult flies that emerged from larvae dipped in Control plants methanol extract (C1M) was 31% while the number of adult flies that emerged from larvae dipped in *H. Lixii* inoculated plant methanol extracts (F31M) was 9.2%. However, dichloromethane extracts did not show no significant differences in the emergence of 2nd instar leaf miner dipped in dichloromethane extracts and the least number of flies emerged from the pupae of larvae treated in *H. Lixii* inoculated exposed plant dichloromethane extracts (F32D) compared to the rest. However, emergence from larvae dipped in fungal extracts did not show much variation from the controls. Methanol and dichloromethane extracts did not show significant differences in their effects in emergence (F = 0.446, Df = 8, P = 0.871). There was also no significant interaction between the treatments and the solvents (F = 0.446, Df = 8, P = 0.871). Exposure of the plants to *Liriomyza* adult flies before extraction did not show variation in the extract's effects to emergence (F = 0.831, Df = 6, P = 0.568). Most flies in the controls emerged successfully while flies treated in extracts failed to emerge because they were stuck in the pupal cases (Figure. 4.15).

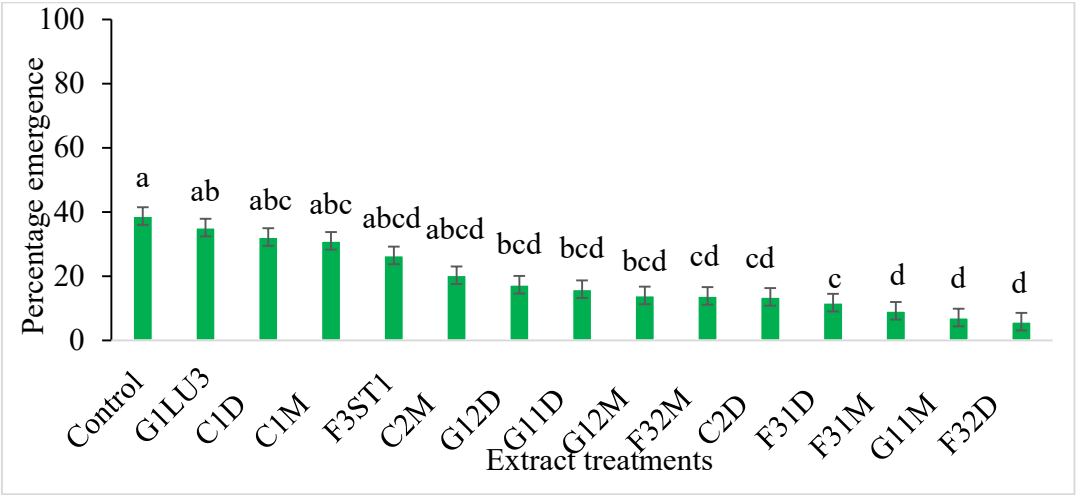


Figure 4.14. Effect of *Phaseolus vulgaris* plant extracts on adult emergence of 2nd instar LMF larvae. Mean emergence of 2nd instar leaf miner larvae dipped in *Phaseolus vulgaris* plant extracts colonized with fungal isolates *Hypocrea lixii and Beauveria bassiana* (F3ST1 and (G1LU3).

Bars denote means ± one standard error at 95 % CI (p = 0.05). Means with the same letter are not significantly different BY LSD test at 95% CI (p = 0,05). Control, Tween 80 solution (Control) (G1LU3) *B. bassiana* fungal extract, (F3ST1) *H. lixii* fungal extract, Control plants methanol extract (C1M), Control plants exposed to insects methanol extract (C2M), Control plants dichloromethane extract (C1D), Control plants exposed to insects dichloromethane extract (C2D), F3ST1 inoculated plants methanol extract (F31M) F3ST1 inoculated plants exposed to insects methanol extracts (F32M), F3ST1 inoculated plants dichloromethane extract (F31D), F3ST1 inoculated plants exposed to insects dichloromethane extracts (F32D), G1LU3 inoculated plants methanol extract (G11M), G1LU3 inoculated plants exposed to insects methanol extracts (G12M), G1LU3 inoculated plants dichloromethane extract (G11D), G1LU3 inoculated plants exposed to insects dichloromethane extracts (G12D).

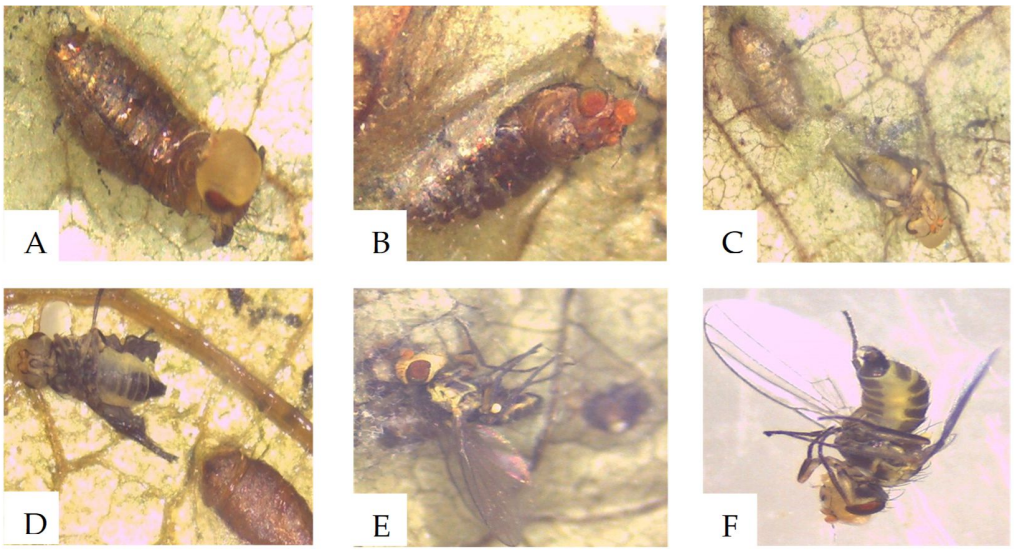
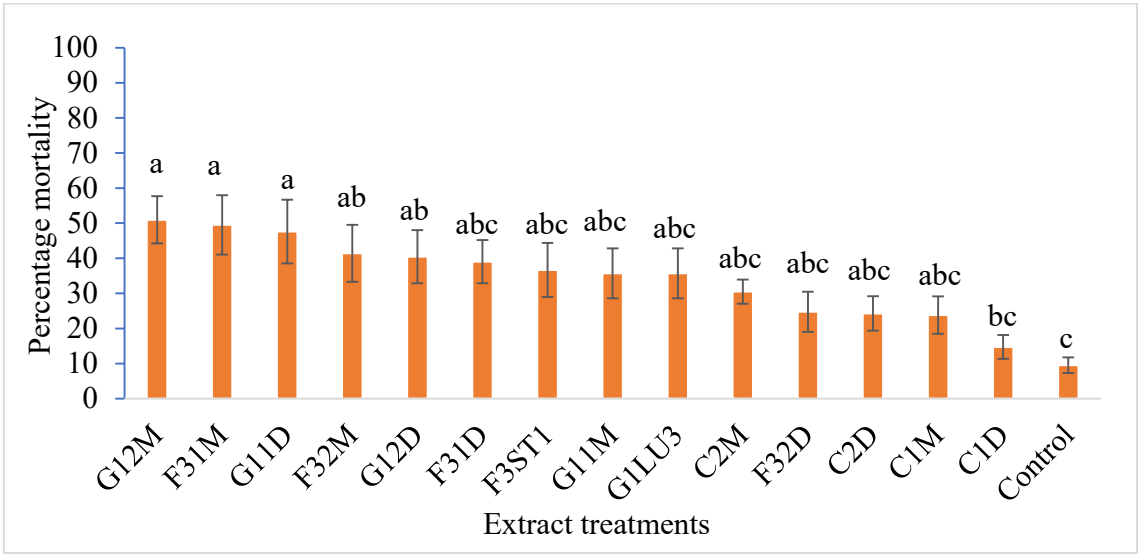


Figure 4.15. Effects of fungi colonized Phaseolus vulgaris plant extracts on Liriomyza huidobrensis pupae, A - insects were stuck (whole part of the body) inside their pupal cases, B – insects died inside the pupae, C – Insects failed to emerge, D, E, F – Insects died before they could fully emerge. (Source; Olivia Ngeno, 2018)

4.9 Effects of Plant Extracts on 1st Instar FAW Larvae

The survival of 1st instar Fall armyworm larvae dipped in inoculated plant extracts was significantly reduced ($F = 8.466$, $Df = 13$, $P < 0.0001$) as compared to the controls (Figure.4.12). Mortality of up to 70% was observed in the treatments within 7days compared to 16% in the controls. The mortality of 1st instar FAW larvae dipped in inoculated plant extracts varied among treatments ($F = 8.466$, $Df = 13$, $P < 0.0001$). For example, at 7days after dipping, mean mortality was 70% for *H. Lixii* inoculated plant methanol extracts (F31M) and 43% for *B. Bassiana* inoculated plant methanol extract (G11M). There were significant differences in the mortality of 1st instar Fall Armyworm dipped in methanol extracts ($F = 3.7$, $Df = 8$, $P = 0.001$). There were also significant differences in the mortality of 1st instar FAW dipped in dichloromethane extracts ($F = 4.01$, $Df = 8$, $P = 0.008$). There were significant differences in the mortality of 1st instar FAW

dipped in non-exposed plant extracts (F = 6.01, Df= 8, P = 0.001) and exposed plant extracts (F = 5.47, Df = 8, P = 0.002).



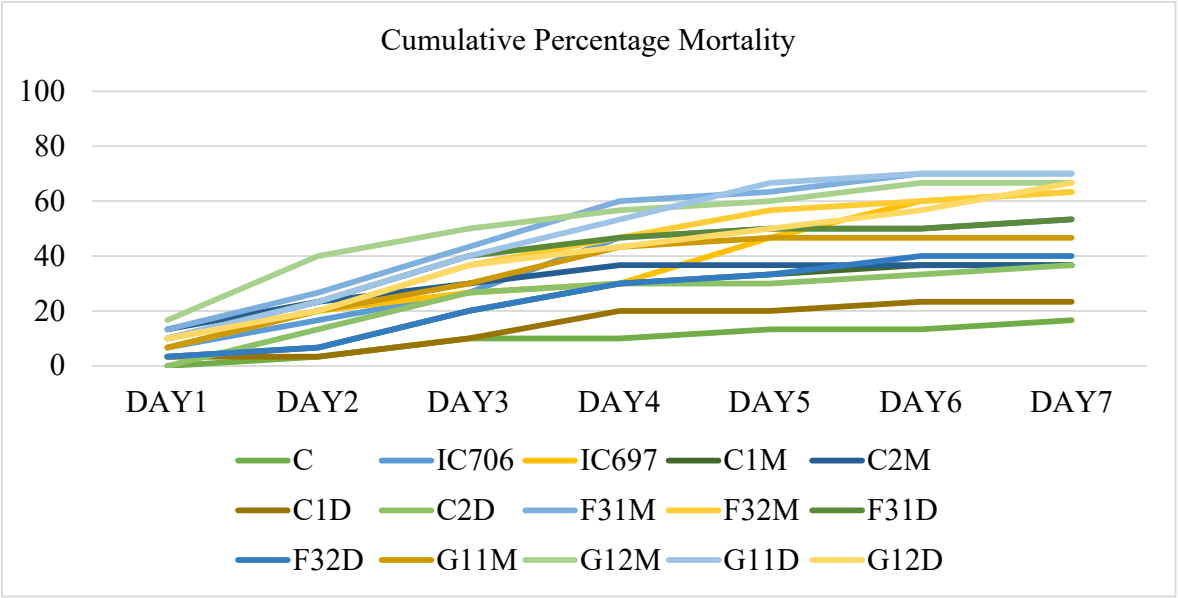


Figure 4.16. Effect of *Phaseolus vulgaris* plant extracts on the mortality of 1st instar FAW larvae. Mean mortality of 1st instar Fall Armyworm larvae dipped in *Phaseolus vulgaris* plant extracts colonized with fungal isolates *Hypocrea lixii* F3ST1 and *Beauveria bassiana* G1LU3.
Bars denote means ± one standard error at 95 % CI (p = 0.05). Means with the same letter are not significantly different BY LSD test at 95% CI (p = 0,05). Control, Tween 80 solution (Control)

(G1LU3) *B. bassiana* fungal extract, (F3ST1) *H. lixii* fungal extract, Control plants methanol extract (C1M), Control plants exposed to insects methanol extract (C2M), Control plants dichloromethane extract (C1D), Control plants exposed to insects dichloromethane extract (C2D), F3ST1 inoculated plants methanol extract (F31M) F3ST1 inoculated plants exposed to insects methanol extracts (F32M), F3ST1 inoculated plants dichloromethane extract (F31D), F3ST1 inoculated plants exposed to insects dichloromethane extracts (F32D), G1LU3 inoculated plants methanol extract (G11M), G1LU3 inoculated plants exposed to insects methanol extracts (G12M), G1LU3 inoculated plants dichloromethane extract (G11D), G1LU3 inoculated plants exposed to insects dichloromethane extracts (G12D).

Methanolic extracts of *B. bassiana* inoculated plants was the most lethal at 4.42 days for the corrected mortality of 1st instar FAW larvae compared to controls which was the least lethal with a survival of up to 17days.

Table 4.7. Median lethal time (LT_{50}) 7 days post treatment of 1st instar FAW larvae dipped in plant extracts

Extracts	Treatments	LT_{50} (Days) (95% FL)
Fungal extracts	*H. lixii* F3ST1	6.24 (6.41-6.07)
	B. bassiana G1LU3	6.73 (6.95-6.51)
Plant extracts	C1M	9.36 (9.83-8.89)
	C2M	16.47 (19.73-13.21)
	C1D	17.06 (19.43-14.69)
	C2D	10.79 (11.56-10.02)
	F31M	4.50 (4.62-4.38)
	F32M	5.58 (5.72-5.44)
	F31D	6.85 (6.89-6.27)
	F32D	8.70 (9.07-8.33)
	G11M	7.87 (8.25-7.49)
	G12M	4.42 (4.59-4.25)
	G11D	4.72 (4.83-4.61)
	G12D	5.76 (5.91-5.61)

All extracts from endophytically colonized plants showed effects on the pupation and emergence of leafminer larvae. Adult flies failed to emerge from their pupal skins in the pupae that were affected by extract treatment. Similar results were reported on LMF exposed to plants inoculated with *H. lixii* F3ST1 and *B. bassiana* G1LU3 by Akutse *et al*., (2013, 2014). In addition to pupal mortality, plant extracts inoculated with fungal endophytes reduced survival of the FAW larvae. Methanol extracts showed overall higher effects compared to dichloromethane extracts. Exposure of plants to insects prior to extraction did not show variation in its effects on pupation and emergence of LMF larvae and mortality of FAW larvae. Mild effects of the extracts against the larvae and pupae points to the use of crude extracts, further work is recommended on isolation and testing of individual compounds to identify the active ingredient.

5 CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

This study demonstrates the potential of endophytic fungi *H. lixii* F3ST1 and *B. bassiana* G1LU3 to induce systemic chemical resistance in the common bean *P. vulgaris* against the leafminer flies and Fall Armyworm.

i. *Phaseolus vulgaris* was successfully colonized by *H. lixii* F3STI and *B. bassiana* G1LU3 through seed inoculation.

ii. Determination of qualitative differences in volatile emissions and liquid extracts between the endophyte colonized plants and non-colonized plants elucidates a possible systemic infection mechanism of the inoculated fungi against the pest.

iii. The active defense compounds extracted from endophytically-colonized *P. vulgaris* plants caused toxicant effects to LMF and FAW larvae and could potentially be used to manage both invasive pests.

5.2 Recommendations

i. Successful colonization of *P. vulgaris* by *H. lixii* and *B. bassiana* allows for recommendation on further research to be done to improve the adoption and application techniques of these fungal endophytes in pest management programs.

ii. Identified volatile compounds were largely terpenes with previous histories as defense compounds for various plants against both microbial pathogens and herbivorous insects. However, further studies are warranted to identify the specific defense compounds causing toxic effects to the pests and validate the results under field conditions. Further work on the confirmation of detected compounds with standards and other analytical methods is also recommended.

iii. The crude extract was tested and found to have effects on the larvae of both the leafminer and the Fall Armyworm. Liquid extracts reduced significantly the pupation and emergence of LMF and the survival of FAW larvae *in vitro*. However, the minimal severity against pupation and mortality of the insects, could be because of the low concentrations of key compounds in the overall extract. This warrants further research on the effects of the individual pure compounds that have been identified against the pests in addition to the dose response bioassays.

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6 APPENDICES

6.1 Mass spectra of identified compounds

Appendix 1: Mass spectrum of meta cresol

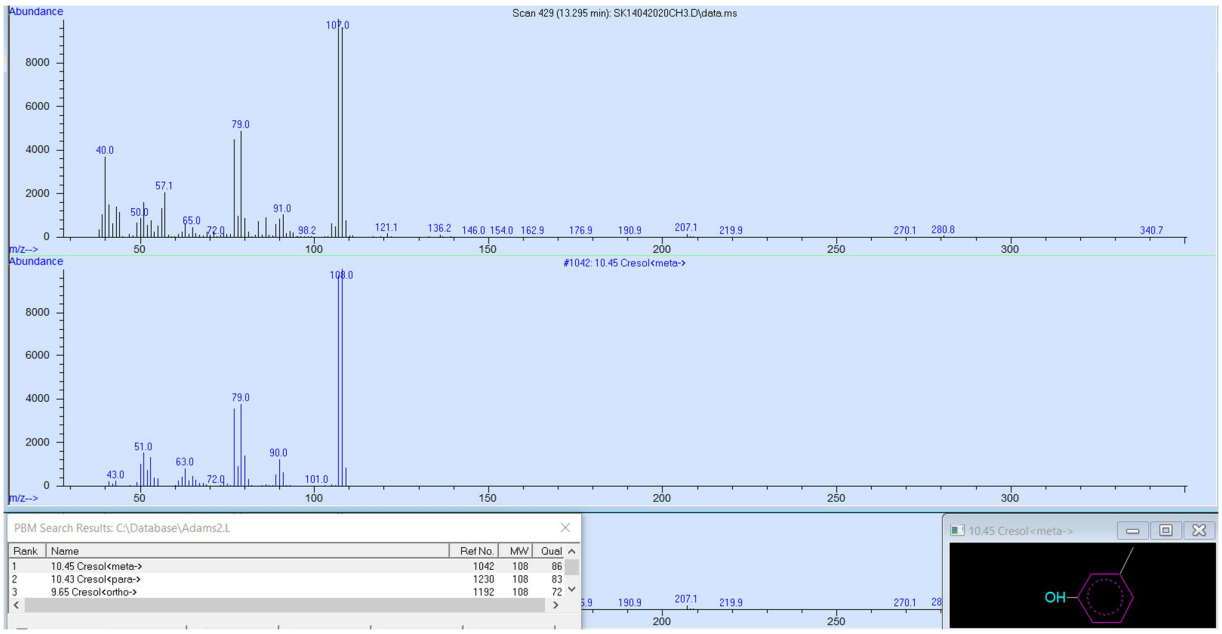

Figure 6.1 Mass spectrum of meta cresol

Appendix 2: Mass spectrum of para cresol

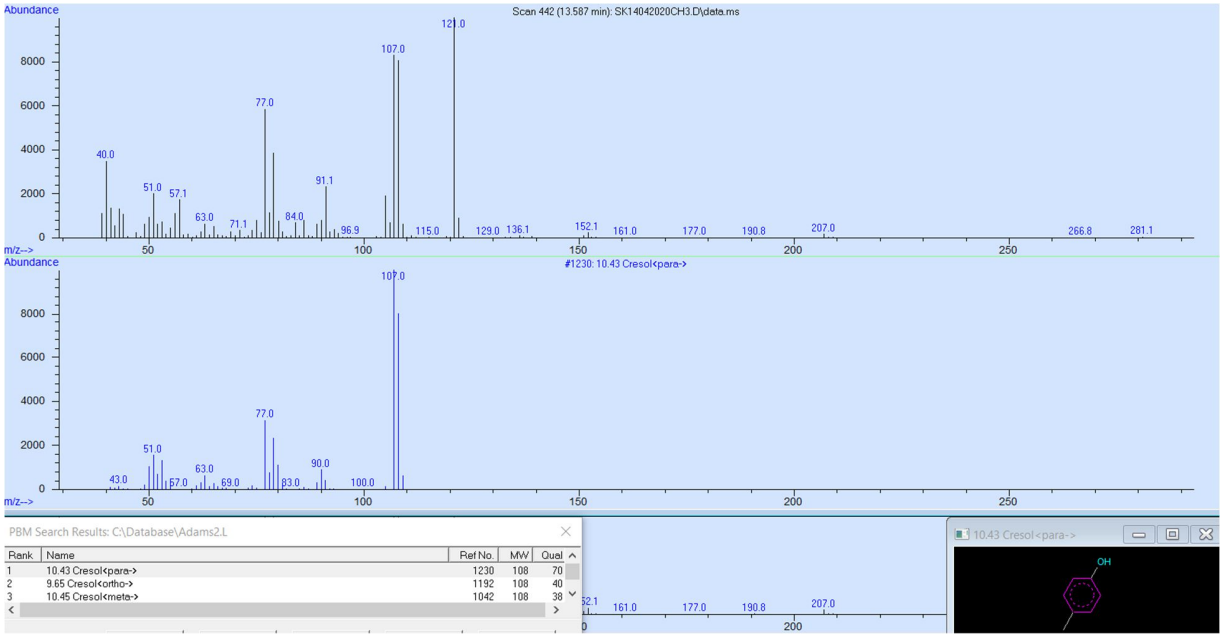

Figure 6.2 Mass spectrum of para cresol

Appendix 3: Mass spectrum of phellandrene

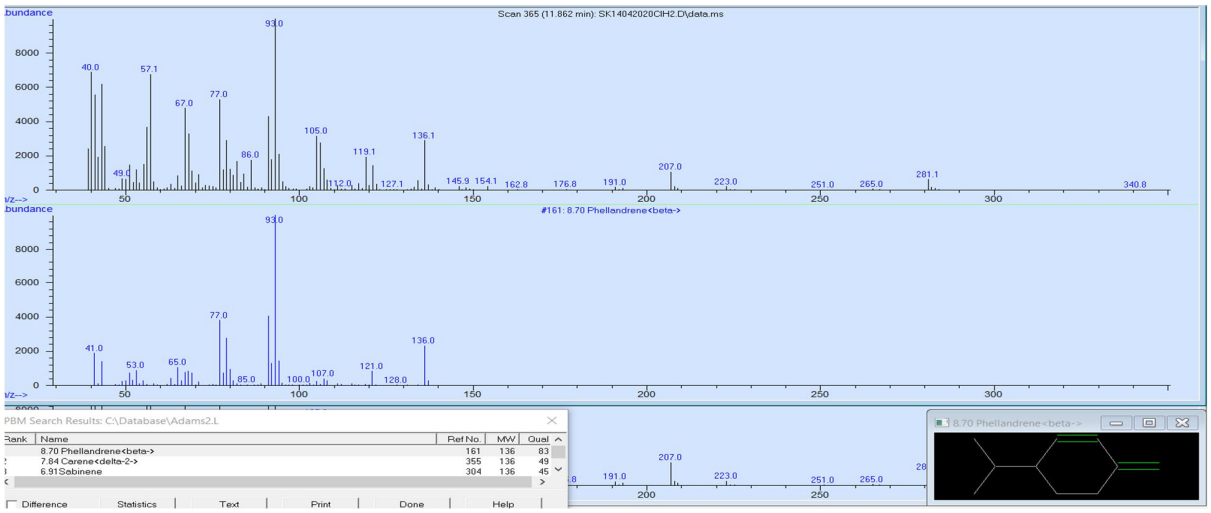

Figure 6.3 Mass spectrum of phellandrene

Appendix 4: Mass spectrum of terpinene

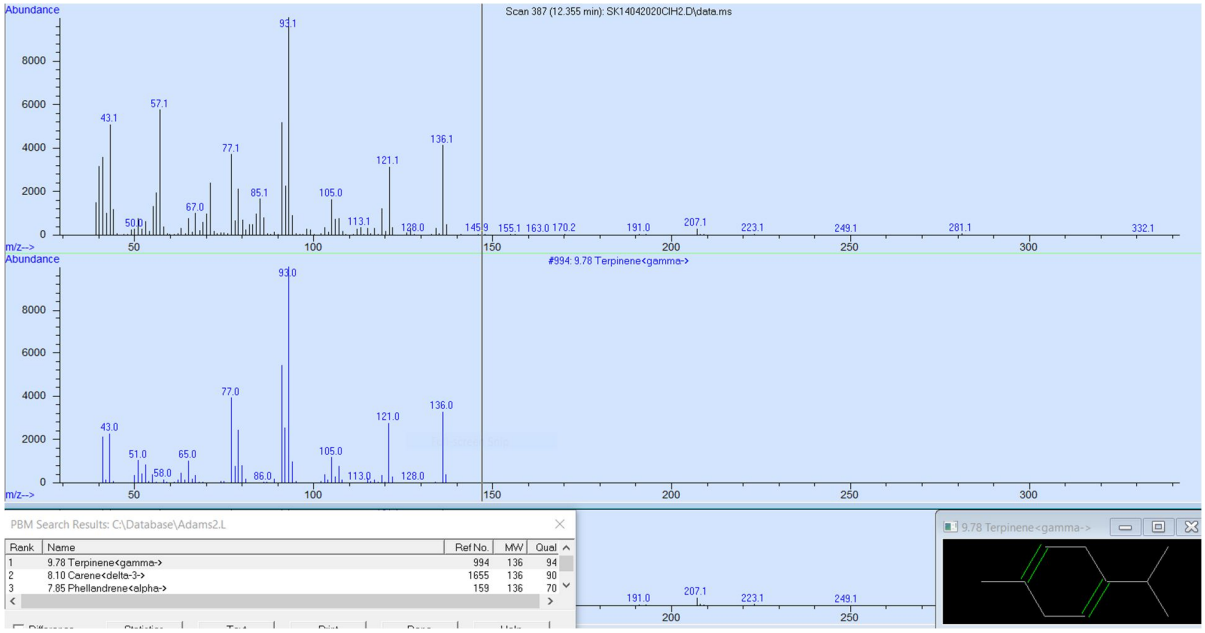

Figure 6.4 Mass spectrum of terpinene

Appendix 5: Mass spectrum of sabinene hydrate cis

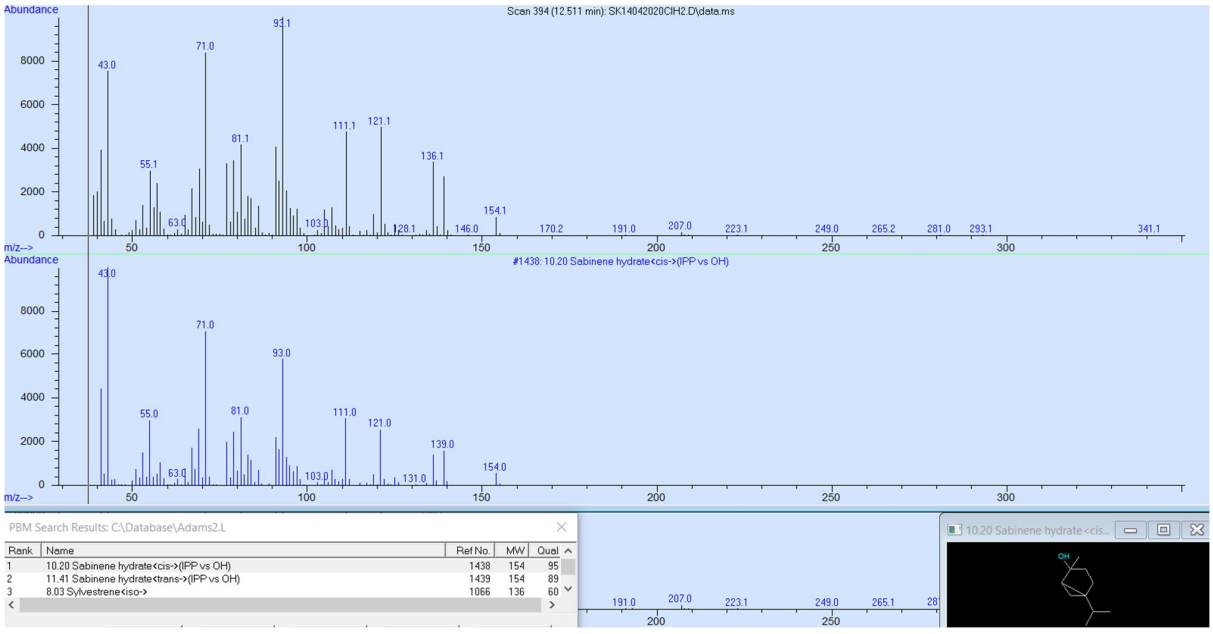

Figure 6.5 Mass spectrum of sabinene hydrate cis

Appendix 6: Mass spectrum of benzaldehyde dimethyl acetal

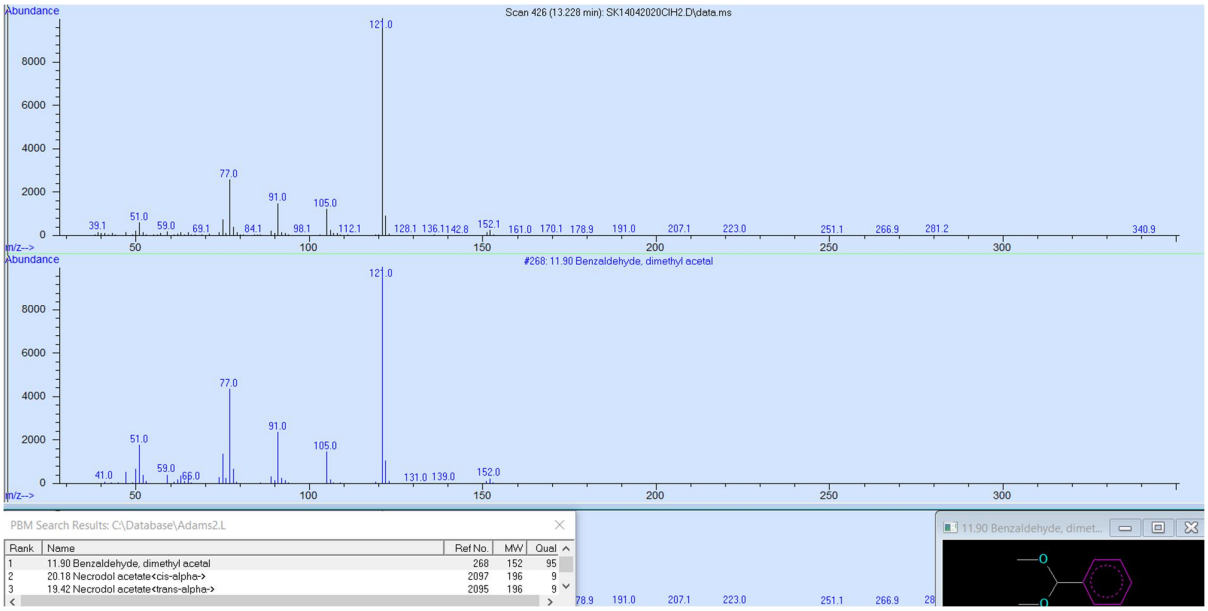

Figure 6.6 Mass spectrum of benzaldehyde dimethyl acetal

Appendix 7: Mass spectrum of camphor

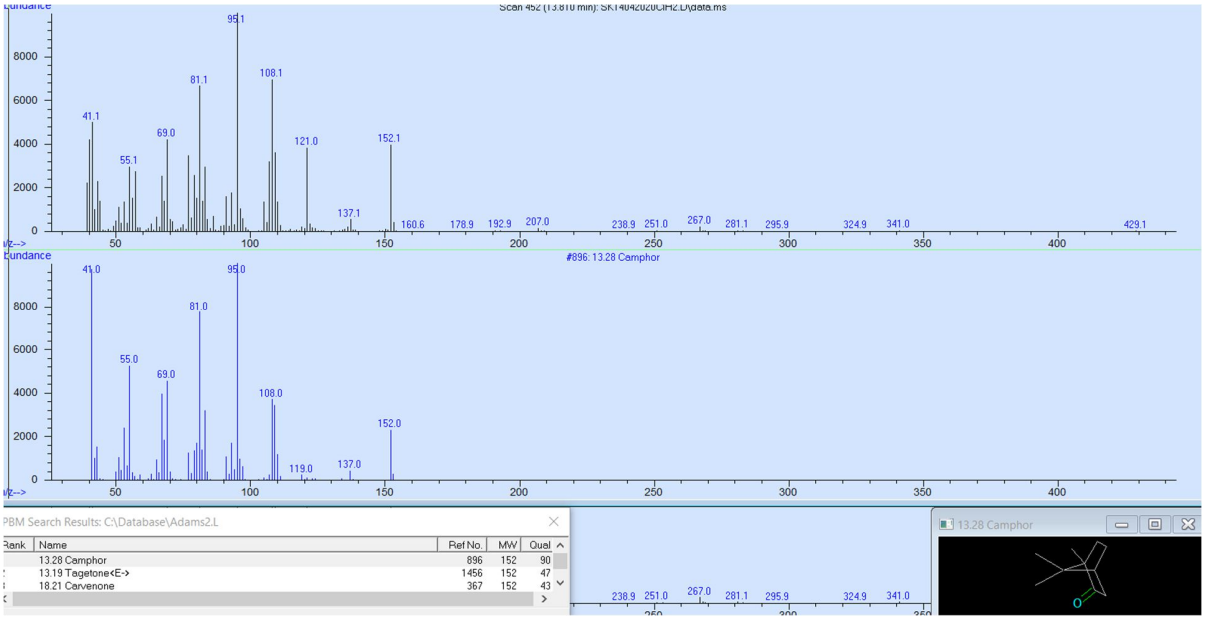

Figure 6.7 Mass spectrum of camphor

Appendix 8: Mass spectrum of terpinene-4-ol

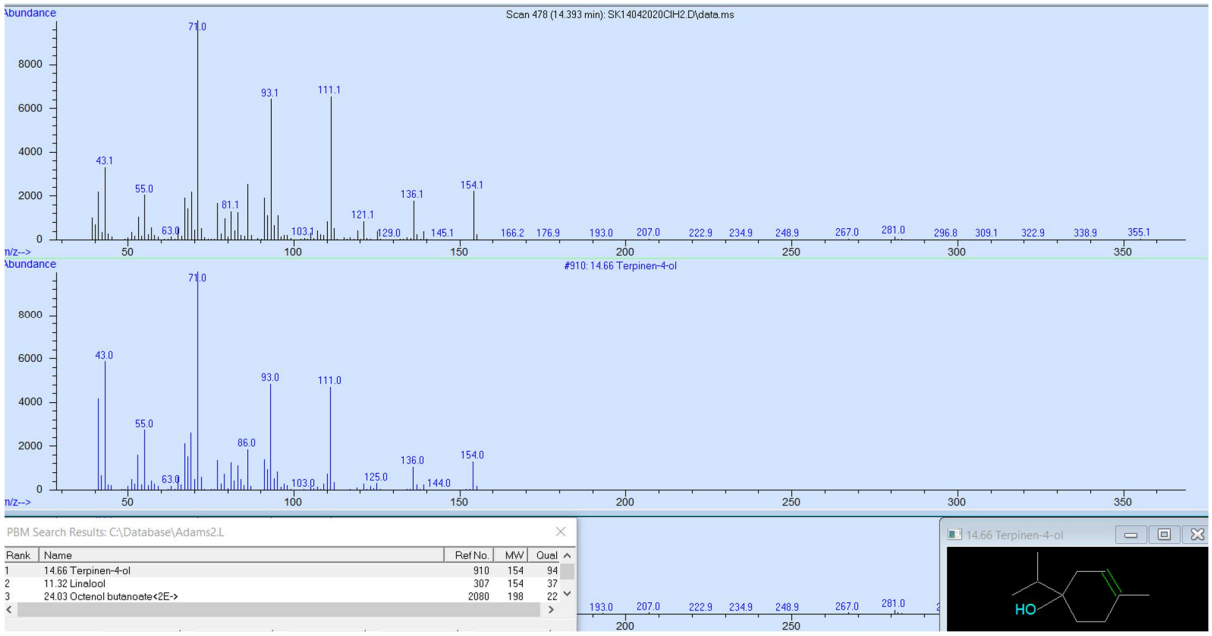

Figure 6.8 Mass spectrum of terpinene-4-ol

Appendix 9: Mass spectrum of heneicosane

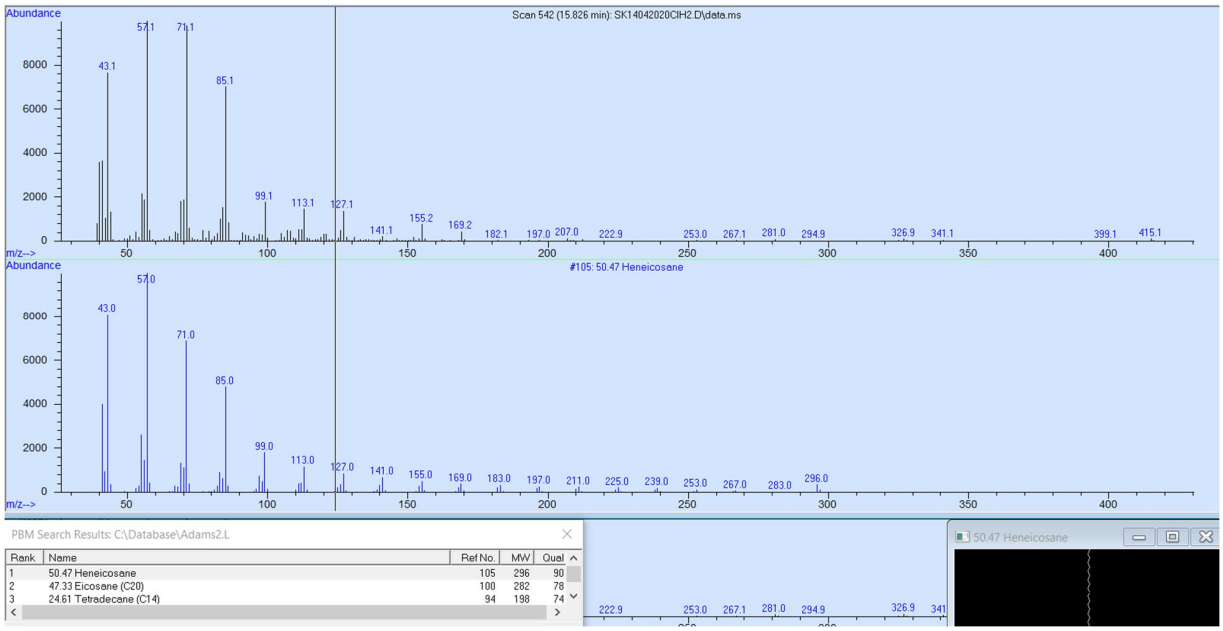

Figure 6.9 Mass spectrum of heneicosane

Appendix 10: Mass spectrum of caryophyllene

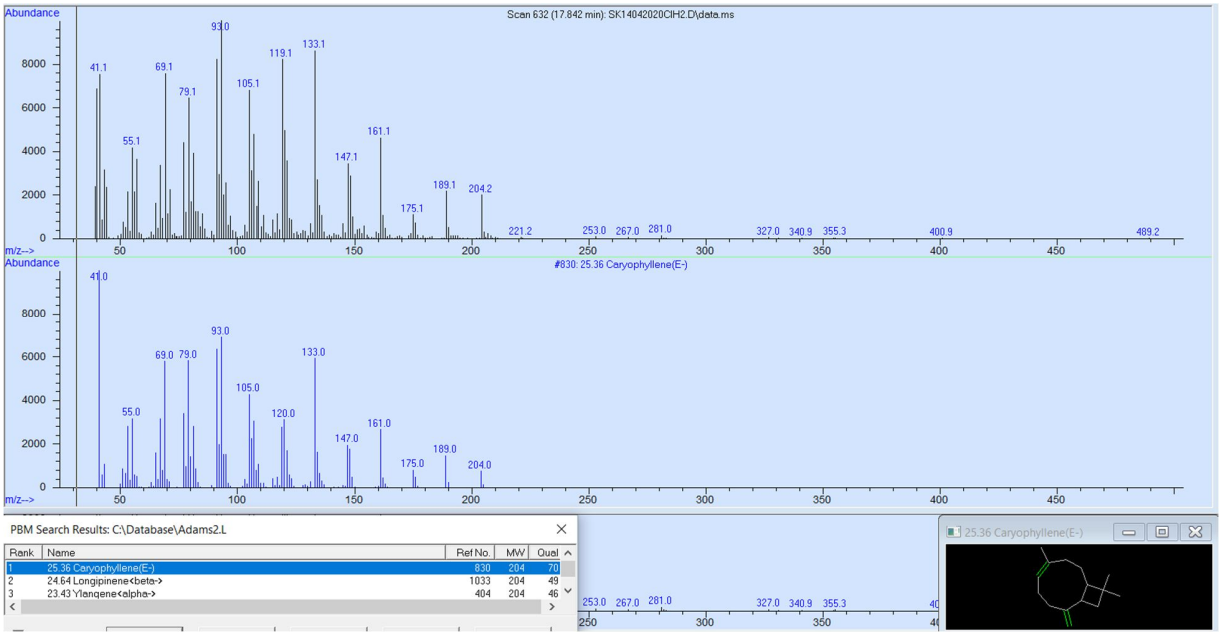

Figure 6.10 Mass spectrum of caryophyllene

Appendix 11: Mass spectrum of butylated hydroxytoluene

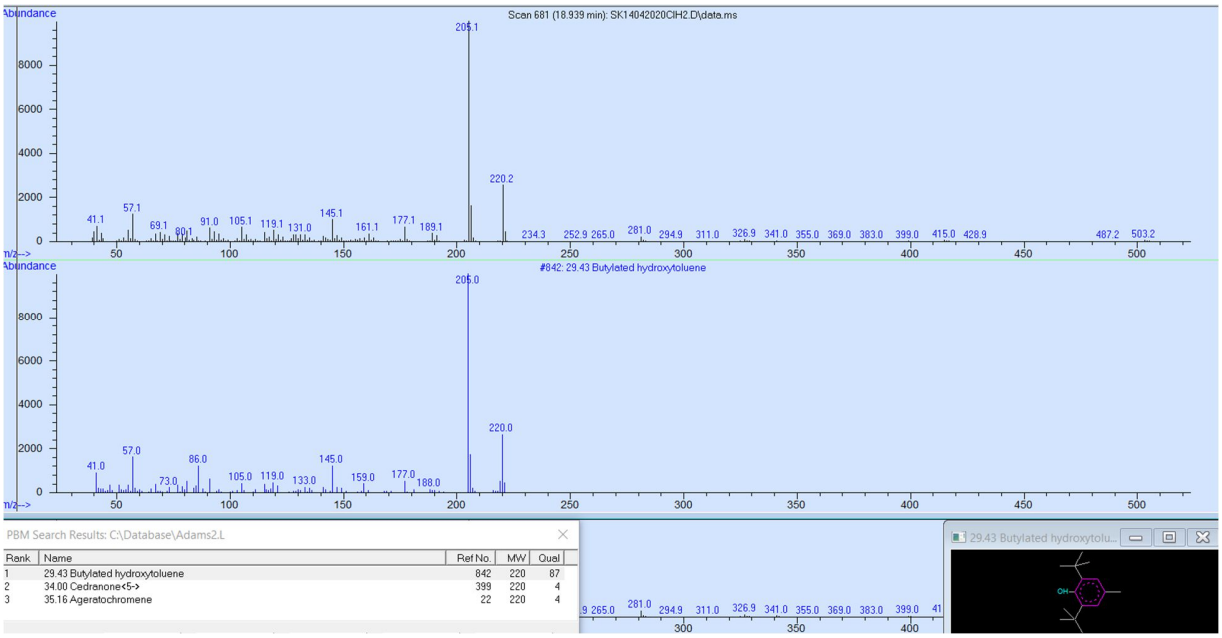

Figure 6.11 Mass spectrum of butylated hydroxytoluene

Appendix 12: Mass spectrum of tetramethyl cyclohexane

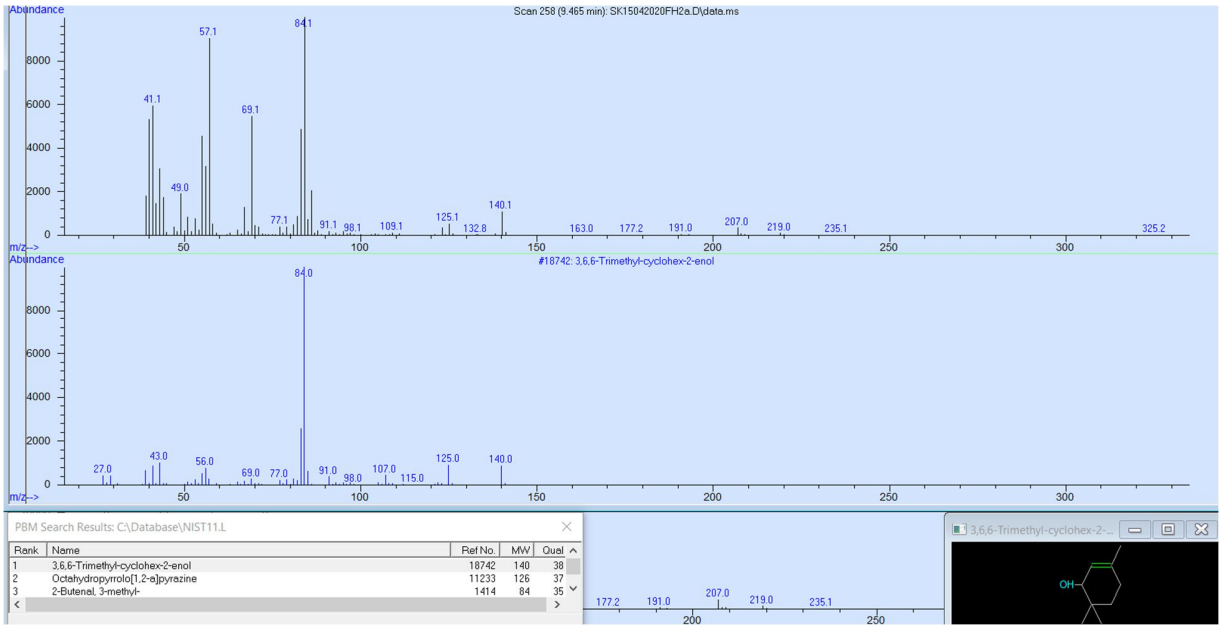

Figure 6.12 Mass spectrum of tetramethyl cyclohexane

Appendix 13: Mass spectrum of phenol

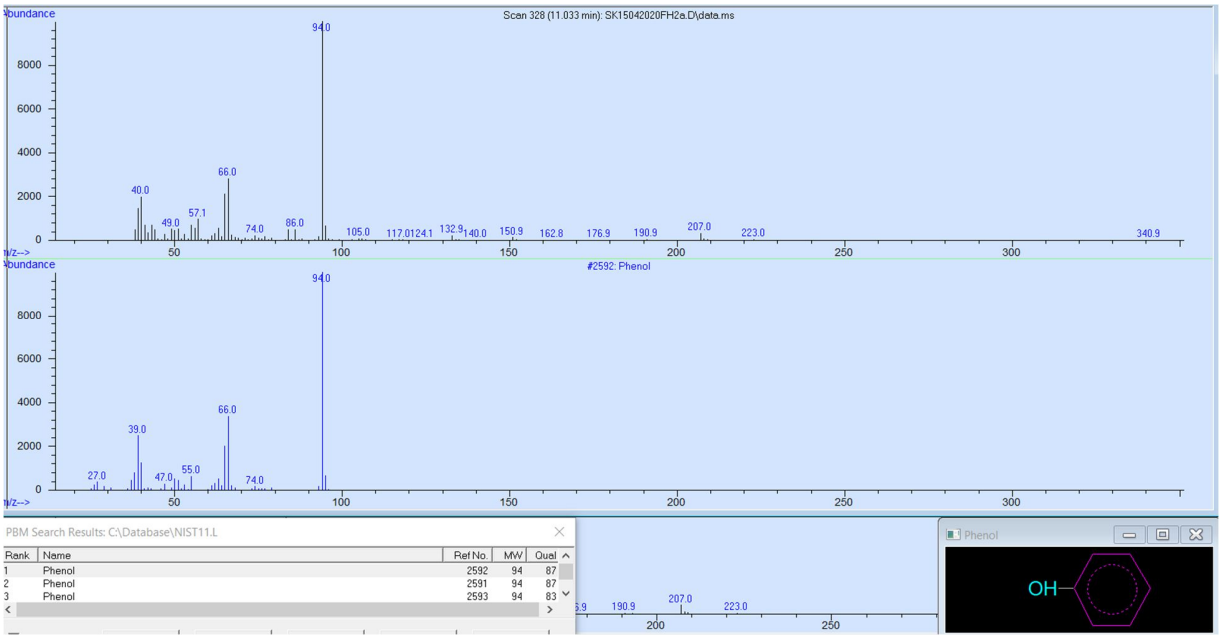

Figure 6.13 Mass spectrum of phenol

Appendix 14: Mass spectrum of benzyl alcohol

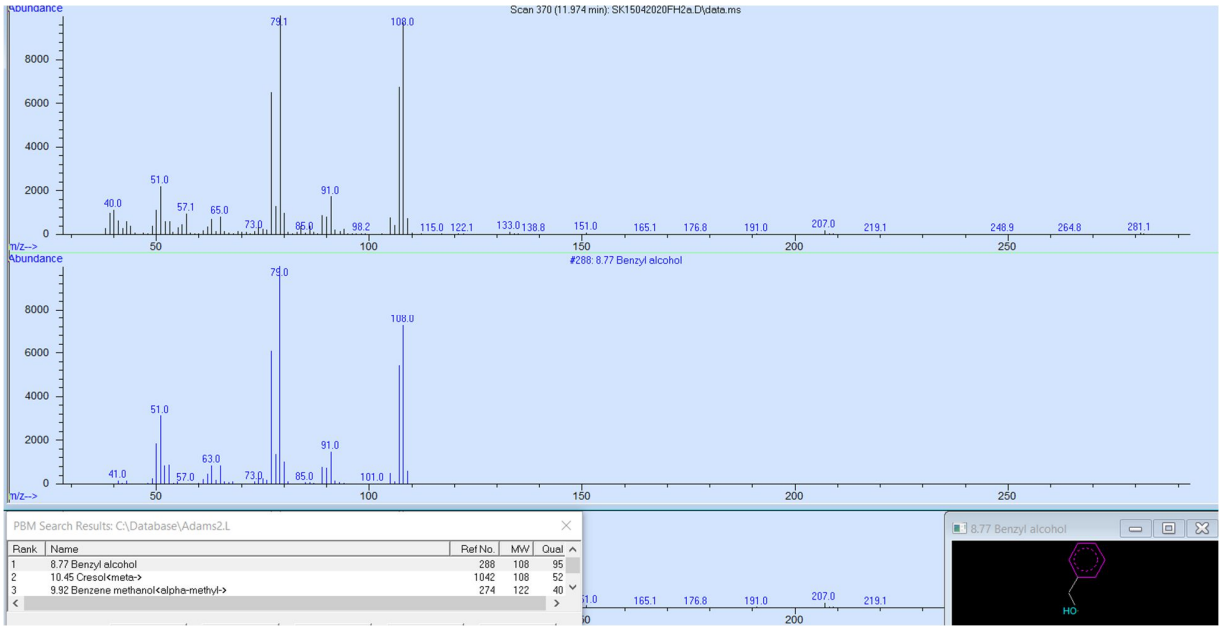

Figure 6.14 Mass spectrum of benzyl alcohol

Appendix 15: Mass spectrum of 3-methylanisole

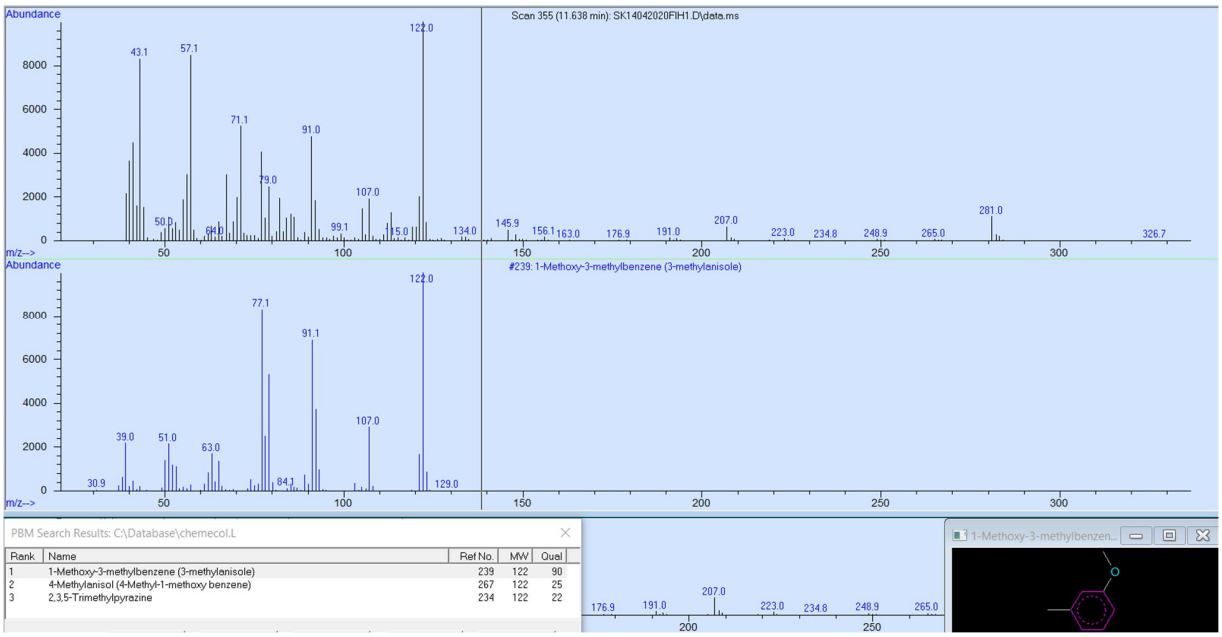

Figure 6.15 Mass spectrum of 3-methylanisole

Appendix 16: Mass spectrum of ocimene

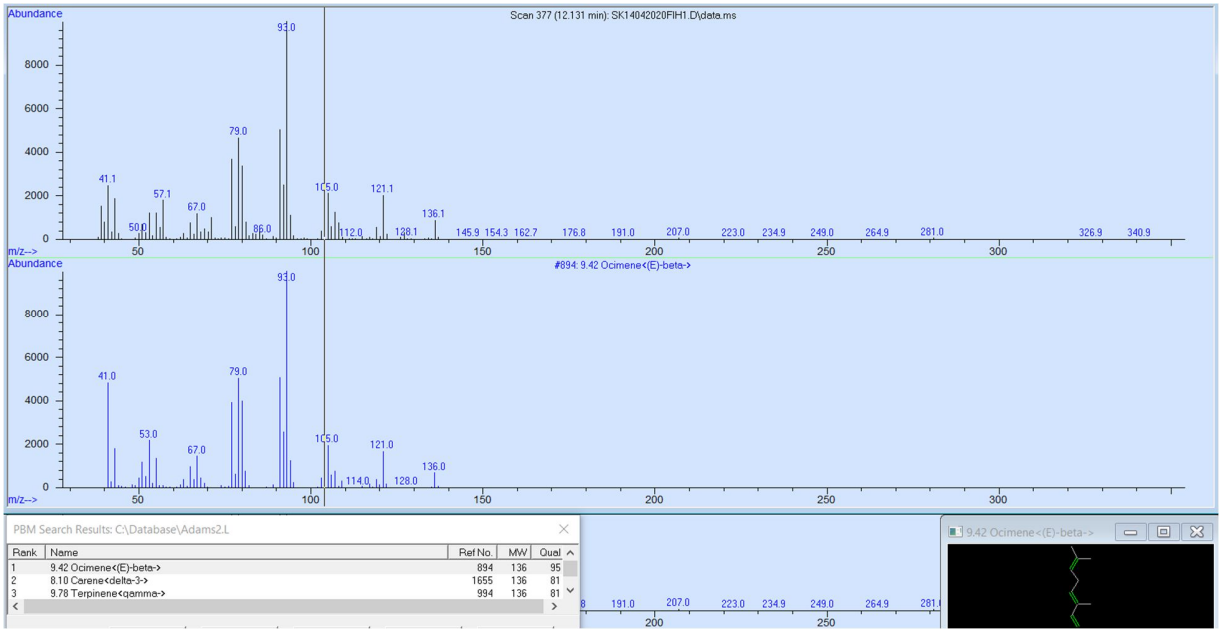

Figure 6.16 Mass spectrum of ocimene

Appendix 17: Mass spectrum of naphthalene

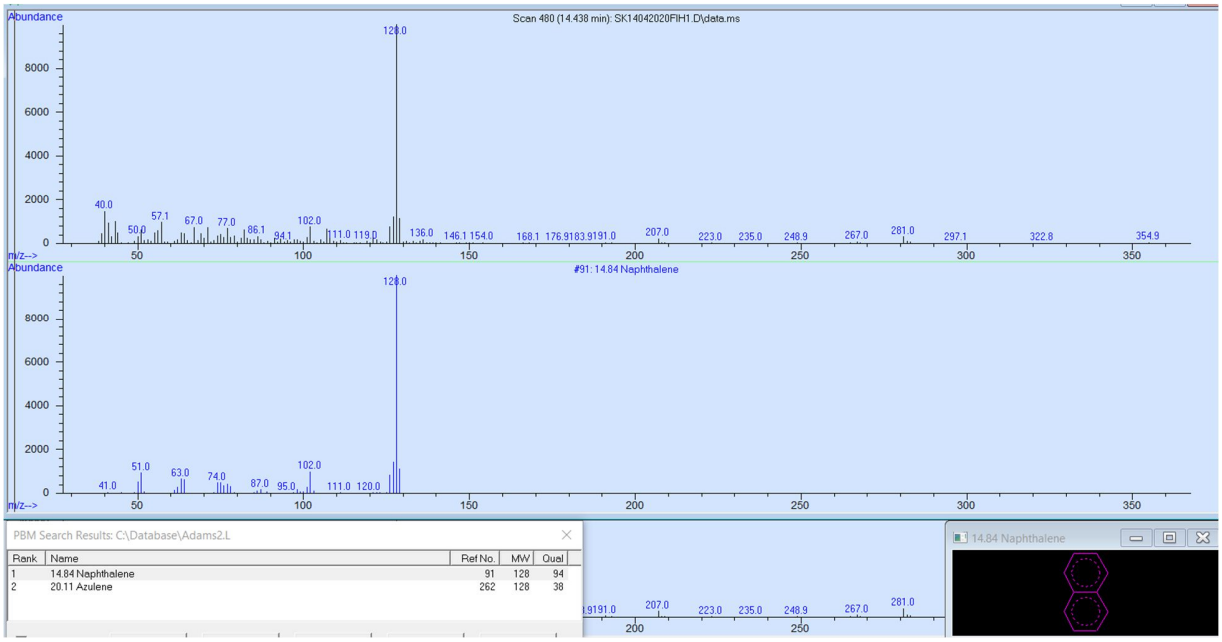

Figure 6.17 Mass spectrum of naphthalene

Appendix 18: Mass spectrum of methyl salicylate

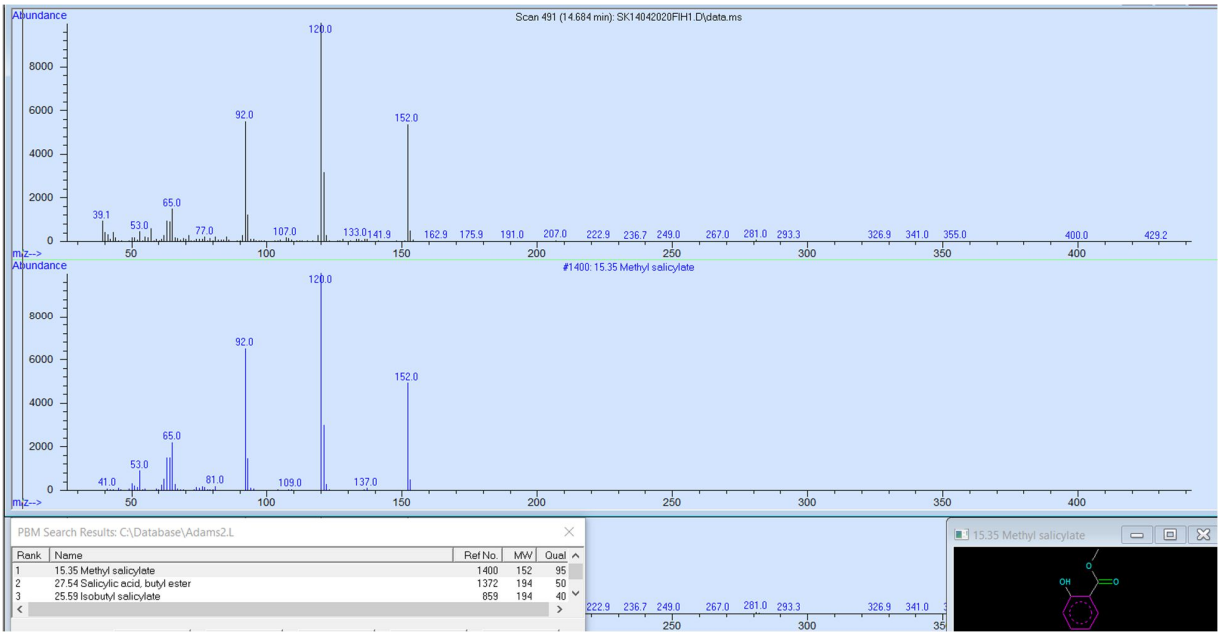

Figure 6.18 Mass spectrum of methyl salicylate

Appendix 19: Mass spectrum of heptadecane

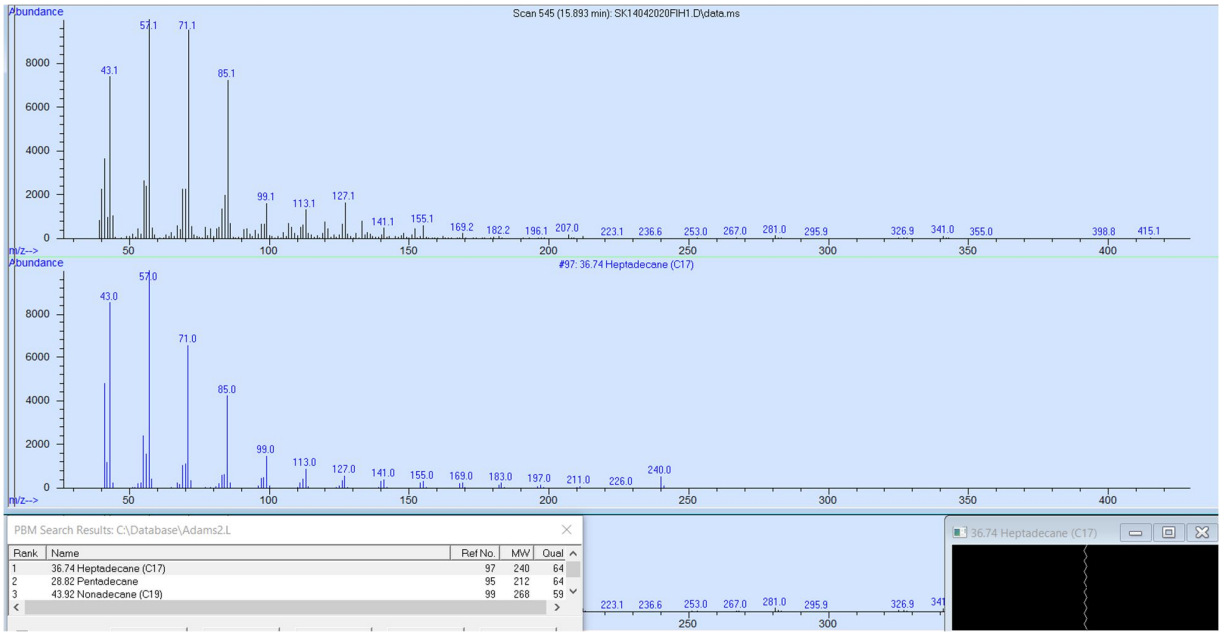

Figure 6.19 Mass spectrum of heptadecane

Appendix 20: Mass spectrum of tetradecane

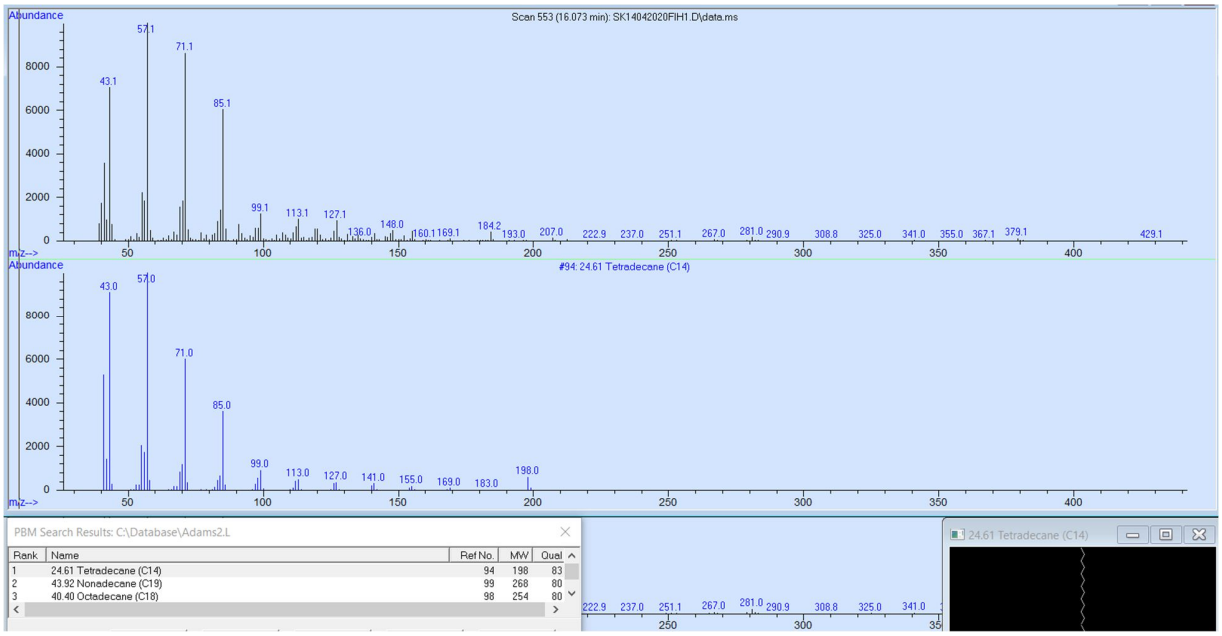

Figure 6.20 Mass spectrum of tetradecane

Appendix 21: Mass spectrum of propyl butanoate

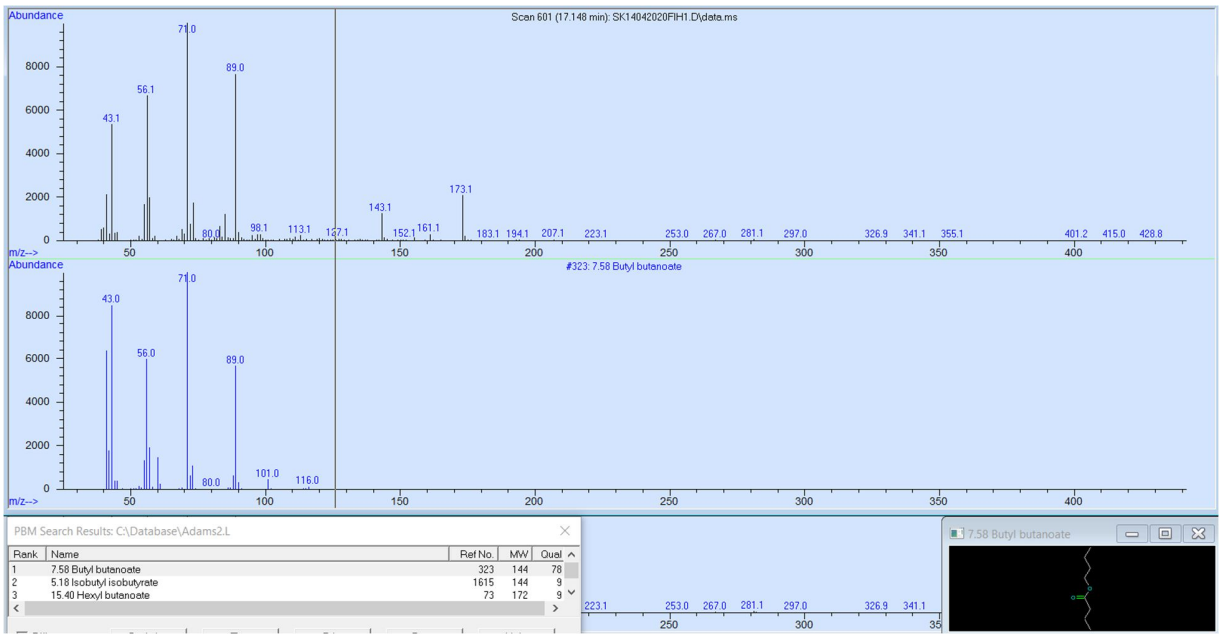

Figure 6.21 Mass spectrum of propyl butanoate

Appendix 22: Mass spectrum of cedrene

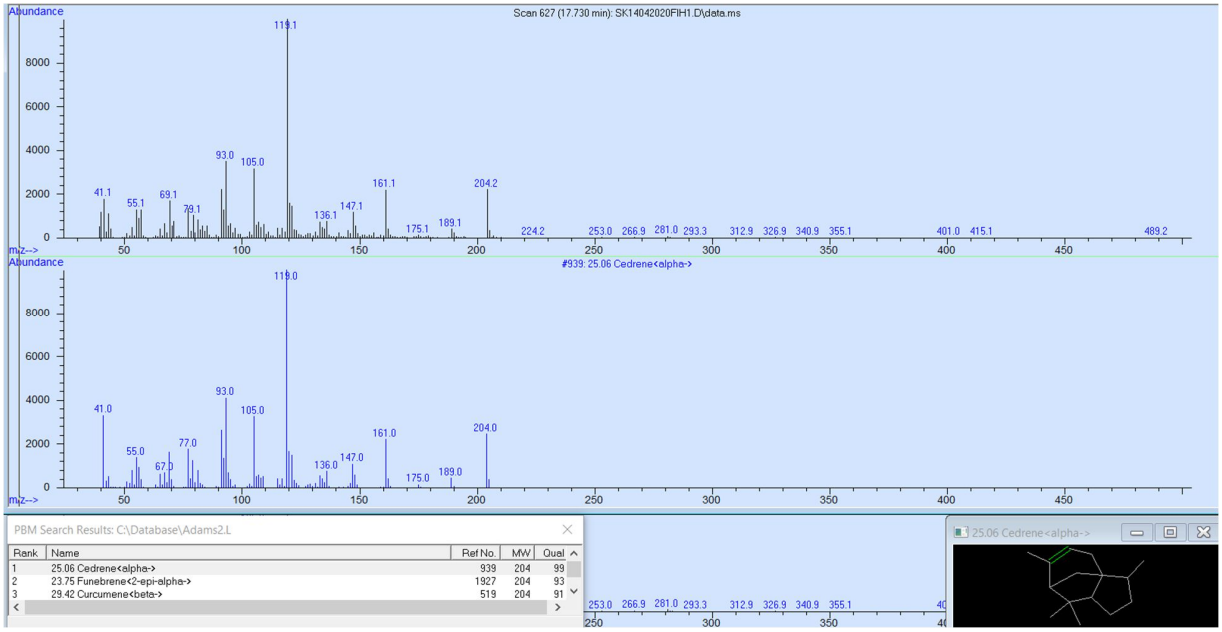

Figure 6.22 Mass spectrum of cedrene

Appendix 23: Mass spectrum of dibutyl phthalate

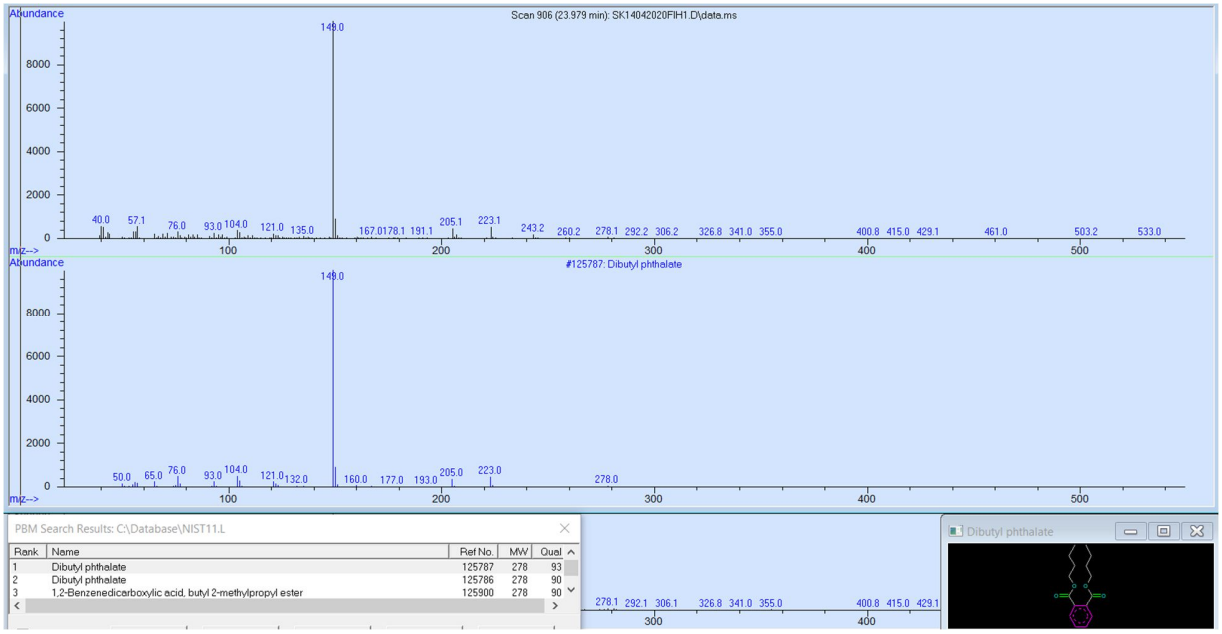

Figure 6.23 Mass spectrum of dibutyl phthalate

Appendix 24: Mass spectrum of bisabolene

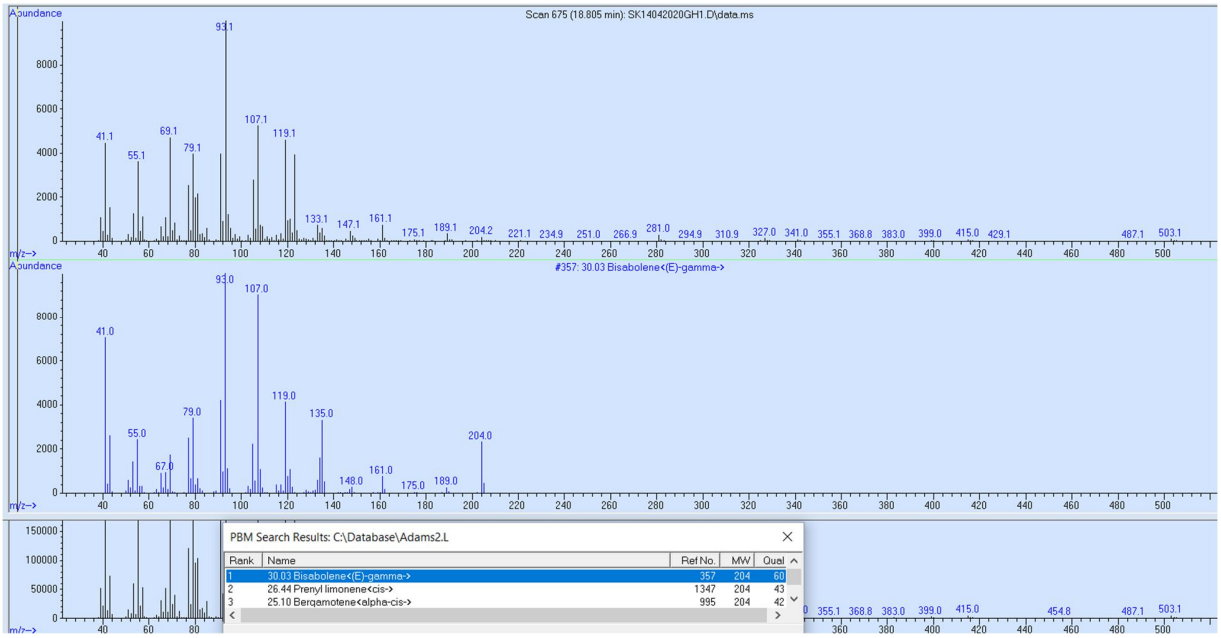

Figure 6.24 Mass spectrum of bisabolene