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Frass from black soldier flies as a valuable fertilizer and biopesticide for crops

By

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"Agriculture is the noblest of all alchemy; for it turns earth, and even manure, into gold, conferring upon its cultivator the additional rewards of health" – Paul Chatfold.

Declaration

I, **Kubayi Consol**, hereby declare that this thesis submitted to the University of Venda for the degree of MSc in Biological Sciences: Zoology (MNMBSZ), has not been submitted previously for any other degree at this or any other institution, and that it is my work in design and execution and that all materials contained therein have been duly acknowledged.

27/05/2022

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Signature Date

Summary

Recently, insect farming has gained recognition globally. This is particularly true for the black soldier fly, *Hermetia illucens*. However, the byproduct of insect feeding, a combination of food residue and frass left by the insects after harvesting, has not been given much attention and even fewer studies focus on the use of frass as a bio-fertilizer. In sub-Saharan Africa, human populations are expected to double by 2050, expensive fertilizers and increased food prices will exacerbate conditions further. This study is divided into three data chapters. Firstly, we conducted a systematic review of published literature on BSF (black soldier fly) larvae frass. In this review we collected literature from across the world, and we compared the research done in Africa with that of the rest of the world. The results show that more studies have been done in developed countries (82 %) as compared to developing countries (18 %). Topics explored around BSF frass included waste disposal, organic fertilizer, bioconversion, chemical composition, and economics. The results from all these studies show that frass can be used as a complementary organic fertilizer or independently. However, none of the papers have explored all the benefits of frass and the impact of different waste streams on frass quality. A better understanding of pH and electrical conductivity (EC) impact on frass as fertilizer are required.

Secondly, the data chapter presents the results from a greenhouse pot trial where we assessed the effectiveness of frass as a potential biofertilizer to improve plant growth. Frass was compared to commercial fertilizer and a control in a greenhouse at the University of Venda. *Amaranthus hybridus* was used in the pot trial, and growth parameters that were measured included number of leaves, plant height, dry biomass, and chlorophyll. The results indicated that dry biomass is highest in the NPK (Nitrogen, Phosphorus, and Potassium) treatment, with the control treatment being the lowest. Frass application at the lowest level did not significantly differ from the control, however, the biomass increased with in response to increased BSFL (black soldier fly larvae) frass addition and commercial fertilizer. The addition of 20g of frass improved all the parameters, and they performed similarly with commercial fertilizers. Frass maturity was performed through phytotoxicity test, and further evaluated the efficiency of frass tea as a biopesticide for crop pests through foliar spray technique. The results further revealed that frass used in the study was not matured enough

as the relative root growth rate was below 50 %, hence it was phytotoxic. Less leaf damage was observed in brewed frass tea compared to boiled frass tea as it was utilized as a foliar spray.

Lastly, ultra-high-performance liquid chromatography- quadrupole time-of-flight mass spectrometry (UHPLC-QTOF-MS) and some chemometric software including XC-MS and R-Software were utilized to characterize different metabolites found in *Amaranthus hybridus*. A total of 30 metabolites have been identified including derivatives of coumaric acid, caffeic acid, ferulic acid, and flavonoids. These metabolites serve different functions in plants including plant defence against pests and cardiovascular diseases in humans. Different frass treatment levels affected the distribution of metabolites. In conclusion, frass remains a promising option to substitute inorganic fertilizers, thus reducing global warming challenges and alleviating poverty in developing countries.

Keywords: Frass, BSFL, LC-MS, UHPLC-QTOF-MS, BSFL bioconversion, biofertilizer, *Hermetia illucens*.

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In Proverbs 3: 5-6, the bible says, “Trust in the LORD with all your heart and lean not on your understanding; in all your ways submit to him, and he will make your paths straight.”. I thank the almighty Lord, all-mighty, for always giving me strength and showing me the way of life in my daily activities. The energy to push until I finished my MSc is forever appreciated.

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Manuscript thesis

This thesis was written in a manuscript style that is in a manner that is suited for publication. Therefore, there might be some repetition.

References for the whole thesis have been consolidated into one list at the end of the thesis.

Chapter 1

A review of BSFL frass in developing and developed countries

Introduction

The African human population is expected to double by 2050 (Boaru *et al.* 2018United Nations 2019; Shumo *et al.* 2019;), leading to increased demands on food production, which places a burden on limited available natural resources. This is especially true for sub-Saharan Africa (SSA), where natural agricultural resources are fragile and prone to degradation, such as soil organic matter depletion, soil erosion, acidification, and nutrient mining (Choi *et al* 2009; Lal and Stewart 2010; Swanepoel *et al*. 2016). The Sustainable Development Goals, as adopted by all the United Nations member states in 2015, aim to find solutions to these environmental problems, and concurrently improve livelihoods. Sustainable and productive agricultural practices could reduce poverty, improve nutrition, and contribute to food security (Barragan-Fonseca *et al* 2017; United Nations 2019).

Farming alleviates poverty and improves food security (Shumo *et al.* 2019). However, the exponential growth in the human population results in a similar increased demand for organic high protein sources such as eggs and fish (Shumo *et al.* 2019). The challenge to meet organic food demands causes indirect competition between animal feed and human food because organic food, including soybean, is directly consumed by humans and forms part of livestock diet formulation for domesticated poultry and fish (Madau *et al.* 2020).

Poverty and food security largely affects developing countries (Shumo *et al.* 2019; Madau *et al.* 2020). About 70 % of food in the global market comes from small-scale farms, and small-scale farmers are looking for alternative animal feed as prices continue to escalate (Nyakeri *et al.* 2016; Zahn *et al*. 2017). Intensive soybean production requires more land, and as a result, leads to natural habitat degradation as this crop can contribute around 30 % of diet formulation for domesticated livestock (Dicke 2018; Shumo *et al.* 2019). Increasing fish and soybean prices will cause a direct increase in animal feed prices (Barragan-Fonseca *et al.* 2017; Shumo *et al.*, 2019; Nyakeri *et al*. 2016).

Rearing of beneficial insects are mainly focused on biological pest control (Barragan-Fonseca *et al.* 2017). However, mealworms (*Tenebrio militor*), house cricket (*Acheta domesticus*), and stink bugs (*Encosternum delegorguei*) are used as human food (Barragan-Fonseca *et al.* 2017; Hlongwane *et al*., 2021a). The black soldier fly (BSF) can be used as animal feed due to its high protein and fats composition (Nyakeri *et al.* 2017; Barragan-Fonseca *et al.* 2020; Van Huis 2020). In recent years, developing countries (i.e Kenya) have faced problems related to limited proteins and the little that is available has risen in market price and reduced affordability (Nyakeri *et al*. 2017).

The performance of BSFL depends on the waste stream used (Holmes 2010; Nyakeri *et al*. 2017). In SSA, a large portion of the waste is reportedly low in nutrient quality (Beesigamukama *et al.* 2020b). Animal manure, some fruits, and vegetables are examples of reported waste that have low nutrient quality (Cickova *et al.* 2015; Beesigamukama *et al*. 2020b; Quilliam *et al*. 2020). Approximately 75% of the produced food for humans, ends up as waste (Menino *et al.* 2020; United Nations 2019). Hence, the abundance of waste streams allows the rearing of BSFL to be continuously reared in different parts of the world by maintaining optimal rearing conditions (Temperature and Relative Humidity, RH as suggested by Holmes 2010.

Compared to other insects, BSF has several advantages when used in animal feed production. BSF also provides services like reducing house fly larvae and can recover nutrients from leachate, poultry, cow, and brewery manure (Green and Popa 2012; van Huis *et al.* 2015; Barragan-Fonseca *et al.* 2017 Xiao *et al.* 2018). The lipids from BSF larvae can also be converted into biodiesel (Li *et al.* 2011). Furhtermore, farming BSF is advantageous in terms of using less land compared to other feed crops (Van Huis *et al.* 2015; Barragan-Fonseca *et al.* 2017; Schmitt and Vries. 2020). During the bioconversion process, they can reduce odor from waste and produce odorless substrates (Cickova *et al*. 2015).

After converting the waste, that which is left over, combined with the insect chitin during metamorphosis are referred to as frass (Schmit and Vries 2020). Frass from BSFL is used as an organic fertilizer for crops (Kataga and Ohgushi 2011). BSF frass includes plant nutrients such as phosphorus and nitrogen (Kataga and Ohgushi 2011; van Huis *et al.* 2015). BSF larvae are very useful because they can modify the microflora of manure (Nguyen *et al.* 2015; Solomone *et al.* 2015; Schmitt and Vries. 2020). Several studies have reported that frass improves crop

yield and can serve as pests repellent after less damage was observed in plants that were supplemented with frass to improve growth (Kawasaki *et al*. 2020; Quilliam *et al*. 2020).

Aquatic animals produce a powder called Chitosan from the chitin (i.e crustaceans), and the chitosan is believed to contain biopesticide properties that promote pest resistance in plants and other plant diseases (Schmitt and de Vries 2020; Quilliam *et al.* 2020). The production of chitosan requires the use of chemicals that can damage the environment. Frass contains insect exuvia that is said to have biopesticide properties, and aid in reducing pathogens with less negative environmental impacts (Quilliam *et al.* 2020; Beesigamukama *et al,* 2020c; Schmitt and de Vries 2020).

The biology of the black soldier fly

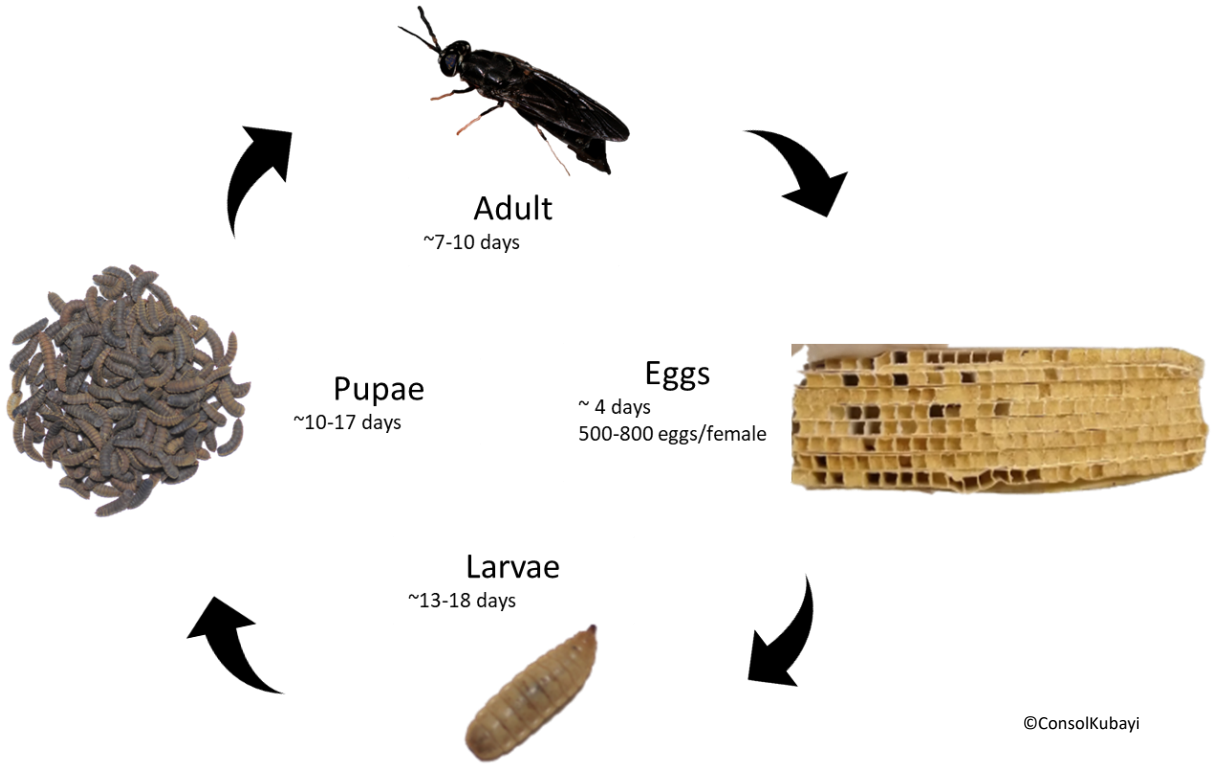

Figure 1: Life cycle of the black soldier fly (BSF) under complete metamorphosis.

BSF undergoes a complete metamorphosis with four major developmental stages (**Fig 1)**; egg, larva, pupa, and adult (Li *et al.* 2011; Vickerson *et al.* 2017). BSFL moults six times, (Holmes

2010; Terfa 2021). During reproduction, females mate four days after emerging and they can lay up to 500 eggs, usually in dry pores, gaps, and cracks next to decomposing organic material such as compost heaps (Sheppard *et al.* 1994; Li *et al.* 2011; Vickerson *et al.* 2017). The female can lay eggs at a minimal temperature of 12 ºC but maximum oviposition takes place at 32 ºC (Holmes 2010; Li *et al.* 2011).

Eggs hatch within four to five days at 24 to 27 ºC (Sheppard *et al.* 1994; Li *et al.* 2011; Vickerson *et al.* 2017). When the conditions are optimal (temperature: 29 - 31 ºC; Solomone *et al.* 2015; relative humidity: 60 - 70 ºC; Setti *et al.* 2019) and the larvae have accumulated enough fats and proteins, it takes two to three weeks to reach prepupal stage but increase by four months to reach mature stage if the optimum temperature is below its range or food is limited (Sheppard *et al.* 1994; Li *et al.* 2011; Solomone *et al.* 2015). During the prepupal phase, BSF larvae stop feeding, empty their digestive tract, their skin darkens, and their mouth part changes from straight to hook-like part called a rostrum, enabling it to climb out of the frass and find a dry place for pupation (Sheppard *et al.* 1994; Solomone *et al.* 2015).

The BSF gut contains enzymes such as amylase, lipase, and protease that allow the fly to break down a variety of different organic streams (Barragan-Fonseca *et al.* 2017; Klammsteiner *et al.* 2020). The black soldier fly digestive system is maintained at 54 °C, and it digests food and kills some bacteria like *E coli* and *Salmonella* spp that can directly kill people (Erickson *et al.*, 2004; Klammsteiner *et al.* 2020; Lalander *et al*. 2014). A fully-grown adult is 13–20 mm long (Nguyen *et al.* 2015; Solomone *et al.* 2015). The adults do not consume food and only drink water (Sheppard *et al.* 1994; Holmes 2010). A mature adult's life expectancy is between seven to ten days and the female adult fly lays a cluster of eggs once in its lifetime (Vickerson *et al.* 2017; Holmes 2010).

The distribution and ecology of black soldier fly

The BSF (*Hermecia illucens*, Linneus 1758; Order: Diptera) is a saprophagous fly in the Stratiomyidae family that is native to the Americas ranging from Boston, and Seattle (Solomone *et al.* 2015; Gujarathi and Pejaver 2013; Barragan-Fonseca *et al*. 2017). BSF is now globally distributed due to transportation by man (van Huis 2019). Martínez-Sánchez (2011)

reported that BSF was first seen in Europe in 1926, and it was then discovered in Switzerland, Spain, and Portugal.

Currently, not much is known about its potential impact on native biodiversity, but De Groot and Veenvliet (2011) reported that BSF larvae have the potential to cause cutaneous myiasis in human skin. This occurred after a European woman took a vacation to Uganda in Africa conducting teaching lessons and BSF larvae was alleged to have caused the disease after microscope examination of the larva (De Groot and Veenvliet 2011).

Objective and importance of the review

In this chapter, studies on BSF and its frass are reviewed. This includes a systematic literature search for peer-reviewed studies, focussing on BSL farming and the use of frass as a soil amendment. We used publication data to explore the temporal and spatial distribution of BSF research and identify the gaps and future research opportunities.

Methods and materials

Selection of studies that focus on black soldier fly rearing

Search strategy

A systematic search was conducted using the following search engines: Science Direct, Web of Knowledge, and Google Scholar (Swanepoel *et al.* 2016). Search words were adjusted according to search engines and databases, and consisted of a combination of 'frass', 'BSFL bioconversion', 'BSFL biofertilizer', 'black soldier fly', '*Hermetia illucens*', 'BSFL'.

Inclusion and exclusion of papers

All three search engines resulted in more than 1000 papers and evaluations stopped at the end of each last page. Google Scholar resulted in more than 1000, Science Direct: greater than 1800, and Web of Knowledge: 1500 records. These papers were extracted and counted as they included search keywords. **Fig** is the flow diagram of the methodology followed. Presentations, dissertations, and book chapters were considered grey literature and were not included in this study. Only peer-reviewed publications were included (**Fig 2**).

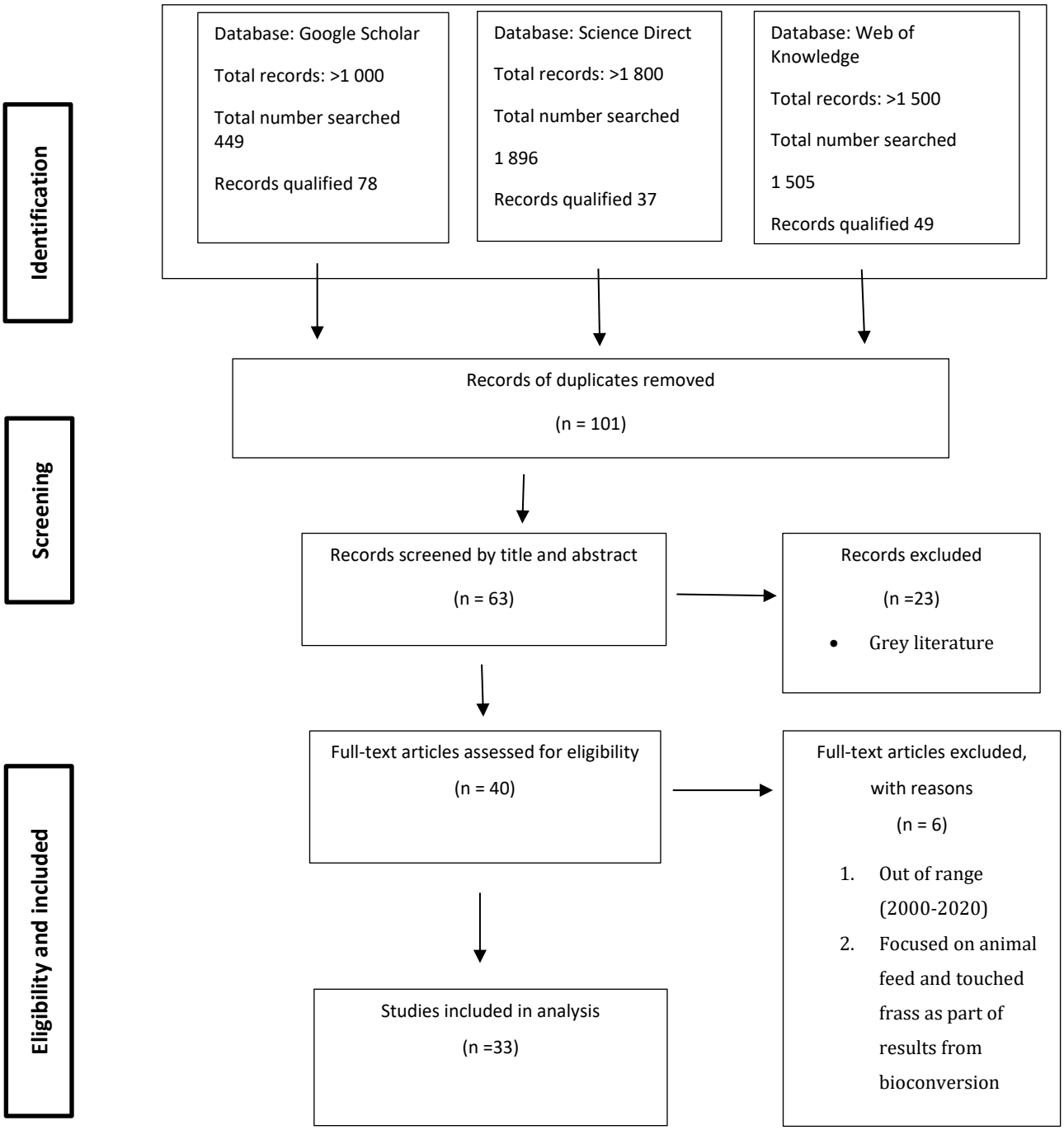

Figure 2: PRISMA diagram showing the summary of information extraction from various databases.

Data extraction and analysis

A total of 164 publications that included duplicates were sourced from the three databases and only 63 papers were considered for screening focusing on frass from black soldier fly larvae. All the papers were published between 2000 and 2020. Forty (40) papers were full articles that strictly fell in the criteria of frass, and 23 papers were excluded due to the grey literature category. After the selection of the publications that met the criteria of this study, more information was extracted as follows: title, authors, date, theme, main result, country, and location of the study. The publication date was used to represent the temporal distribution of the studies.

The country where the study was done, was used to determine the spatial distribution of studies. Some publications, such as reviews, did not have specific countries, and thus the number of countries does not correspond with the number of papers. The themes of data were categorized using search categories in databases: organic fertilizer, bioconversion, economy, waste recycling, and chemical composition. The data was further divided into two categories, distinguishing between developed and developing countries.

Results

Eligibility of studies

Over 4 300 studies were extracted from three databases using the search criteria. A total of 101 (68 %) were duplicates and 63 (38 %) met the criteria of screening by title and abstract for further screening. Full-text articles assessed for eligibility were 40 (63 %) and 23 (37 %) studies were excluded with reasons (**Fig 2**). Studies that met the full-text articles for eligibility criteria were further screened to remove grey literature and out-of-range (outside 2000 – 2020) studies. A total of 34 (85 %) studies were included for analysis and 6 (15 %) were excluded.

Geographical footprint

A total of 33 studies that focused on BSF frass were included in the construction of spatial distribution (**Fig 3** and **Fig 4**). One study was excluded as no locality was identified. Of these, 27 (82 %) were from 12 developed countries namely: Austria, China, Germany, Indonesia, Italy, Japan, Korea, Netherlands, Romania, France, Sweden, and the USA. Six (18 %) of these publications were from two developing countries namely: Ghana and Kenya respectively. In developed countries, China (30 %), Romania (15 %), and the USA (11 %) contributed most studies followed by Italy, Netherlands, Sweden (7 % each) and lastly, Austria, Germany, Japan, Korea, Romania, France all contributed 4% each. In developing countries, Ghana contributed a third and the remaining ones were from Kenya.

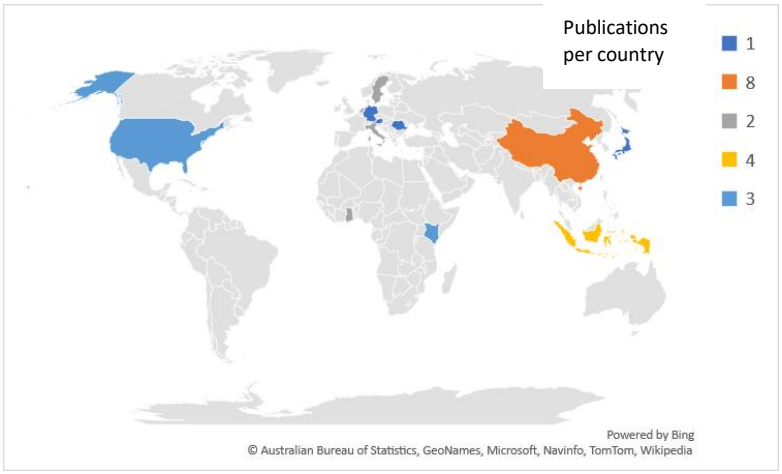

Figure 3: Spatial distribution of BSF studies globally as ranked by countries that conducted studies about frass from black soldier fly as an organic fertilizer.

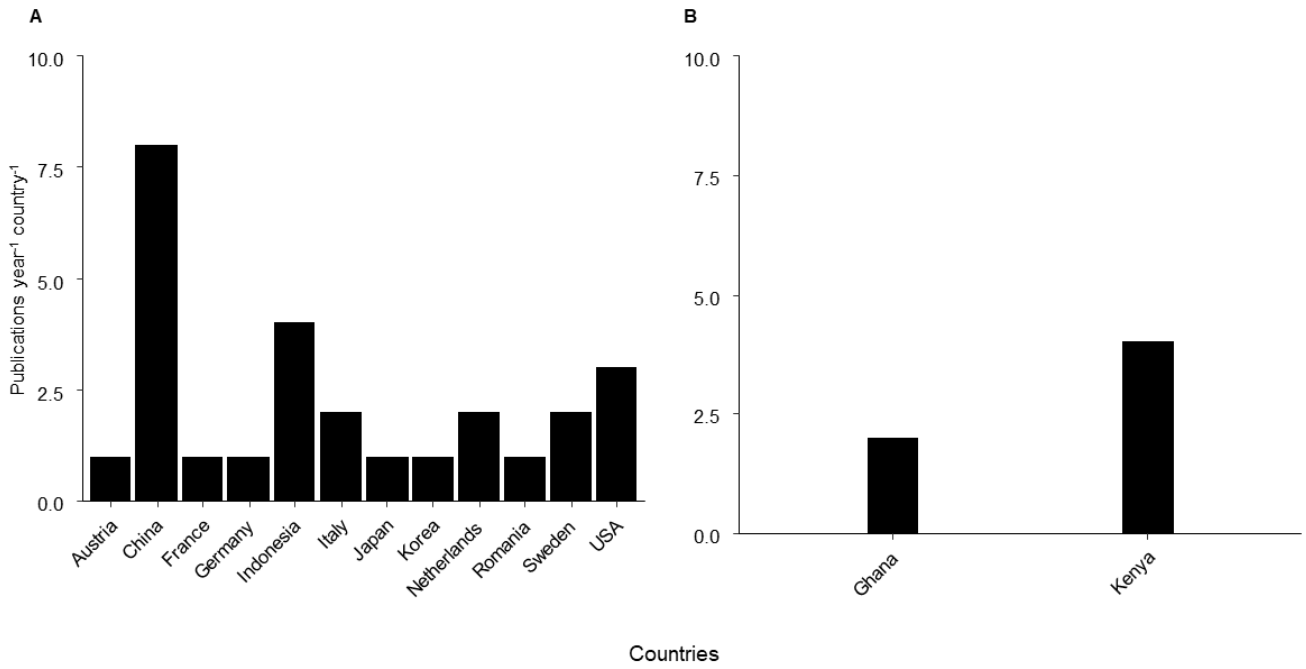

Figure 4: Number of published articles per country according to three databases using keywords (December 2020) in (A) developed and (B) developing countries.

Temporal trends

Over the estimated range between 2000 – 2020, in the first eight years, no studies focused on frass until 2009. Between 2009, 2012, 2013, and 2016, there was an average of one publication (3 %). However, in 2020, the topic gained considerably more interest, 17 (50 %) studies were published followed by 2019 with seven (21 %) studies. 2015, 2017, and 2018 all had two (6 %) published studies each (**Fig 5**).

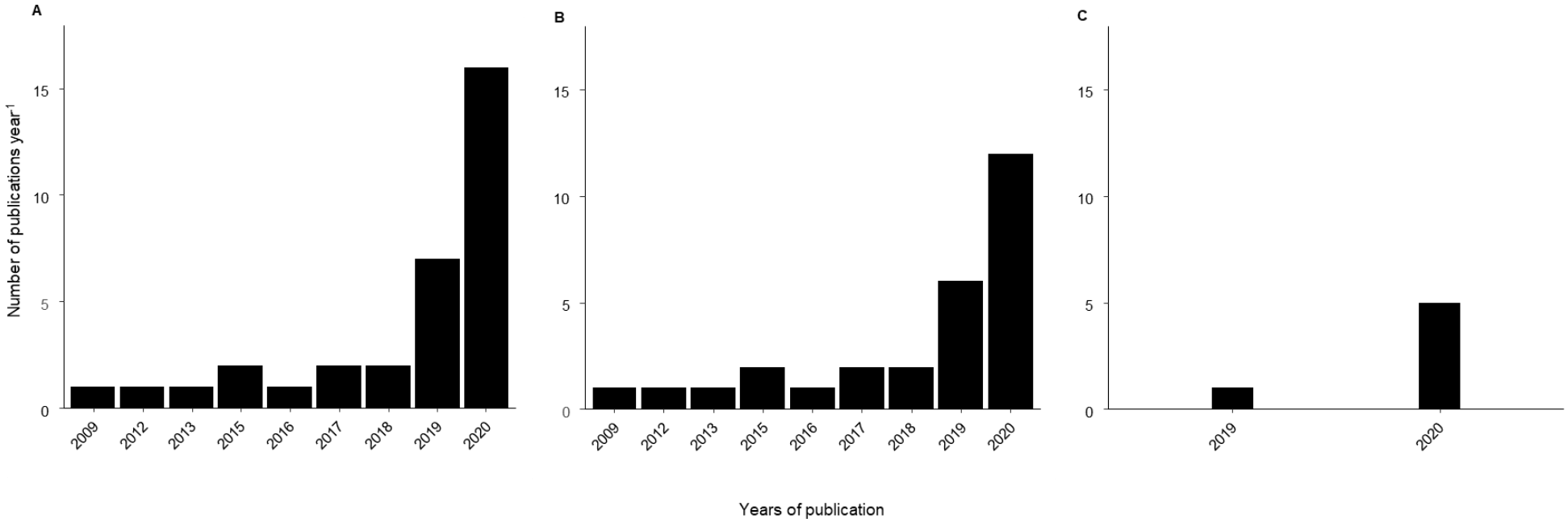

Figure 5: Number of published articles per year (2009-2020) in (A) overall publications, (B) developed countries, and (C) developing countries.

Study themes

Developed countries have conducted more research and published more papers on BSF frass compared to developing countries in all the six themes identified in this study (**Fig 6**).

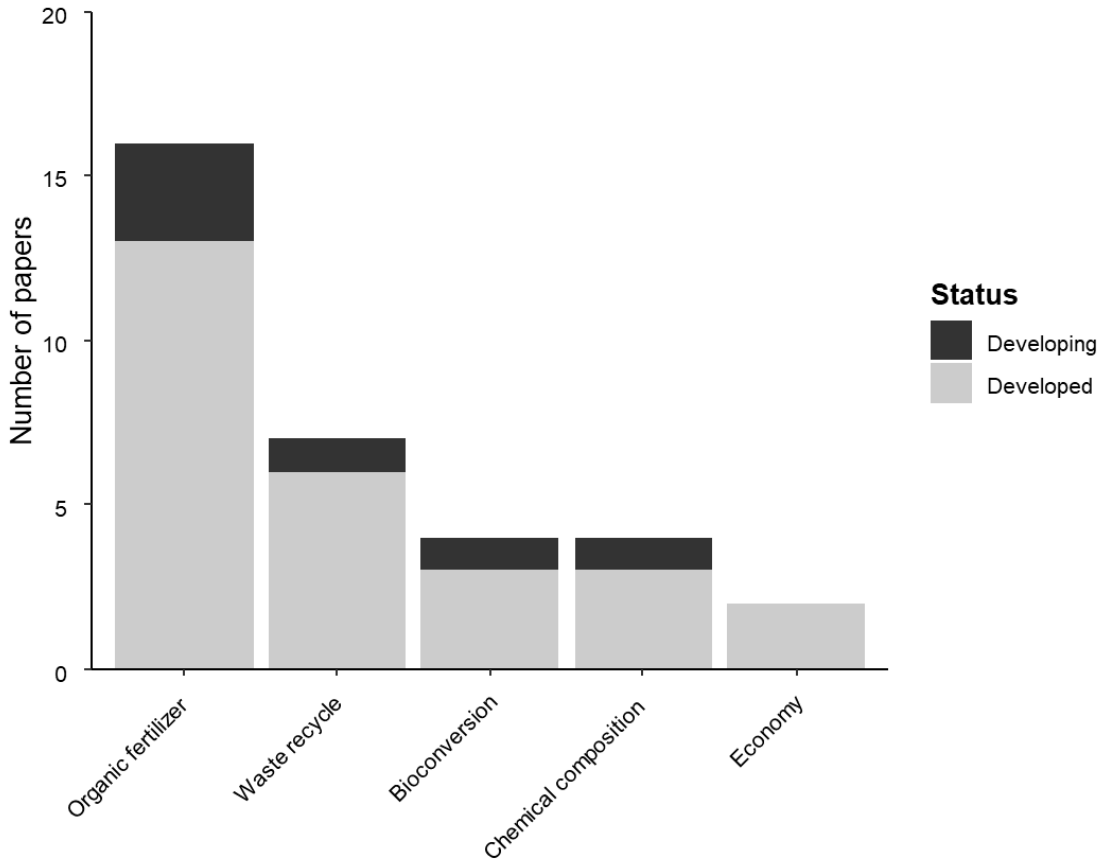

Figure 6: Number of publications in different themes in developing and developed countries.

Discussion

Rearing conditions of BSF

Historically, BSF survival is known to be influenced by several abiotic factors that include relative humidity, temperature (Barragan-Fonseca *et al.* 2017), and the substrate used to feed the larvae (Nyekeri *et al.* 2017). A few studies have reported the optimal conditions to rear BSF colonies (**Table 1**).

Table 1: Optimal rearing conditions and waste stream for product-specific black soldier fly (BSF) production. RH: Relative Humidity.

Waste stream	Temperature (°C)	RH (%)	Photoperiod	By-product	Reference
Poultry manure and chabazite	26 ± 0.5	60 - 70	-	Animal feed and Biofertilizer	Bartolini *et al*., 2020
Poultry manure and chabazite	22 - 28	40 - 60	-	Biofertilizer	Boaru *et al*., 2018
Corn, alfalfa, wheat bran	27 ± 0.5	60 - 70	-	Biofertilizer	Setti *et al*., 2019
Brewery spent grain	28 ± 2	60 - 70	L12:D12	Biofertilizer	Beesigamukama *et al*., 2020[a]

Fermented poultry, rabbit manure, fruits	28 ± 2	60 - 70	L12:D12	Biofertilizer	Beesigamukama *et al.*, 2020[b]
Brewery waste, fruits, vegetables, and kitchen waste	28 ± 2	60 - 70	L12:D12	Biofertilizer	Beesigamukama *et al.*, 2020[d]
Municipal organic solid waste	25.5 - 26.8	60- 85	L12:D12	Biofertilizer	Sarpong *et al*., 2019
Mushroom waste	25 - 30	70		Animal feed and Biofertilizer	Cai *et al*., 2017

Temperature and the substrate (organic waste) used to feed insects interact synergistically to improve the development of insects such as the black soldier fly (Harnden and Tomberlin 2016). The optimal temperature growth condition for BSF is 27 ºC and the female adults can lay eggs at temperatures as low as 12 ºC (Tomberlin *et al.* 2009; Holmes 2010). BSF eggs can hatch at 12 and 15 ºC while the hatchlings at 15 and 18 ºC can survive up to three days (Holmes 2010). At low temperatures, it remains unknown whether BSF mating is affected or not (Holmes 2010).

Relative humidity (RH) is a key abiotic factor to be controlled when rearing insects as it affects the developmental stages (Holmes 2010). BSF does well in subtropical and tropical regions and Holmes (2010) determined the threshold RH levels for the fly. An increase in relative humidity increased the chances of eggs and pupae hatching to the adult stage. The impact of low relative humidity on the fecundity of adults remains unclear. However, there is evidence that adults reared in RH above 70 % live two to three days longer as compared to adults reared on lower relative humidity (Holmes 2010).

The waste stream for BSFL has an impact on life cycle development. Development time at the prepupal stage was shorter in pig liver and kitchen waste as compared to vegetables and manure as reported Nguyen *et al*. (2013). Adults also had a short developmental time in the same waste stream, but larvae grown in fish and manure took longer to reach the adult stage (Nguyen *et al*. 2013). Vegetables and manure have calories and fish have more fats, but too much fat can hinder metabolization of fats during metamorphosis (Nguyen *et al*. 2013). Contamination with heavy metals has negative effects on insects and some insects are unable to grow in contaminated feed (Nguyen *et al*. 2013). Pupation of larvae is affected by the density of the substrate as high densities can increase pupation time, because the larvae dig deep into the substrate (Harnden and Tomberlin 2016).

Geographical footprint

The rearing of insects is practiced globally for human and animal consumption. However, the rearing of insects in some developing countries is mostly done at a small scale for household purposes (Nyakeri *et al*. 2017; Chia *et al*. 2019). Although developing countries rear BSF very little scientific research has been done in these countries (Nyakeri *et al*. 2017; Chia *et al.*

2019). but there is a gradual increase in awareness and knowledge (Barragan-Fonseca *et al.* 2017; Nyakeri *et al*. 2017; Beesigamugakama *et al.* 2020ab**)**. Developed countries mainly focus on BSF as a waste management tool to convert food waste and manure (Sarpong *et al*. 2019).

Temporal trends

Research on BSF has increased since 2009, with only one or two papers published worldwide per year, to seven publications in 2019, and 16 publications in 2020 (**Fig 5**). This is evidence that frass has gained increased attention as a potential soil improver (Barragán-Fonseca *et al.,* 2020). After 2009, 32 % of the screened publications focused on BSF frass application as biofertilizer. The first reported study on BSF frass was by Choi *et al.* (2009) who tested their properties as a soil improver. In Africa, studies focus on frass in Sub-Saharan Africa due to factors such as soil degradation, and population expansion (Quilliam *et al.* 2020; Swanepoel *et al.* 2016).

Theme of study of BSFL frass between developed and developing countries

The categories of papers were divided into papers focusing on a) the bioconversion of organic products, b) performance of frass as an organic fertilizer, c) the effectiveness of BSFLto reduce waste products, d) economic aspects of BSF farming and e) the chemical composition of frass. The most researched theme is frass as an organic fertilizer (16 papers, or 48%), followed by BSF's role in the reduction of waste products (8 papers or 23% of the total). Sustainable farming (5 papers), bioconversion (4 papers), chemical composition (4 papers), and economy (2 papers) were less common research topics (**Fig 6**).

Organic fertilizer

Organic fertilizers are in high demand in horticulture to promote plant growth (Chavez and Uchanski 2021). Frass as an organic fertilizer remains an interesting option to farmers that are concerned with environmental challenges caused by synthetic fertilizers that may lead to eutrophication and nutrient leaching of soil (Schmitt and Vries., 2020; Chavez and Uchanski 2021). Vegetable production remains the most essential, cultivated, and sold crops in Sub-Saharan African countries (Terfa 2021). Globally, most countries have failed to meet the crop production for the ever-increasing human population, and intensive cultivation of vegetable crops remains an option to cater to this challenge and alleviate poverty (Terfa 2021).

Organic fertilizers are environmentally friendly and release nutrients into the soil for plants to absorb at a gradual rate (Kataga and Ohgushi 2012). Nutrient availability varies with the type of fertilizer used (Putra *et al*. 2017). Nutrients in inorganic fertilizers are labile as they are already in inorganic form, and in organic fertilizers, gradual mineralization must take place before minerals are available for plants (Beesigamukama *et al*. 2020c; Kataga and Ohgushi. 2012).

Nutrient utilization by plants from coffee husk-derived frass and cow manure varies (Putra *et al*. 2017). Lettuce (*Lactuca sativa* Var. Crispa) grown in cow manure yields high dry and wet biomass, thus improving the height, number of leaves, leaf area, and chlorophyll content relative to cow manure in the above-mentioned growth indicators (Putra *et al*. 2017; Rosmiatti *et al*. 2017). Nitrogen was mostly utilized by plants grown in manure, and more phosphorus and potassium were utilized by plants grown in frass (Putra *et al.*, 2017). Both soil improvers play a similar role at different rates, it is, therefore, important to combine both to complement each other for an improved crop yield.

The use of frass does not only improve plant growth, but frass has biopesticide properties as well (Kawasaki *et al*, 2020; Quilliam *et al*. 2020). In a comparative study, plants under different frass treatment levels responded differently, the number of plants attacked by fusarium wilt decreased with an increase in frass fertilizer (Quilliam *et al*, 2020). Komatsuna (*Brassica rapa var. perviridis*) green leafy vegetable was grown in frass and cow manure treatments, the crop yield was higher in frass grown komatsuna compared to cow manure treated komatsuna (Kawasaki *et al*. 2020). The crop yield in frass was not affected but 10 % of proteobacteria that attacks plants was detected (Kawasaki *et al*. 2020). Frass can suppress bacteria and other plant diseases and improve crop yield.

Studies have shown that different parts of a plant (i.e stem, roots, and leaves) respond differently depending on the waste stream (Choi *et al*. 2009; Bartolini *et al*. 2020; Rosmiati *et al*., 2017). Bartolini *et al*. (2020) reported that frass improved plant height but did not affect stem diameter and fresh weight. Rosmiati *et al*. (2017) and Putra *et al*. (2017) reported that all the plants grown in pots treated with manure were higher, had larger leaf areas, and more leaves than those produced using frass.

Chlorophyll content of plants grown in frass improves in various plants, including maize, lettuce, to rice, relative to animal manure, and commercial fertilizer (Beesigamukama *et al*. 2020; Putra *et al*. 2017). A study reported that the overall highest grain biomass production was achieved in plots treated with 7.5 t ha^{-1} and 100 kg N ha^{-1} of Black soldier fly frass fertilizer (BSFFF) compared to equivalent commercial fertilizers (Beesigamukama *et al*, 2020b).

Waste recycling

BSF larvae can reduce fecal sludge by 50to 55% (Lalander *et al*. 2014; 2015). The remaining waste products undergo a physico-chemical change, including change in total ammonium-nitrogen concentration, increased pH, total solids, volatile solidsand , increased available phosphates (Lalander *et al*. 2014; 2015), and dissolved organic carbon (Liu *et al*., 2020). Organic nitrogen decreases in the waste product, but increases in inorganic nitrogen forms, such as ammonium and nitrate (Lalander *et al*. 2014, Liu *et al*. 2020).

Harmful pathogens are reduced during BSF larvae inoculation (Lalander *et al*. 2015). Lalander *et al*. (2014) measured a decrease in harmful bacteria, such as Salmonella. Viable virus concentrations were shown to decrease below a detectable limit within 14 days (Lalander *et al*. 2015), while bacteria in manure waste was shown to be drastically reduced within nine days (Awasthi *et al*. 2020). Although harmful pathogens can be reduced, BSF larvae treatment is not an adequate method for total fecal waste conversion that is intended to be re-used in agricultural land (Lalander *et al*. 2014). Another advantage of using BSF as waste treatment is that it can self-harvest and reduce the use of extensive equipment (Cickova *et al.* 2015). Biorefineries remain a promising tool to clean up waste and produce biofertilizers and oil as a byproduct that can be marketed (Habiburrohman *et al*. 2019).

Bioconversion

BSF has the potential to convert different waste streams (Cickova *et al.* 2014). The saprophagous characteristic of the BSFL enabled more scientific research to be conducted and waste with rich nutrients and some that have low nutrients that can be accumulated by the larvae has been used such as rice straw, municipal organic solid waste, chicken, cow, and chicken manure, banana, and cassava peel waste (Sarpong *et al*. 2019; Gao *et al*. 2019; Liu *et al*. 2019[a]; Rahmi *et al*. 2020). Animal manure is a problem to the environment as they release methane and greenhouse gases into the atmosphere and can be bio-converted using BSF larvae (Beesigamukama *et al*. 2020; Liu *et al.* 2019a). Converting manure waste can increase

total P and N suitable for plant growth, reduce volatile fatty acids and organic matter (Liu *et al*. 2019a).

Some organic wastes are hard to digest by animals, and bioconversion can be used to facilitate the digestion of inorganic compounds found by using enzymes and bacteria that break down those compounds (Bloukounon-Goublon *et al*. 2019). Chicken manure has been converted with the inoculation of *Bucillus subtilis* bacteria reducing the conversion time to 13 days (Xiao *et al*. 2018). *Aspergillus oryzae*, and *Trichoderma reesei* fungi were used to ferment maize straw and the larvae did not experience mortality (Gao *et al*. 2019).

Various substrates can be added to the waste to improve conversion rate thus producing an agricultural end-product such as animal feed and organic fertilizer. Municipal waste contains heavy metals that can be accumulated in the body through food webs. BSF larvae can convert waste that is contaminated by heavy metals to produce frass and larvae that have none or less of the metals as compared to waste before the bioconversion process (Sarpong *et al*. 2019).

Chemical composition

A large amount of waste streams contains cellulose, hemicellulose, and lignin that cannot be easily digested, but a synergistic relationship between microbes and insect larvae to efficiently convert waste at a fast rate by making the waste palatable to the larvae remains an alternative (Bloukounon-Goublon *et al*. 2019). BSF can potentially convert such waste with assistance of bacteria introduced in waste to promote palatability of the waste (Xiao *et al*. 2018). Breaking down sources of energy is not all, but the challenge remains the by-product, frass. Frass fertilizer is faced with low nutrient content challenges from the ammonium-ammonia equilibrium where most of the nitrogen is lost through evaporation as ammonia during the biodegradation process by BSF larvae (Beesigamukama *et al*. 2020a).

Temperature, pH, and electrical conductivity (EC) are physical properties that affect the insect larvae and the by-product during the biodegradation process (Beesigamukama *et al*. 2020a). Moreover, these properties are the main drivers of the above-mentioned equilibrium (Palma *et al*., 2020; Bloukounon-Goublon *et al*. 2019). The substrate experiences changes in pH from neutral to alkaline levels between nine and ten days when the frass gets dried up, and such

trends were observed in several studies (Gartling *et al*. 2020; Palma *et al*. 2020; Bloukounon-Goublon *et al*. 2019, Beesigamukama *et al*., 2020b).

Most parts of the soils in Sub-Saharan Africa are degraded and lack nitrogen which is essential for plant growth. The larvae assist with the ammonification process that promotes the conversion of nitrogen into plant-available nitrogen forms, such as Ammonium ($N\text{-}NH_4^+$) and Nitrite ($N\text{-}NO_3^-$) (Bloukounon-Goublon *et al*. 2019).

Economy

The European Union, under novel food regulations 2015/2283, has approved the use of insects as feed and their byproducts (Houben *et al*. 2020). Frass from insects is gaining much attention in agricultural industries as larvae are increasingly being produced on a large scale, as well as frass which is sold as organic fertilizer to local farmers (Houben *et al*. 2020). Ÿnsect, French insect farming improves the livelihood of people by creating jobs, and they have managed to raise $125 million in funding to initiate and develop activities that are part of the circular loop economy. In developing countries, the use of insects to generate frass has been noted to create job opportunities and thus bring some household income for local farmers (Quilliam *et al*. 2020).

Scientific studies have initiated the practice of modelling a large-scale production of insect farming that will yield BSF larvae and frass that can be used as a soil improver, and they also monitored the safety of frass from pathogens to be used as clean fertilizer (Bartolini *et al*. 2019; Kawasaki *et al*. 2020; Quilliam *et al*. 2020). The protein source from the larvae has more economic value in nutrition, thus equally matching the value of frass in the economy to be essential as it can be profitable (Gao *et al*. 2019).

Local and rural farmers experience income challenges to purchase fertilizers, it is a challenge that is difficult to resolve due to money for transportation of synthetic fertilizers to their farms that are located far from towns (Zahn *et al*. 2017; Qulliam *et al*. 2020). These challenges can be alleviated by initiating the farming of BSF in local farms, as proposed by Nyakeri *et al*. (2017) as it does not require any special equipment to rear.

Studies have indicated that frass alone can be used as a soil improver, but it needs to be supplemented by commercial fertilizer for more yield (Beesigamukama *et al*, 2020ac; Quilliam *et al*. 2020), and at a high conversion rate of the waste, introducing bacteria to assist with

digestion can be effective and feasible (Xiao *et al*. 2018). Contamination of soil by heavy metals is a concern in degraded soil and metal such as Cd gets accumulated by plants that negatively affect plant growth, some bacteria get abundant during frass maturation, but after application of frass from bioconversion, it shows that frass can be used without impairing the soil hygienization (Klammsteiner *et al*. 2020).

Chapter 2

Frass from black soldier flies as a valuable fertilizer and biopesticide for crops

Abstract

The rearing of *Hermetia illucens* yields high protein larvae suitable for animal feed. This reduces waste, recovers nutrients, and improves livelihoods. Frass is one of the by-products of these systems, potential organic fertilizer and biopesticide. This study aims to (a) assess the effectiveness of frass as a potential biofertilizer to improve plant growth by experimental assessment of growth parameters of *Amaranthus hybridus* in response to increased BSFL frass addition and commercial fertilizer, (b) assess the maturity of the frass used in this study through phytotoxicity test, and (c) evaluate the efficiency of frass tea as a biopesticide for crop pests. The assessment was done in pot trial. The results suggest that Frass 20g did not differ significantly with commercial fertilizers in growth parameters. At Frass 20g, the nutrient content was relative to the commercial fertilizer. Chlorophyll content was highest in plants grown in Frass 20g. The results further revealed that frass used in the study was not sufficiently matured enough as the relative root growth rate was less than 50 % and phytotoxic. Seed germination rate in all the replicates was above 94 %. Brewed and boiled frass teas did not show to differ compared to the control, whereas more damage was observed from boiled frass tea. In conclusion, frass can be a viable alternative for organic fertilizer, but efficacy may vary based on the waste streams used and the application of frass tea as a biopesticide can further be assessed using a different method to determine its efficacy.

Keywords: BSFL bioconversion, BSFL biofertilizer, *Hermetia illucens*, BSFL

Introduction

The exponential growth of the human population, climate change, and loss of biodiversity could negatively influence food production (Beesigamukama *et al*. 2020b). It is necessary to develop sustainable and alternative food sources to improve food security and reduce the impact of food production on the environment. The use of organic waste (such as manure or vegetables) to grow Black Soldier Fly Larvae (BSFL), *Hermetia illucens* Linneus 1758, is a cheap and effective way of producing frass for sustainable agriculture in Sub-Saharan Africa (Beesigamukama *et al*. 2020a; Quilliam *et al*. 2020). If the by-product of this BSFL, the frass, can be used as an organic fertilizer, the process can significantly contribute to sustainable and cheap food production in regional areas.

Flies in general are associated with poor sanitation and considered a nuisance. Insects such as flies are common and widespread globally (Hussein and John *et al*. 2014). House flies and black soldier flies are commonly known species of flies that are abundant in different waste streams and human-populated areas such as markets, hospitals, restaurants, and livestock farms (Cickova *et al*. 2015; Khamesipour *et al*. 2018). Domestic flies are a potential vector of disease carriers to humans (i.e diarrhea) and livestock (i.e typhoid), but the same is not true for black soldier flies. (Nguyen *et al*. 2015; Barragan-Fonseca *et al*. 2017; Van Huis 2019).

The consumption of insects is a traditional practice in Africa as many countries rely on indigenous fruits and insects for sustainability and to alleviate poverty (Hlongwane *et al*., 2021a). South Africa is one of the countries in South Africa that practices the consumption of edible insects such as mopane worms (*Imbrasia belina*), Termites (*Macrotermes* species), and Stink bug (*Encosternum delegorguei*) (Hlongwane *et al*., 2021ab). These insects play a significant role in rural communities because they have high nutritional composition, they can be used as a coping strategy during dry seasons when there is a shortage of food, and most importantly, they can be seasonally harvested for household income revenue by selling them in local towns (Hlongwane *et al*., 2020; Dube *et al*., 2013).

Insects such as Black soldier fly larvae (BSFL) have the potential to be used as animal feed and is a source of high protein, fats, and vitamins (Bessigamukama *et al.* 2020 ad; Chiam *et al.* 2021; Xiao *et al*. 2018). The production of animal feed requires about 70 % of agricultural land

and has negative environmental effects such as the emission of greenhouse gases (Dicke 2018). These challenges affect human food production that is used as animal feed. For example, in three years (between 2012-2014), 838 megatons including 34 % of the global production of cereals were used as animal feed instead of food for humans (Dicke 2018). Insects can be used as an alternative high source of protein for animal feed thus reducing the costs of animal feed price (Dicke 2018; Van Huis 2019). Mass rearing of insects can be done using organic waste streams.

Human societies produce large amounts of solid waste, fruits and vegetables, and kitchen waste, and about 60% of organic food wastes are discarded (Boaru *et al*., 2018; Sarpong *et al*. 2019; Rahmi *et al*. 2020). In developed and developing countries, food waste is a serious concern as millions of U$ dollars are spent to treat waste (Choi *et al*. 2009). In 2012, an amount of $205.4 billion was spent globally to treat solid waste in cities that also affect local communities, and the amount spent to treat the waste was expected to increase up to $375.5 billion in 2015 (Sarpong *et al*. 2019). The implementation of black soldier fly larvae as a bioconversion tool can reduce the costs to treat waste efficiently because the fly is a voracious insect that feeds on various organic waste streams ranging from brewery waste, poultry, pig and cow manure, human faeces, kitchen waste, green waste, and leachates (Beesigamukama *et al.* 2020[b]; Quilliam *et al* 2020; Xiao *et al*. 2018).

Organic fertilizers are natural organic materials that are a product of animal manure, compost, or plant materials and are in great demand in horticulture (Menino *et al.* 2020). (Ouda and Mahadeen 2008). Organic fertilizers can also be produced by the conversion of waste using insects, and the final product is called frass or biofertilizer (Sarpong *et al*., 2019). Inorganic fertilizers that are insects derived are environmentally friendly, however, the release of nutrients occurs gradually and takes time (Kataga and Ohgushi 2012).

Frass is a combination of insects exuvia, feces, and uneaten residuals mixed with the microbial community that carries out fermentation (Schmitt and Vries 2020). Frass improves soil nutrients and the impact of frass on plant growth has been explored for mealworms, house fly, and black soldier fly (Cickova *et al*. 2014; Houben *et al*. 2020; Bloukounon-Goubalan *et al*. 2019).

Waste streams used to feed the larvae have an impact on the frass and affect plant responses (Schmitt and Vries. 2020). Experimental work on frass waste streams includes Brewery waste (Beesigamukama *et al.* 2020), vegetables (Quilliam *et al.* 2020), ryegrass (Menino *et al.,* 2020), sewage sludge (Liu *et al*. 2020), Coffee husks (Putra *et al*. 2017; Rosmiatti *et al.* 2017) and poultry manure (Xiao *et al*. 2018). Therefore, conclusions on frass as a soil improver cannot be made as frass quality depends on the substrate used as feed to the BSFL (Menino *et al.* 2020; Schmitt and Vries 2020).

Frass is associated with anti-pathogen properties in plants (Quilliam *et al*. 2020; Kawasaki *et al*. 2020). Chitin of insects is known to contain plant defense mechanisms and at times increase resistance to pests (Quilliam *et al.* 2020). A study conducted by Quilliam *et al*, (2020), showed that frass one of the trials of the fertilizers on cowpea plant was severely attacked by Fusarium wilt (*Fusarium oxysporum*) and the trial was terminated. Cowpeas were less attacked by Fusarium wilt compared to commercial fertilizer. The total number of dead plants decreased with an increase in frass treatment level, yet the damage was below that of the commercial fertilizer. Kawasaki *et al.* (2020) assessed the microbial load in all the treatments, frass was found to contain more Bacillaceae (22.91 %), *Sporosarcina* (13.21 %), and Xanthomonadaceae (9.82 %) respectively. Xanthomonadaceae contains a genus that attacks plants, and it was the third-highest in the microbial classification. However, plants grown in frass yielded high fresh weight compared to other treatments. This is evidence that frass can work as an anti-pest or pathogen agent for plants.

Pot trials conducted by Rosmiati *et al.* (2017) observed impact on growth on lettuce (*Lactuca sativa* Var. Crispa) using cow dung manure, coffee husk, and frass derived from coffee husk through BSFL bioconversion. All the plants grown in pots treated with manure significantly had high yield in relative to frass. Putra *et al.* (2017) conducted a similar experiment to assess nutrient utilization and harvested biomass. Lettuce grown in manure yielded the highest dry, and fresh biomass and utilized the most nitrogen, while plants grown in frass utilized the most phosphorus and potassium. It can be said that frass derived from coffee husk can be used as a biofertilizer, but more attention should be given to the bioconversion process to further balance nutrient utilization.

Three field-scale trials were performed by Quilliam *et al.* (2020) where frass by-products were applied at different treatment rates and the response of yield as a measuring parameter. Frass

as an organic fertilizer performed well, but it needed to be supplemented with commercial fertilizers to achieve the desired yield. A high yield of shallots and peppers was achieved in brewery waste supplemented by a commercial fertilizer in three different harvest stages, followed by brewery biofertilizer and poultry biofertilizer that performed better than the commercial fertilizer alone. Poultry biofertilizers and commercial fertilizers combined did not yield different amounts compared to the stand-alone biofertilizers. The highest grain yield was achieved at 10 t ha^{-1} of frass supplemented with 300 kg ha^{-1} of commercial fertilizer and above-ground dry biomass was achieved at 10 t ha^{-1} supplemented with 150 kg ha^{-1}. These results are in line with Beesigamukama *et al.* (2020a) who achieved the highest grain yield at the highest frass application rates.

Microbes and nutrients are found in solid compost, manure, and biofertilizers (Schmitt and Vries 2020). The availability of the nutrient and microbes can be transferred from solid soil improvers to an aqueous solution. (Farb 2012; Edwards *et al*. 2016). These aqueous solutions or compost teas have been regarded as plant health, growth, and crop yield improvers yet the primary mechanism of the aqueous solution is not yet fully understood (Pant *et al*. 2011; Tan *et al*. 2021). Compost teas are the aqueous solutions obtained from infusion or decoction of compost materials in water for a longer period (Tan *et al*. 2021).

Compost teas can be prepared using different methods depending on the aim of the experiment, and mostly, they can be prepared using aerated or non-aerated methods (Edwards *et al*. 2016; Tan *et al*. 2021). The preparation of non-aerated tea involves low oxygen conditions when extracting the tea, while aerated tea involves the exposure of maximum oxygen during the extraction processes (Pant *et al.* 2011). Microbial activity in the final product of aerated tea can be enhanced by adding sugars, grain, humic acid, and molasses. There is limited information regarding the effectiveness of these additives on the quality of the frass tea responding to growth (Tan *et al*. 2021)

According to literature, frass tea from BSFL frass has not yet gained popularity to be utilized as a liquid biofertilizer or biopesticide compared to vermicompost tea derived from different substrates (Edwards *et al*. 2016; Renčo and Kováčik 2012; Surrage *et al*. 2010; Pant *et al*. 2011). In addition, Frass tea from the BSFL frass is receiving attention as a biofertilizer for the soilless cultivation of leafy vegetables using aerated, non-aerated, and aerated additives (Tan *et al*., 2021). The application of frass tea improved crop yield, total carotenoids, and

glucosinolates in plant tissues (Tan *et al*. 2021). More studies on frass tea are required to evaluate frass tea's properties as a potential biopesticide and plant disease suppressant. In this study, the frass tea used was non-aerated without any additives as mentioned above.

Given the above, this study aims to (a) to determine if BSFL frass can be used as organic fertilizer by comparing growth parameters of *Amaranthus hybridus* in response to increased BSFL frass addition relative to commercial fertilizer and control, and (b) evaluate the efficiency of frass tea as biopesticides for crop pests. We hypothesized that **(**a) an increase in frass application levels does not have an impact on the plant yield, height, the number of leaves, chloroform content compared to control and commercial fertilizer, and (b) frass tea has no effect on crop pest control when used as a biopesticide, and lastly, with the following research questions: (a) Can black soldier fly frass be used as an effective organic fertilizer?, and (b) Can frass tea reduce or control pests from leafy vegetables?

Methods

Experimental layout and trial maintenance

. In this study, Amaranthus plant was also used because they germinate fast and are quite hardy. *Capsicum L*. was used to test for the phytotoxicity of the frass. The response of amaranth plants to frass was assessed using a randomized block design. There were four treatment levels: commercial fertilizer (NPK), Frass 5g, Frass 10g, and Frass 20g, and controls that were replicated ten times. River sand was sieved through a KingTest sieve (2.0 mm) and air-dried (**Table 1**). The soil was transferred into pots (16.5 cm x 24 cm x 19 cm), lined with mesh to prevent soil loss, and five kilograms of soil was added to each pot. Treatments were mixed into the top 10 cm of the soil. Seeds were propagated for two weeks on seedling trays before being transplanted to the pots. Each pot contained one seedling. Preparation and experiments were done in a greenhouse.

Irrigation took place on alternate days with pots receiving an equal amount of water and irrigating until saturated. Weeds were removed every time irrigation took place (Zahn and Quilliam *et al.* 2017). The number of leaves was counted four weeks after the transplant and on the final day of harvest. Plant height was measured at four- and eight-weeks intervals. Chlorophyll content was also measured once a week for eight weeks. Plant height was measured in centimetres. Chlorophyll content was measured using a chlorophyll content meter (CCM-200). Plants were harvested after eight weeks, dried, and weighed (Temple *et al*. 2013).

Fertilizer application rate

The optimal NPK application ratio for Amaranthus is 1:1:1, with an application rate of 100 kg/ha for each of the macro nutrients (nitrogen, phosphorus, and potassium) as described by Abayomi and Adebayo, 2014.. A similar application rate for the pot trials was calculated by assuming that the cultivation depth is 0.2 m and bulk density (BD) of the soil is 1.5 $g.cm^{-3}$ (McKenzie *et al*. 2002). Firstly, the volume of soil in one hectare was calculated (Equation 1), secondly, the volume was converted into mass by using BD (Equation 2). Lastly, the ratio of the mass of the soil in the pot to the mass of field soil was used to convert the nutrient load (Equation 3). To determine the mass of fertilizer to be added to the pot, the ratio of nutrient per mass of fertilizer as indicated on the fertilizer package was calculated (Equation 3). The

frass that was used in this experiment was from kitchen waste which included tomatoes, onions, cabbage, lettuce, and carrots. Frass composition and soil analysis were done separately in an accredited lab (Labserve Analytical Services and EnviroTek Labs).

Table 1: Selected nutrient composition of the soil and frass.

Selected nutrient composition of the soil and frass.

Soil nutrient composition								
pH	Bulk density	N	P	K	Na	Ca	Mg	
	g cm^{-3}			mg kg^{-1}				
6.2	1.5		1	23	31	534	109	
Frass nutrient composition								
pH	Moisture	N	P	K	C	Ca	Mg	C:N
				mg kg^{-1}				
9	248 000	23 000	22 000	65 000	790 000	11 000	3 000	350 000

Volume of the soil 1 ha with field depth of 0.2m.

$$Lenght \times Width \times Depth \quad (1)$$

$$= 100m \times 100\,m \times 0.2m$$

= 2 000m^3

From the volume of soil and bulk density of sandy soil, the mass of the soil in 1 ha is obtained.

$$BD \times Vol \quad (2)$$

$$= 1.5\,Mgm^{-3} \times 2000m^3$$

$$= 3\,000Mg$$

$= 3\,000\,000\,000\,g$ of soil in 1 hectare and 0.2 m soil depth

$$\frac{Pot\ soil\ (g)}{Field\ soil\ (g)} \times N\ application\ per\ hectare\ (g) = N\ application\ per\ pot\ (g) \quad (3)$$

$$\frac{5\ 000\ g}{3\ 000\ 000\ 000\ g} \times 100\ 000g \qquad = 0.167g\ \ of\ N\ fertilizer\ per\ pot$$

Fertilizer **x** with a nutrient ratio of 1:0:1 (180g/kg N, 0 P and 180 g/kg K), together with Superphosphate (50g/kg P), were applied at the following rates:

$$\frac{N\ (g)\ applied\ per\ pot}{N(g) in\ 1\ kg\ fertilizer} \times 1000g\ of\ fertilizer\ = Total\ g\ of\ fertilizer\ needed$$

(4)

$\frac{0.1667}{180} \times 1000\ = 0.93\ g\ 1{:}0{:}1\ fertilizer$ for N and K nutrients

$$\frac{0.1667}{50} \times 1000\ = 3.3\ g\ of\ Superphos\ for\ P\ nutrient$$

For the frass treatment, the following calculations were made:

Optimal frass content was aligned with the N and P content in the NPK treatment.

N content is 0.1667 g per treatment. The average N content in the frass is 2.26%, dry mass.

$$\frac{0.1667\ g\ N\ per\ pot}{2.26\ g\ N\ in\ 100g\ frass} \times 100\ g\ frass\ = 7.38g\ of\ dry\ frass\ per\ pot$$

The average moisture content of frass is 24.8%, so to correct for the moisture content, we adjust as follows:

$$x - x(0.248) = 7.38$$

$$0.752x = 7.38$$

$$x = 10\ g\ \ frass\ to\ apply\ per\ pot$$

Frass at half rate is 5 g, and frass as the double rate is 20 g (see **table 2**)

A rate of 9.8 g of frass per pot, equates to about 6 tons of frass per hectare:

$$\frac{3\,000\,000\ kg\ of\ soil\ in\ 1\ hectare}{5\ kg\ of\ soil\ per\ pot} \times 0.01\ kg\ of\ frass\ per\ pot$$
$$= 6\,000\ kg\ of\ frass\ per\ hectare$$

Table 2: Summary of treatments per pot. Values are read per column.

Nutrient	Control	NPK fertilizer	Frass, ½ load	Frass, optimal	Frass, x2 load
Application rate kg/ha	0		3000 kg	6000 kg	12000 kg
Application rate g/pot	0		5 g	10 g	20 g
Nitrogen ($g\ \ ha^{-1}$)	0	100	50	100	200
Phosphorus ($g\ \ ha^{-1}$)	0	100	50	100	200
Potassium ($g\ \ ha^{-1}$)	0	100	150	300	600

Frass maturity testing using phytotoxicity analysis.

Phytotoxicity is a simple technique that can be used to understand the quality or condition of substances that can pose negative effects to plant growth (Baral and Parade., 2011). This technique is simple, cost-effective, it can be performed in a short period, and it can be replicated (Wang *et al*. 2001). Furthermore, phytotoxicity is used to test composting procedures that involve decomposition and maturity from composts or any form of inorganic or organic fertilizers (Baral and Paradelo 2011). Phytotoxic on compost can be potentially caused by factors such as phenols from waste, and acids (acetic, propionic, and butyric) (Baral and Paradelo 2011; Song *et al*. 2021).

Capsicum L. seeds were used to assess the toxicity of the frass following Xiao *et al.*, (2018) method. The samples of the frass were prepared in triplicate (Xiao *et al.*, 2018). A total of 100 *g* of frass was mixed with deionized water at a ratio of 50 *g* per 1 liter (Song *et al.,* 2020). The solution was left to shake for 24 hours at 130 rpm and left for a day to cool down. The supernatant was separated from the pellet after 10 minutes of centrifugation (40 ml) at 6 000 rpm (Xiao *et al.*, 2018). Twenty seeds of *Capsicum L*. were evenly distributed in a 15 cm diameter petri-dish and Whatman no.1 filter paper with a 5 ml aliquot of the frass supernatant (Vaid *et al.*, 2011; Xiao *et al.*, 2018; Setti *et al.*, 2019). Deionized water was used as a control in triplicate (n=3) and the Petri-dishes were sealed with a sellotape. The seeds were kept in a

controlled environment at room temperature (25 °C and 63.5 % RH) and 24-hours photoperiod (16h Light: 8h Dark) for seven days following Vaid *et al*, (2011). Root length and the number of germinated seeds were assessed to calculate the Germination Index (GI%) following Beesigamukama *et al.*, (2020[b]) and Palma *et al.*, (2020). The root length of the germinated seeds was measured in millimeters (mm) using a light microscope (Carl Zeiss Discovery V12, Jena, Germany).

$$GI\% = \frac{(GI_{rsg}(\%)\ x\ GI_{rrg}\ (\%))}{100} \qquad (4)$$

Where:

$GI_{rsg}(\%)$ is the relative seed germination and $GI_{rsg}(\%)$ is the relative root growth of *Capsicum L*.

$$GI_{rsg}(\%) = \frac{germinated\ seeds\ in\ \ frass\ aliquot\ treatment}{germinated\ seeds\ in\ control\ treatment} \text{ x } 100 \qquad (5)$$

$$GI_{rrg}\ (\%) = \frac{mean\ root\ length\ in\ frass\ aliquot\ treatment}{mean\ root\ length\ in\ control\ treatment} \text{ x } 100 \qquad (6)$$

The seed germination index is evaluated through the percentage of the seeds germinated in a single petri-dish. GI% less than 50% is phytotoxic, 50% - 80% is moderately phytotoxic and 80% and above is not phytotoxic (Beesigamukama *et al.*, 2020b; Xiao *et al.*, 2018).

Liquid bio-fertilizer preparation.

The frass was stored to dry up for more than a year at room temperature 25 °C. One kg of frass was prepared and mixed with distilled water in a 20L bucket at a ratio of 1:20 following the method described by Ramya *et al*, (2015). Brewed frass was soaked in distilled water for 24 hours, while Boiled frass was boiled until it reached boiling point. The extracts were left for a day to cool down and thrown through a 2.0 mm sieve to separate the solution from the residuals after boiling and filter the solution using a Whatman No. 42 filter paper. The aliquot was centrifuged at 10 000 rpm for 10 minutes and stored as described by Monisha and Rameshaiah. (2016).

The aliquots were sealed tight after centrifugation and stored at room temperature, 25 °C for three months to reach maturity (Monisha and Rameshaiah 2016; Churilova and Midmore 2019). The wetting agent added at 0.15 ml of wetting agent (G49 WETTER, Surfactant**)** was added in a proportion of 0.15ml:1 in the 20 liters of the liquid biofertilizer. The frass liquid biofertilizer was applied as a foliar spray to the plants (both sides of the leaves) until the solution started dripping to the ground of the pot. The foliar spray was applied at a three-day interval after transplanting the seedlings into the pots (Ramya *et al*, 2015). Leaf damage was calculated using the proportion of damaged leaves per replicate.

Statistical Analysis

All the analyses were done using R v. 4.0.4 (R Core Team, 2021). All the data were tested for normality using the Shapiro-Wilks test and homogeneity of variances underwent a series of tests using Bartlett's test. Treatment effects were tested using ANOVA when all assumptions were met, and the non-parametric Kruskal-Wallis test was used when response variables were not normal or homoscedastic (Hollander and Wolfe., 1973). Multiple comparisons between treatment levels were performed using the Kruskal-Wallis test. A generalized Linear Model (GLM) under the "lme4" package (Bates *et al*., 2015) was applied to compare leaf damage between boiled, brewed frass treatments and a control.

Results

Growth parameters

All dependent variables varied significantly in response to the treatments (**Table 3**). The dry biomass of *Amaranthus hybridus* in Frass 20g was almost three times that of the control, while NPK was more than six times. A similar trend was observed for the dry leaf biomass when NPK and Frass 20g were compared with Frass 5g. There were also significant differences between leaf count after four weeks. The NPK had twice the number of leaves relative to the control, Frass 5g, and Frass 10g. After eight weeks of leaf count on frass treatments, Frass 20g significantly differed from the Frass 5g. Moreover, the number of leaves in Frass 20g was twice that of the Frass 5g and three times that of the control (**Table 3**).

Plant height after four weeks differed significantly between treatments and a similar trend was observed after eight weeks (**Fig 3**). After four weeks of height measurements, plants grown in Frass 20g differed significantly from plants grown in Frass 5g and control. Frass 20g had high chlorophyll content (10.65 ppm) compared to all the treatments and the highest frass treatment level significantly differed from Frass 5g and control (**Table 3**).

Table 3: Dry biomass, leaf count, height, and chloroform of each applied treatment (mean ± se). The experiment was terminated after eight weeks. Different letters represent a significant difference in pair-wise comparisons according to the Kruskal Wallis Test. Asterisks `*` represents a significant impact on all the responses within each column. FWLC- Four weeks leaf count, EWLC- Eight weeks leaf count, FWH- Four weeks height, EWH- Eight weeks height, N- number of replicates, Mean- mean value of replicates per treatment.

		Dry weight	**Dry leaves**	**FWLC**	**EWLC**	**FWH**	**EWH**	**Chloroform**
Treatment	**N**	**Mean ± SE**	**Mean ± SE**	**Mean ± SE**	**Mean ± SE**	**Mean ± SE**	**Mean ± SE**	**Mean ± SE**
P-value		***	***	***	***	***	***	***
Control	8	$1.53^{a} \pm 0.13$	$0.61^{a} \pm 0.04$	$13.67^{a} \pm 0.60$	$23.38^{a} \pm 1.66$	$13.53^{a} \pm 0.59$	$26.93^{a} \pm 1.72$	$5.97^{a} \pm 0.36$
Frass 5g	10	$2.07^{a} \pm 0.14$	$0.88^{a} \pm 0.06$	$12.80^{a} \pm 0.51$	$25.00^{a} \pm 1.25$	$13.34^{a} \pm 0.49$	$30.39^{a} \pm 1.16$	$7.60^{ac} \pm 0.54$
Frass 10g	10	$3.00^{ab} \pm 0.25$	$1.31^{ac} \pm 0.10$	$16.10^{a} \pm 0.90$	$37.30^{ac} \pm 2.32$	$14.78^{a} \pm 0.53$	$34.73^{ab} \pm 1.42$	$9.19^{bc} \pm 0.51$
Frass 20g	10	$5.23^{bc} \pm 0.24$	$2.30^{bc} \pm 0.23$	$21.50^{ab} \pm 2.01$	$55.70^{bc} \pm 2.04$	$17.00^{ab} \pm 0.79$	$44.43^{bc} \pm 2.80$	$10.65^{b} \pm 0.75$
NPK	10	$10.05^{c} \pm 1.38$	$3.92^{b} \pm 0.46$	$37.60^{b} \pm 3.45$	$67.00^{b} \pm 3.40$	$24.36^{b} \pm 1.52$	$52.52^{c} \pm 2.39$	$9.39^{bc} \pm 0.82$

Signif. codes: *** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$

Treatments also had an overall significant effect on dry biomass (X^2= 40.29, Df= 4, p < 0.001). Multiple comparisons indicated that the control, Frass 5g, and Frass 10g were significantly smaller than NPK. However, Frass 20g showed a significant effect on the control and Frass 5g (**Fig 1**). A similar trend was observed in dry leaf biomass, (X^2= 28.36, Df= 4, p < 0.001, **Fig 2**)

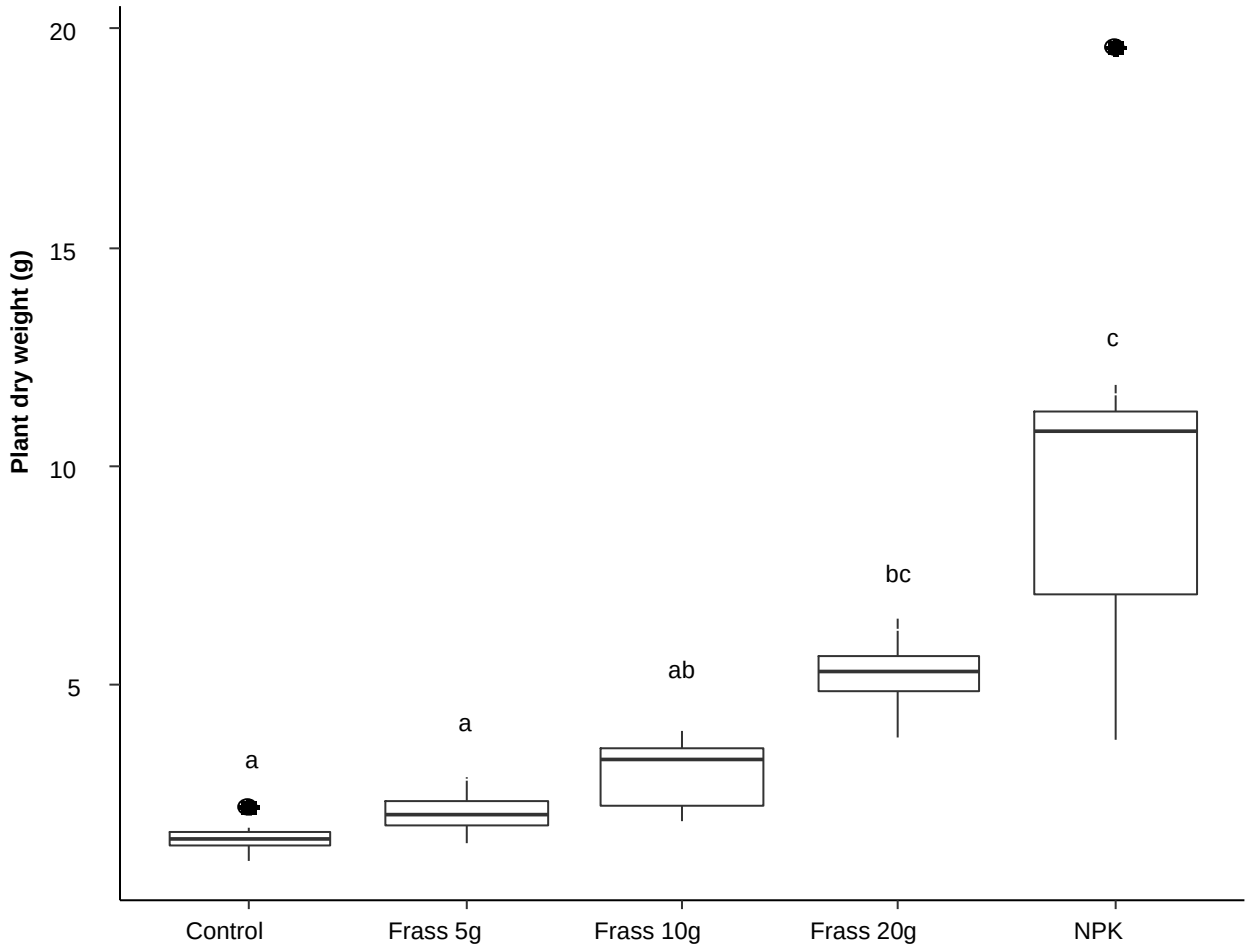

Figure 1: Dry biomass of *Amaranthus hybridus* grown for eight weeks in pots at different application rates of BSF frass and inorganic fertilizers. Different letters represent a significant difference in pair-wise comparisons.

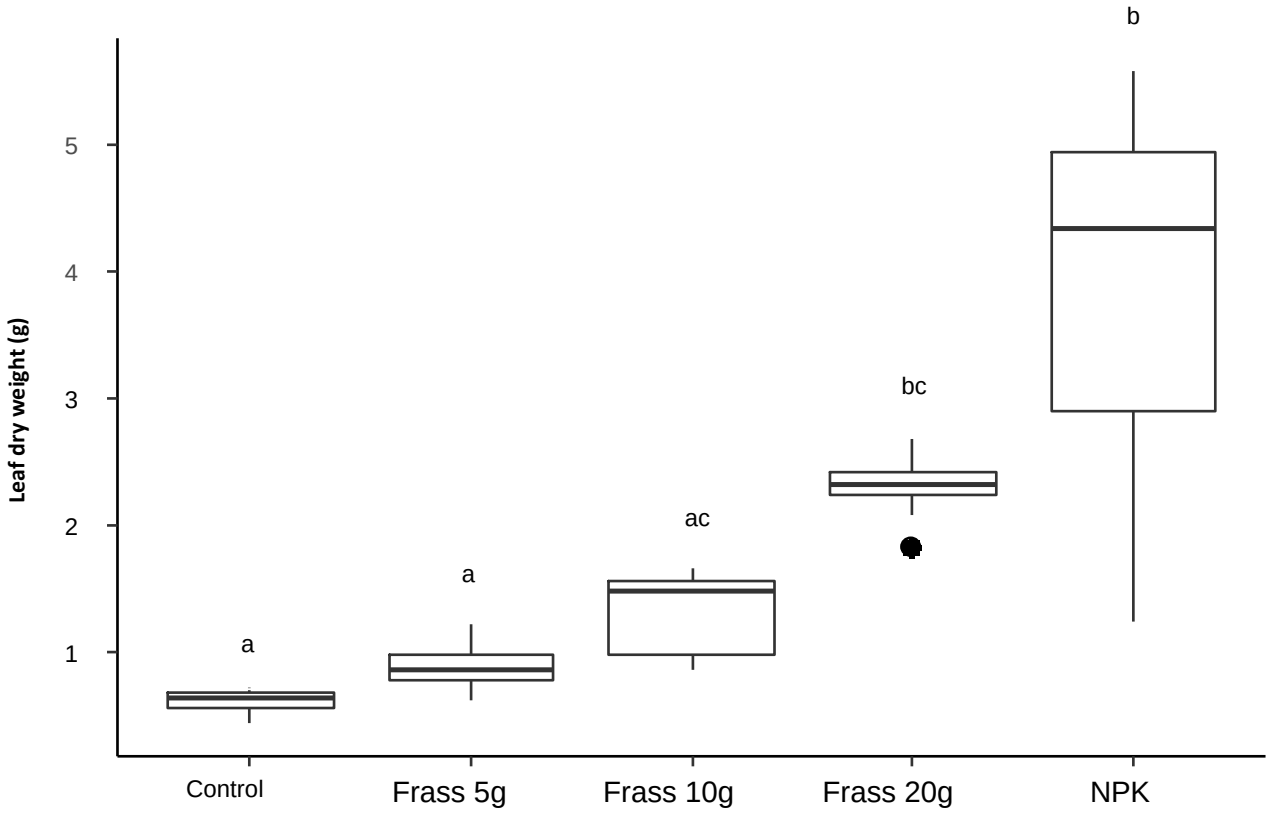

Figure 2: Dry leaf biomass of *Amaranthus hybridus* grown for eight weeks in pots at different application rates of fertilizers. Different letters represent a significant difference in pair-wise comparisons.

The height of *Amaranthus hybridus* responded positively to all treatments in the first four weeks (X^2= 30.711, Df= 4, $p < 0.001$) and after eight (X^2= 33.51, Df= 4, $p < 0.001$) weeks. In the first four weeks, only NPK were significantly higher than all, except Frass 20g treatments (**Fig 3A**). After eight weeks. Frass 20g was significantly higher than the control and Frass 5g. The NPK treatment kept a similar trend as compared to four weeks height (**Fig 3B**).

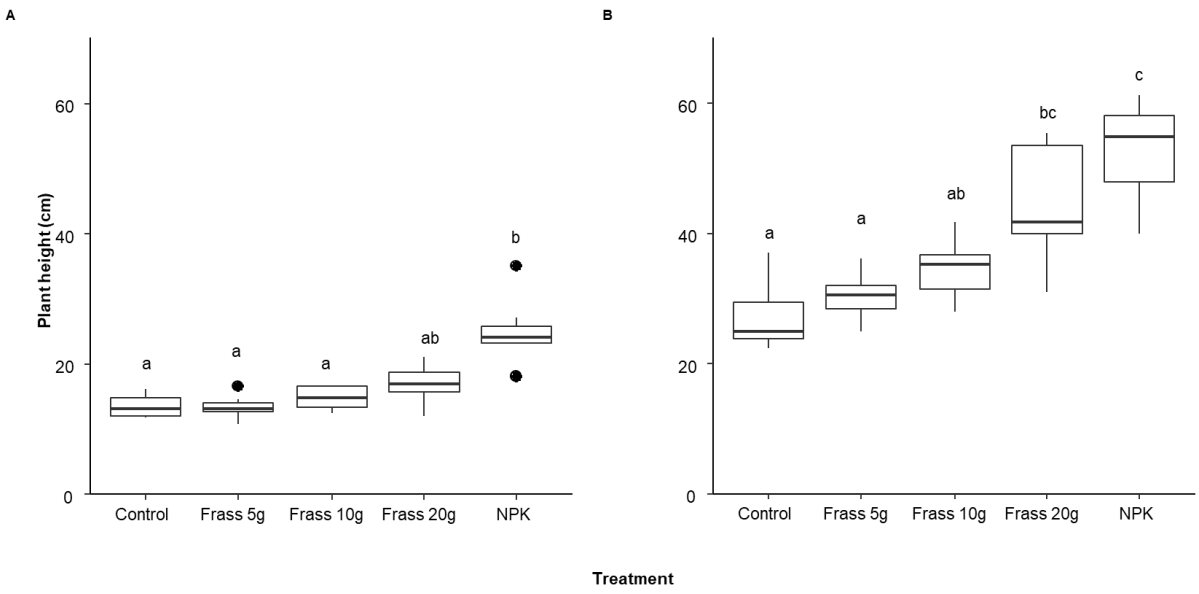

Figure 3: Height of *Amaranthus hybridus* after four (A) and eight (B) weeks at different treatment application rates of fertilizers. Different letters represent a significant difference in pair-wise comparisons.

The fertilizers had a significant effect on the number of leaves after four (X^2= 32.15, Df= 4, p< 0.001) and eight (X^2= 40.15, Df= 4, p< 0.001) weeks. Statistically, NPK treatment differed significantly from the control, Frass 5g, and Frass 10g after four weeks (**Fig 4A**). After eight weeks, a similar trend with a significant effect was observed in the control, and Frass 5g compared to Frass 20g. The NPK remained constant as observed in the first four weeks (**Fig 4B**). Frass treatments performance improved during the last four weeks relative to the NPK. Leaf count almost tripled in Frass 20g while it only doubled in the NPK

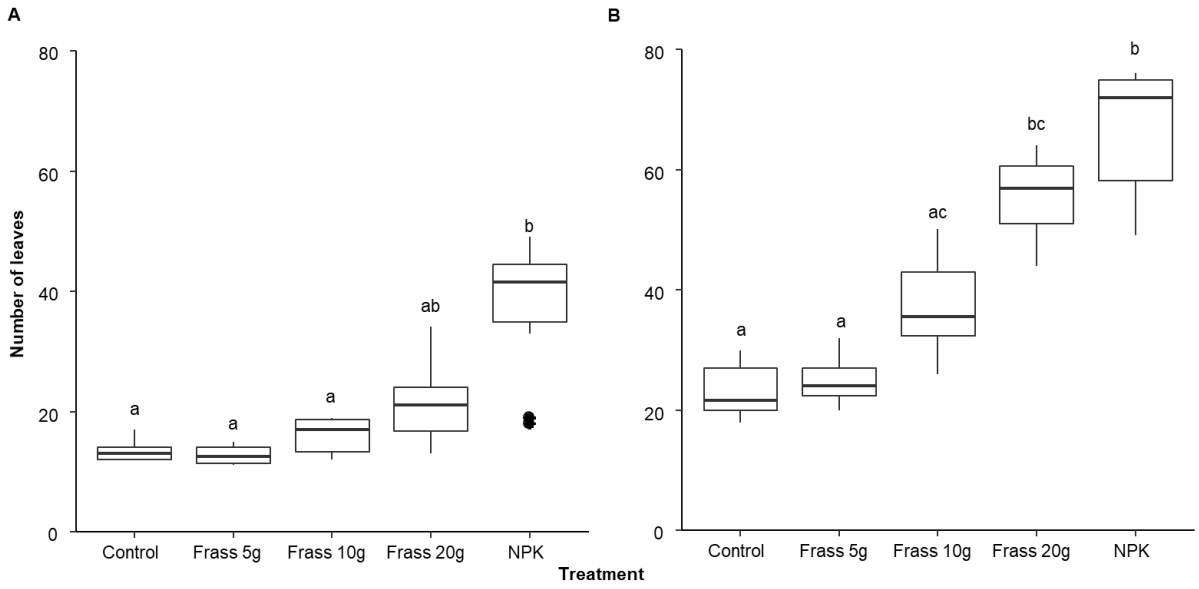

Figure 4: *Amaranthus hybridus* leaf count after four (A) and eight (B) weeks leaf count after eight weeks in pots. Different letters represent a significant difference in pair-wise comparisons.

The chlorophyll responded significantly to the treatments (X^2= 22.97, Df= 4, $p < 0.05$) in all the treatments. Frass 20g significantly differed from Frass 5g and the control (**Table 3** and **Fig 5**).

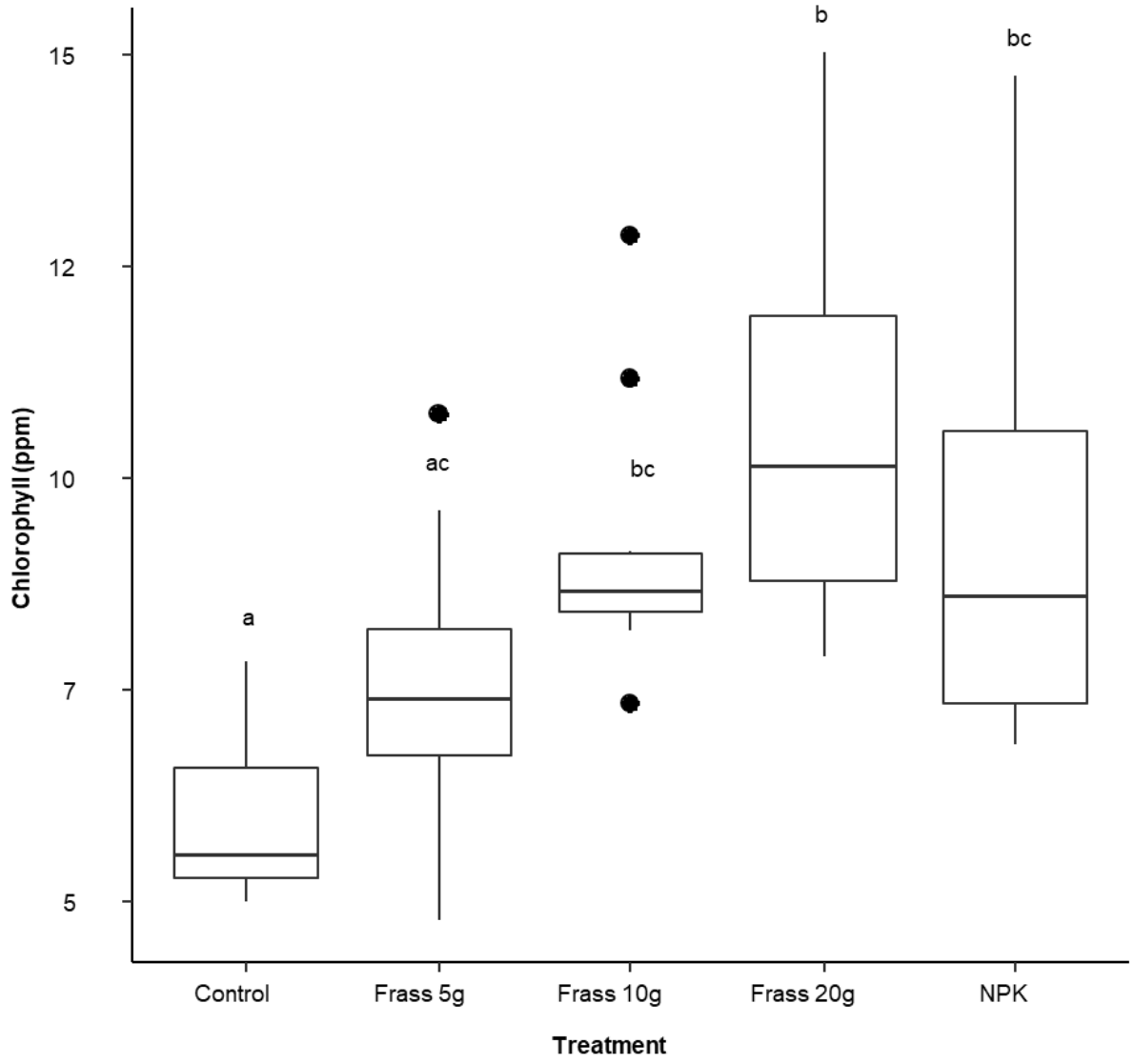

Figure 5: Chlorophyll content of *Amaranthus hybridus* grown for eight weeks in pots at different treatment application rates. Different letters represent a significant difference in pair-wise comparisons.

Frass maturity test through phytotoxicity

Root length responses differed significantly between the control and frass treatment (X^2= 51.298, Df= 1, p< 0.001) with an average of 10.38 and 3.07 millimeters respectively (Figure 6a). Seed germination of *Capsicum L.* in frass aliquot did not show any significant effect (X^2= 0.051, df= 1, p = 0.082) with an average of 17.72 compared to 17.75 for control (**Fig 6b**).

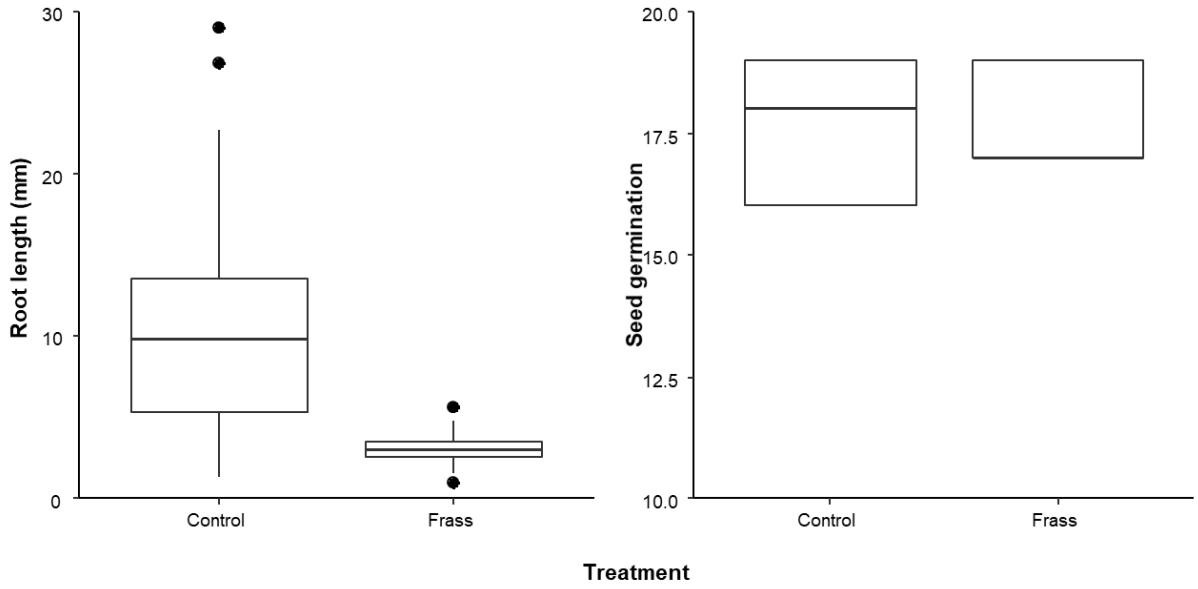

Figure 6: Mean root length (A) and seed germination (B) of *Capsicum* L. grown in Petri dishes. The control is distilled water, and frass is the aliquot extracted from frass organic fertilizer. Error bars represent the standard error of the mean (n= 3). Different letters represent a significant difference in pair-wise comparisons.

Frass tea applied as a foliar spray

All frass tea treatments did not have a significant impact when compared to the untreated sample (control). However, although our treatments were not significant, estimates from the Generalized Linear Model (GLM) suggest that in both treatments leaf damage was less compared to the control (**Table 4**).

Table 4: Comparison of leaf damage inflicted on *Amaranthus hybridus* under different treatments of frass tea when compared to a control. Asterisks `*` represents a significant impact.

	Estimate	Standard error	z value	P(>|z|)
Intercept	-0.774	0.458	-1.69	0.09 .
Boiled frass	-0.098	0.83	-0.119	0.90
Brewed frass	-0.627	0.754	-0.832	0.40
Significant codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1				

The boiled frass had more damage than the control and brewed frass (**table 4**). The proportion of the leaf damage and leaf count was performed to enable the percentage calculations of the total number of leaves that are damaged and run a GLM model with a binomial distribution (**Table 5** and **Fig 7**).

Table 5: Leaf count and leaf damage of Amaranthus hybridus. The experiment was terminated after five weeks. Different letters represent a significant difference in pair-wise comparisons according to the Kruskal Wallis Test.

Treatment	**N**	**Leaf count**	**Leaf damage**	**Damage (%)**
		Mean ± SE	**Mean ± SE**	
P-value		0.65	0.25	
Control	7	$12.57^{a} \pm 0.57$	$3.43^{a} \pm 0.86$	27.28
Boiled frass	8	$16.50^{a} \pm 3.26$	$6.75^{a} \pm 1.72$	40.10
Brewed frass	8	$12.50^{a} \pm 0.76$	$2.88^{a} \pm 0.77$	23.04

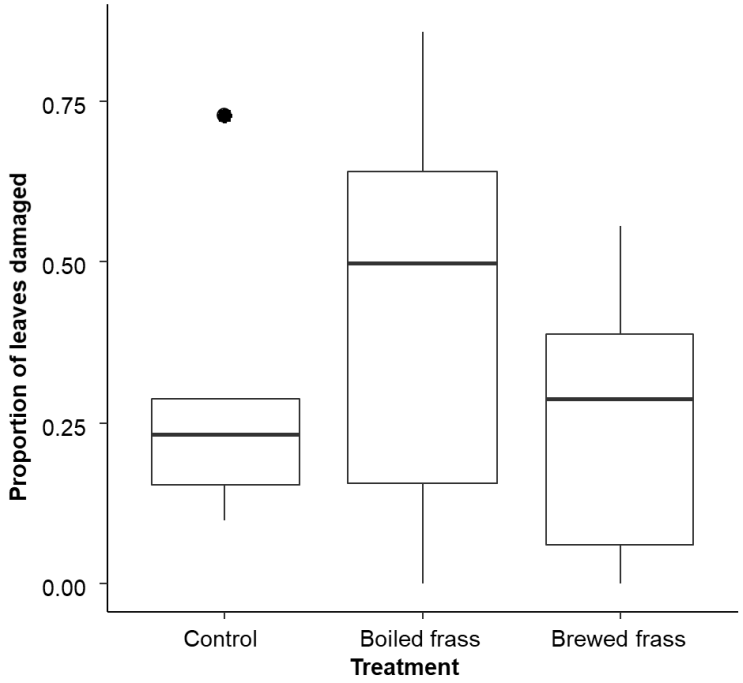

Figure 7: Leaf damage after five weeks comparing two different frass teas and an untreated sample (control). Different letters represent a significant difference in pair-wise comparisons.

Discussion

Black soldier fly larvae frass can be used as an organic fertilizer and improve plant growth. All the growth indicators increased monotonically with increased frass application. In general growth, parameters performed better with the addition of inorganic fertilizer, except when compared to Frass 20g which represents double the nutrient addition of the inorganic fertilizer.

Nutrients in inorganic fertilizer are available immediately as it is already in an inorganic form that can be utilized by plants. Organic fertilizer, however, has nutrients in organic compounds, and this needs to be mineralized first before the nutrients can become available to the plant (Beesigamukama *et al*. 2020[c]). As mineralization is a microbially mediated process, aspects that will influence the rate of mineralization include soil temperature, soil moisture, chemical environment (such as pH), and the quality of the organic material (Azeez and Van Averbeke 2010). Due to the slow release of nutrients, eight weeks was not enough to get adequate nutrients released for plant growth. This was observed by Azeez and Van Averbeke (2010)

after they conducted a mineralization trial and found that nitrite was immobilized in the first 10 days and on days 70 and 90, a high concentration of nitrite was recorded, and N also followed the same pattern. Nitrogen mineralization in the soil is estimated to peak 55 days after manure application (Azeez and Van Averbeke 2010).

The C:N ratio is another aspect that must be considered when interpreting the results of this study (Bloukounon-Goubalan *et al*, 2019; Beesigamukama *et al*. 2020ab). The C:N ratio of the frass was 35, which is relatively high. Any C:N ratio that is below 20 (a relatively high concentration of nitrogen), will allow organic nitrogen to mineralize in the soil and release excess nitrogen into the soil, where it can be utilized by plants (Sarpong *et al*. 2019). High C:N ratios above 20-25, can cause slower mineralization rates, as there is not enough nitrogen to fulfill microbial needs. In very high C:N ratios, a nitrogen negative period can be observed, where microbe activity is stimulated by the added carbon source, but the organic material does not provide enough nitrogen for microbial needs, and therefore the microbes can extract nitrogen from the surrounding environment (the soil), and the nitrogen in the soil decrease or is immobilized, with less available nitrogen for plants to grow (Kagata and Ohgushi 2012; Bloukounon-Goubalan *et al*. 2019; Houben *et al*. 2021; Beesigamukama *et al*, 2020b; Garttling *et al*. 2020).

This nitrogen will be released again later, but it would be advisable to add inorganic N in the early phase when a nitrogen-negative period is experienced. In our study, the lower plant parameters observed for some of the frass treatments can be indicative of slow nutrient release and would be expected in organic matter with a relatively high C:N ratio. The coarse soil substrate that was used, would typically not have a high microbial load, and thus the mineralization could be slow and ineffective (Kagata and Ohgushi 2012). Herein, if the nutrients were available in organic form, the mineralization process was slow and did not convert organic nitrogen into plant-available inorganic nitrogen forms (Bloukounon-Goubalan *et al*. 2019).

Inorganic fertilizer can greatly enhance soil fertility and has been responsible for high-yield crop production. However, inorganic fertilizers have several negative environmental impacts such as eutrophication and acidification of terrestrial ecosystems that alter biodiversity and their functioning (Choi *et al*. 2009; Schmitt and Vries 2020). The use of organic fertilizers has gained popularity in the past decade as an alternative to increase soil fertility with less or no

negative environmental health problems (Quilliam *et al*. 2020; Alatter *et al*. 2016). Vegetable crops require labile nutrients such as carbon, nitrogen, and phosphorus for proper growth, and the introduction of insect frass into the soil assist with soil nutrient that is ready to be absorbed by plants, trigger microbial activities and root formation responsible for plant growth (Houben *et al*. 2020, Terfa 2021).

Frass quality should vary depending on the substrate (i.e waste stream) used to feed the BSFL. The activation of microbes in soil amended with frass, bacteria like *Bacillus,* and nitrifying bacteria that are found in the nitrogen cycle assist with converting organic nitrogen into plant-available forms that are required by plants for photosynthesis (Terfa 2021; Houben *et al.* 2020; Paillat *et al.* 2020). Nitrification is essential for agricultural purposes to boost plants with nitrogen as a limited, important nutrient in the environment (Terfa, 2021). Frass and the commercial fertilizer were not combined to test synergistic ability to acquire nutrients for optimal plant growth, instead, they were tested independently. However, synergistic effects were observed in other studies (Quilliam *et al*. 2020; Houben *et al*. 2020).

In the first few weeks, nutrient supply was inadequate to provide plant growth (Azeez and Van Averbeke 2010, Agustiyani *et al*. 2021, Xiang Wu *et al*. 2021, Song *et al*. 2021). Dry biomass of *Amaranthus hybridus* was achieved at the highest frass treatment rate (5.23 g). In contrast to Zahn *et al*. (2017), dry biomass of spring onions was achieved at low frass treatment rates (150 mg). Xiang Wu *et al*. (2021) also found that frass at low application rates, improves rice plant height by 49.59 %, and at the highest application rate, growth was inhibited and reduced plant height, above-ground biomass, and dry matter by 9.98 %, 22.59 % and 22. 66 respectively. Alatter *et al*. (2016) found that maize under-performed with the addition of frass showing depressed traits compared to other treatments that went through aerobic and microaerobic fermentation. It is likely BSFL frass lacked key nutrients or poor aeration in the soil that led to phytotoxic components.

The highest chlorophyll was observed in *Amaranthus hybridus* leaves in frass 20 g treatment. These results are comparable with that of Agustiyani *et al*. (2021) who treated Pak choi plants. Moreover, maize plant leaves showed a similar tendency of high chlorophyll content following (Cai *et al*, 2020). Increased frass addition improved nutrient absorption that resulted in improving chlorophyll biosynthesis optimization. More photosynthesis pigments could have improved plant growth and crop yield (Agustiyani *et al*. 2021; Purbajanti *et al*. 2019).

Overall, there was no significant difference between the highest frass application rate and the commercial fertilizers. In an agroecological context, it is important to consider the input and output optimization of frass as a soil amendment to make it feasible for local farmers since the pricing of synthetic fertilizers is quite high for small-holder farmers in Sub-Saharan Africa (SSA) (Zahn *et al*. 2017).

The phytotoxicity results suggest that frass aliquot is phytotoxic. All the results were below the phytotoxicity threshold (<50 %). The relative seed germination in all the frass replicates was above 94 % and the relative root growth was above 25 %. Song *et al*. (2021) performed a phytotoxicity test in three different frasses. Fresh frass out of the three frasses yielded low GI indices. Frass used in this experiment was stored in a dry area at a room temperature of 25 °C for over a year. In contrast to Song *et al*. (2021), the fresh frass could be affected by other factors such as composting and the availability of phenols. Poor GI in fresh frass did not have phenols as phenols take up to 12 months to be at least 95 % degraded.

Lower seed germination indices are associated with high electroconductivity (EC) and ammonium concentration that hinder plant growth. EC and ammonium concentration were not analyzed in the frass nutrient composition (Beesigamukana *et al*. 2020b). Radical elongation from the *Capsicum L.* plant was observed, and the relative seed germination was higher in control than frass replicates. This can be explained by the overall GI that was below the moderate phytotoxic indices. We recommend that the key parameters stated above (i.e pH and EC) should be quantified in future studies.

The preparation of frass tea as a biopesticide was done without adding any additives such as sugars or molasses that can potentially cause environmental damage (Edwards *et al*. 2016). For example, *Salmonella* bacteria are found in animal manure that can be processed to produce compost tea (Edwards *et al*. 2016). The production of teas as biofertilizers or liquids to suppress plant diseases has been done mostly in vermicompost processing (Renčo and Kováčik 2015; Surrage *et al*. 2010; Edwards *et al*. 2016). Solid frass and vermicompost tea from earthworms have been used as biofertilizer and biopesticide for crops (Quilliam *et al*. 2020; Klammsteiner *et al*. 2020; Surrage *et al*. 2010) as well as the use of vermicompost tea as plant disease suppressor and nematodes repellent (Edwards *et al*. 2016; Renčo and Kováčik 2015).

Edwards *et al*. (2016) showed that the vermicompost tea applied at different rates suppressed *Verticillium* wilt significantly. The fact that most microorganisms from brewed frass passed to the tea while boiling frass could have killed some microorganisms and might explain why brewed frass performed better than boiled frass hen boiling that is capable of repelling pests and other microorganisms responsible for plant defense against pests (Edwards *et al*. 2016).

Teas produced can have reduced or no significant effect on disease and parasitic nematodes, but they can be effective as soil improvers. Vermicompost tea applied as a biopesticide to control two nematodes (*Globodera rostochiensis* and *Globodera pallida*) species was reported to have no significant impact on leaf damage, but improved stem weight and stem height of potato plants (Renčo and Kováčik 2015).

Understanding how frass improves plant growth and reduces plant damage from pests is important for horticulture purposes to improve crop yield production. This was a short-term (i.e few weeks) study and future studies should focus on longer-term (seasons) trials of growth and nutrient release impacts. In this study, the sandy soil did not provide any additional nutrients, but also possibly reduced mineralization rate, due to low microbial load. Soils with more microbes can facilitate mineralization much more effectively, and as such, can provide more nutrients for plant growth. Further studies that will pay attention to frass that is being produced from other waste streams could provide viable alternatives to inorganic fertilizers. Frass tea remain a promising pest management tool in agriculture after the leaf damage in this study was less in all the treatments. The methods used to prepare these teas should be considered an important aspect to improve the effectiveness of frass teas.

Chapter 3

Evaluation of the effects of frass treatments on *Amaranthus hybridus* through UHPLC-QTOF-MS and multivariate (chemometrics) statistical models

Abstract

Frass from insects has gained popularity in the past decade owing to its potential to be used as an organic fertilizer, and at times it consists of biopesticide properties for crops in horticulture. Those biopesticides could be secondary metabolites that serve as a plant defense mechanism. Frass is the combination of insect feces, exuvia, and uneaten residual waste. Leafy vegetables such as *Amaranthus hybridus* contain secondary metabolites that are necessary for different functions in protecting themselves against pests, and other functions in humans that exhibit various pharmacological activities such as protection against cardiovascular diseases, cancer, and diabetes. The metabolite distribution of green leafy vegetables has not been reported before. Therefore, it is essential to establish the effect of frass on the secondary metabolite distribution, if there is any, on the metabolite composition and distribution. This study aims to (a) evaluate the effect of frass on the metabolite distribution of a green leafy vegetable, and (b) to apply chemometric models to evaluate the latent metabolite perturbation in *Amaranthus hybridus* exposed to various frass treatment levels. Herein, ultra-high-performance liquid chromatography- quadrupole time-of-flight mass spectrometry (UHPLC-QTOF-MS) and some chemometric Software including XC-MS and R-Software were utilized to characterize different metabolites found in *Amaranthus hybridus*. A total of 30 metabolites were identified, including derivatives of coumaric acid, caffeic acid, ferulic acid, and flavonoids. In the PCA plot, samples were grouped according to frass treatment thereby suggesting a differential metabolite distribution pattern. Further studies are required to assess the number of metabolites within each treatment level and to establish the relationship between metabolite levels and frass treatments. Frass remains a promising option to substitute inorganic fertilizers, reducing global warming challenges and alleviating poverty in developing countries.

Keywords: Frass, BSFL, Black soldier fly, LC-MS, UHPLC-QTOF-MS,

Introduction

Most of the countries in Africa are suffering from hunger and poverty due to poor land management and low nutrients in the soil (Beesigamukama *et al*., 2020a). Agriculture in Sub-Saharan Africa (SSA) is constrained by soil quality challenges that include soil degradation and excessive use of inorganic fertilizers (Terfa 2021; Swanepoel *et al*. 2016). To tackle these challenges, strategies that will improve food security and alleviate poverty should be prioritized as the population size in SSA grows exponentially (Beesigamukama *et al*. 2020a).

Globally, inorganic fertilizers are used to improve plant yield and plant nutrients (Augastiyani *et al*. 2021). However, there are negative effects associated with the use of inorganic fertilizers such as being the catalyst of algal bloom and eutrophication in rivers caused by surface runoff of inorganic fertilizers from nearby crop fields (Schmitt and Vries 2020). Secondly, eutrophication alters the functionality and balance of biodiversity (Schmitt and Vries 2020). Inorganic fertilizers lead to soil acidity which results in poor crop yields (Schmitt and Vries. 2020). Approximately 51 % of soils in SSA are suffering from poor soil nutrients because of soil acidity, nutrient leaching, and phosphorus fixation (Bessigamukama *et al*. 2020a).

Organic fertilizers can be utilized as soil quality improvers (Houben *et al.* 2020). These nutrients are also found in organic fertilizers produced from insects such as black soldier fly (*Hermicia illucens*) and mealworm (*Tenebrio molitor*) (Houben *et al.* 2020; Zahn *et al*. 2017). Frass is the combination of insect feces, ingested, and uneaten waste (**Fig 1**) (Xiao *et al*. 2018). Moreover, the use of organic fertilizers (or frass) remains a growing interest globally because of the potential properties associated with soil fertility (Bloukounon-Goublan *et al*. 2019; Ohgushi and Kataga 2012). Organic fertilizers are environmentally friendly as they improve plant growth and reduce greenhouse gases emission compared to animal manure (Beesigamukama *et al*. 2020b).

Frass has been tested and applied in different edible plants such as lettuce (*Luctiva sativa* var. crispa), Pakchoi (*Brassica rapa* L.), and chilli pepper (*Capsicum annumi* var. 'Cayenne') to test the impact of frass on growth (Rosmaitti *et al.* 2017; Quilliam *et al*. 2020) Furthermore, it has

also been tested as a biopesticide (Klammsteiner *et al*. 2020; Kawasaki *et al*. 2020). The application of inorganic pesticides and fertilizers has been shown to have a negative effect on non-targeted species and genetic modification that result in emergence resistance pest species (Adeyemi 2010).

Figure 1: Byproduct, frass, from black soldier fly used as biofertilizer.

Pests are a major problem in agriculture as they affect crop yield (Kortbeek *et al*. 2018). To adapt to the environment, plants develop defense mechanism strategies to protect themselves against insects' infestation (Kortbeek *et al*. 2018). Over 2 000 species of plants in Africa have been reported to contain secondary metabolites including flavonoids that exhibit various pharmacological activities such as protection against cardiovascular diseases, cancer, and diabetes in humans (Steffensen *et al*. 2011; Adeyemi 2010; Ramabulana *et al*. 2020). Pseudocereals such as amaranth species have been shown to exhibit enhanced nutraceutical properties (Pasko *et al*. 2008). Phenols that have been reported from different parts of amaranth species are quercetin, *p*-coumaric, kaempferol, rutin, and ferulic (Pasko *et al*. 2008; Adeyemi 2010).

Amaranthus hybridus (**Fig 2**) belongs to the Amaranthaceae family with 70 amaranth species of which twenty of the amaranth species are edible (Steffensen *et al*. 2011). These species are widely distributed globally due to human travel and the shipment of goods from one place to another (Saker and Oba 2019). In the SSA, these plants grow naturally, mostly in cow kraals due to the availability of cow manure that improves plant growth (Dlamini *et al*. 2020). These plants also get cultivated for harvest at a matured stage (Ayodele *et al*. 2008).

Figure 2: *Amaranthus hybridus* experiments terminated eight weeks after transplant in pots. This is an image of the plant used this study. (Free nature images).

Animal manure or organic fertilizers, frass, are plant pathogen repellents that may affect secondary metabolite distribution in plants (Adeyemi 2010; Kawasaki *et al.* 2020; Schmitt and Vries 2020). The effects of these fertilizers are important in horticulture to improve crop yield and reduce plant pathogens (Kawasaki *et al.* 2020). Therefore, it is essential to establish the effect of frass on the secondary metabolite distribution. Metabolomics are therefore important to establish metabolite perturbation post-treatment with these organic fertilizers.

Several techniques such as Liquid Chromatography-Mass Spectrometry (LC-MS) have been used to characterize a vast number of natural compounds including polyphenols that are found in plants (Makita *et al*. 2016). LC-MS has allowed researchers to collect different plant samples for comparison through targeted and non-targeted approaches (Makita *et al*. 2016;

Summer and Hall 2013). The accuracy and robustness of this technique provide accurate analysis of hundreds of thousands of metabolites simultaneously (Gbashi *et al*. 2016).

Due to high dimensional data obtained from LC-MS, chemometric statistical models are used to give the biological meaning of the data (Makita *et al*. 2016). This sensitive mass spectrometry method coupled with chemometric models (SPSS) has been used to evaluate the effect of temperature on metabolites composition and it was shown that an increase in extraction temperature is directly proportional to the yield of the extracted flavonoids (Gbashi *et al*. 2016).

In this study, ultra-high-performance liquid chromatography- quadrupole time-of-flight mass spectrometry (UHPLC-QTOF-MS) and chemometric models including XC-MS and R-Software were utilized to characterize different metabolites found in *Amaranthus hybridus*. Furthermore, it was hypothesized that different frass treatment levels affect the metabolite distribution of *Amaranthus hybridus.* This study aims to (a) evaluate the effect of frass on metabolite composition of a green leafy vegetable, and (b) to apply chemometric models to evaluate the latent metabolite perturbation (distribution) in *Amaranthus hybridus* treated with various frass treatment levels.

Methods

Metabolite extraction

Metabolites were extracted from ground leaf powder following a method done by Nengovhela *et al.* (2020). The fine ground powder (2g) was mixed with 20 mL of 80 % aqueous methanol and sonicated for 20 minutes at room temperature (25 °C). The samples were centrifuged at 10 000 rpm for three minutes to separate the supernatant from the pellet. The supernatant was transferred to new clean tubes. A volume of 2 mL vials fitted with a 0.2 µmm conical bottom glass was used to insert samples. The samples were filtered using a 0.22 µmm microfilter into the vials.

LC-MS analysis

LC-QTOF-MS model LC-MS 9030 instrument fitted with a Shim Pack Velox C_{18} column (100 mm × 2.1 mm, 2.7 µm particle size) (Shimadzu, Kyoto, Japan) was used for metabolite separation. The column oven was set at 40°Cand a binary solvent mixture consisting of 0.1% formic acid in water (Eluent A) and 0.1 % formic acid in acetonitrile (Eluent B), at a constant flow rate of 0.3 mL/min was used for separation. The following gradient method was used: 0-2 min, 2% B, 2-24 min, 60% B, 24-25 min, 95% B, 25-27 min, 95% B, 27-28 min, 2% B, 28-30 min, 2% B and back to 0% B in 1 minute. Analyte elutions were monitored using a mass spectrometer detector under the following conditions: ESI negative modes; 3.5 kV interface voltage; nitrogen gas was used as nebulizer at flow rate 3L/min, heating gas flow at 10 L/min; heat block temperature at 400 °C, CDL temperature at 250 °C; 1.70 kV detector voltage and the TOF temperature at 42°C. For tandem MS (MSMS) experiments, sodium iodide was used as a mass calibration solution to obtain typical mass accuracies with a mass error below 1 ppm. Argon gas was used as a collision gas for MS/MS experiments using collision energy levels between 0 and 50 eV.

Statistical Analysis

Initially, LC-MS-derived data was used to conduct a principal component analysis (PCA) of the fertilizers. (Smith *et al*. 2006). All the Analyses were done in R, V. 4.0.4 (R Core Team 2021). All the data were tested for normality using the Shapiro-Wilks test and homogeneity of variances tested using Bartlett's test. Means and standard error of the mean were calculated using the package `Rmisc` in R software (Hope 2013). Treatment effects on metabolite

distribution were tested using ANOVA when all assumptions were met, and the non-parametric Kruskal-Wallis test was used when response variables were not normal or heteroscedastic (Hollander and Wolfe. 1973). Multiple comparisons between treatment levels were performed using the Kruskal-Wallis test.

Results and Discussion

Herein, UHPLC-QTOF-MS was utilized to separate and measure the masses of metabolites from the methanol extracts of a green leafy vegetable, The leafy vegetable showed metabolite distribution and variation in peak intensities at different frass treatment levels (**Fig 3**). Metabolite distribution in *Amaranthus hybridus* at different frass treatment levels, inorganic fertilizer, and the control were revealed. Chemometric models (Metabo-analyst) were used to unearth the latent metabolite perturbation in the *Amaranthus hybridus* plant treated with various frass treatment levels (**Fig 8** and **9**). As shown in Table 1, metabolites were identified in all the treatment levels and the control following metabolite identification keys presented elsewhere (Ramabulana *et al*. 2020; Nengovhela *et al*. 2020). Herein, a total of 30 metabolites were reported and include noticeable organic acids such as Coumaric acid, Caffeic acid, Ferulic acid, and flavonoids such as those containing the Kaempferol aglycone.

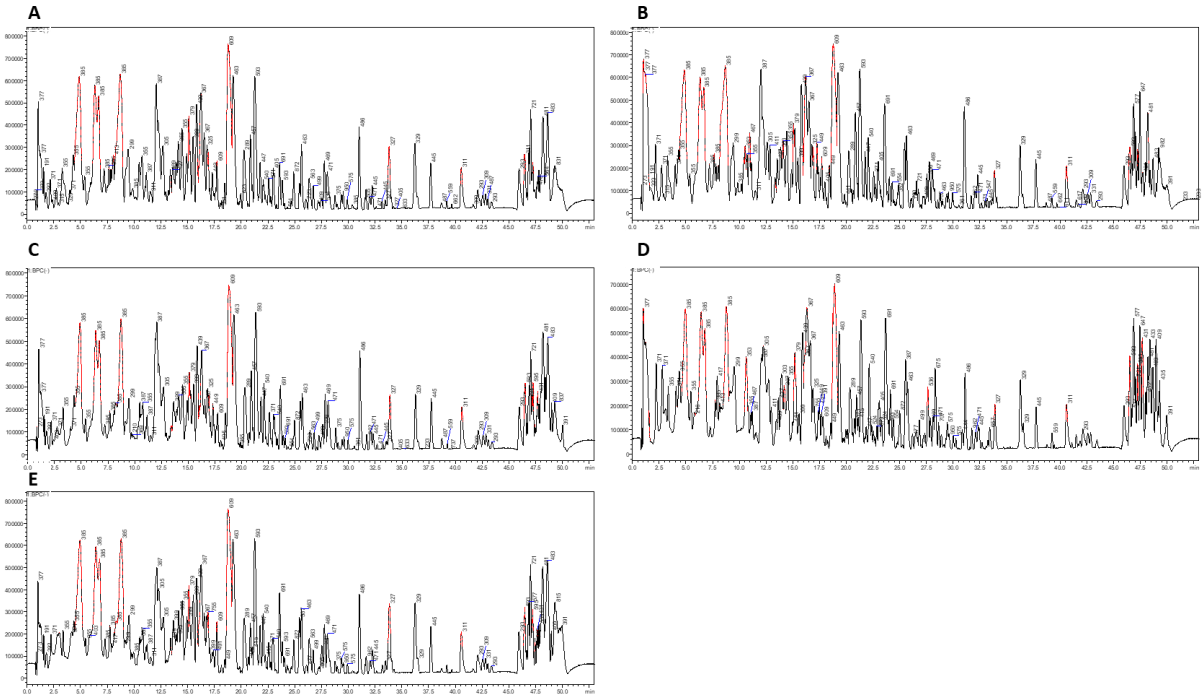

Figure 3: Representative UHPLC-QTOF-MS base peak ion chromatograms showing distribution patterns of metabolites in different frass treatment levels. (a) Control, (b) Frass 5g, (c) Frass 10g, (d) Frass 20 g and (e) NPK. The NPK acronym represent- commercial fertilizer.

Table 1: Tentatively annotated metabolites extracted from a green leafy vegetable, *Amaranthus hybridus*.

Number	m/z	Rt (min)	Fragment ions	Molecular Formula	Metabolite
1	371.06	1.24	209.0289, 191.0180, 133.0129, 129.0185	$C_{15}H_{16}O_{11}$	5-O-trans-Caffeoylgalactaric acid (Isomer 2)
2	371.06	3.37	209.0289, 191.0180, 133.0129	$C_{15}H_{16}O_{11}$	5-O-trans-Caffeoylgalactaric acid (Isomer 1)
3	385.075	4.184	134.0363, 191.0187, 147.0288	$C_{16}H_{18}O_{11}$	Feruloyl glucaric acid (Isomer 1)
4	385.076	4.866	134.0363, 191.0187, 147.0288	$C_{16}H_{18}O_{11}$	Feruloyl glucaric acid (Isomer 2)
5	385.076	8.123	134.0363, 191.0187, 147.0288	$C_{16}H_{18}O_{11}$	Feruloyl glucaric acid (Isomer 3)
6	385.076	8.614	134.0363, 191.0187, 147.0288	$C_{16}H_{18}O_{11}$	Feruloyl glucaric acid (Isomer 4)
7	355.1	8.64	193.0489, 178.0262, 149.0603, 160.0161, 134.0360	$C_{16}H_{20}O_{9}$	1-O-Feruloyl-beta-D-glucopyranose
8	385.076	8.797	134.0363, 191.0187, 147.0288	$C_{16}H_{18}O_{11}$	Feruloyl glucaric acid (Isomer 5)
9	353.09	8.81	191.0553, 135.0438	$C_{16}H_{18}O_{9}$	5-O-caffeoylquinic acid
10	609.14	13.79	447.0915, 446.0824, 327.0489, 285.0386	$C_{27}H_{30}O_{16}$	Kaempferol 3,7-digalactoside
11	337.09	14.19	191.0553, 163.0391, 119.0494	$C_{16}H_{18}O_{8}$	3-O-*p*-Coumaroliquinic acid
12	337.09	14.2	191.0552	$C_{16}H_{18}O_{8}$	5-O-*p*-Coumaroliquinic acid
13	367.1	14.6	193.0498, 134.0363, 191.0551	$C_{17}H_{20}O_{9}$	3-Feruloylquinic acid
14	403.16	15.58	241.1059, 197.1168, 181.0853, 134.1197, 137.0956	$C_{18}H_{28}O_{10}$	6-Deoxylamioside
15	625.14	15.99	463.0867, 343.0439, 301.0335, 300.0255	$C_{27}H_{30}O_{17}$	Quercetin 3-glucosyl-(1->6)-galactoside
16	609.14	18.72	343.0441, 301.0337, 300.0258	$C_{27}H_{30}O_{16}$	Quercetin-3-rhamnosyl-glucoside (Isomer 1)

17	609.14	18.79	591.1325, 243.0436, 301.0334, 300. 0256, 178.9969, 151.0022	$C_{27}H_{30}O_{16}$	Quercetin-3-rhamnosyl -glucoside (Isomer 2)
18	609.14	19.38	343.0436, 301.0339, 300.0264, 178.9981, 151.0020	$C_{27}H_{30}O_{16}$	Quercetin-3-rhamnosyl -glucoside (Isomer 3)
19	609.14	19.4	343.0436, 301.0339, 300.0264, 178.9981, 151.0020	$C_{27}H_{30}O_{16}$	Quercetin-3-rhamnosyl -glucoside (Isomer 4)
20	289	19.54		$C_{22}H_{10}O$	Catechin
21	593.15	19.69	285.0389, 284.0311	$C_{27}H_{30}O_{15}$	Kaempferol 3-O-rutinoside/robinobioside
22	505.1	20.55	301.0348, 300.0258	$C_{23}H_{22}O_{13}$	Quercetin 3-(6-acetylgalactoside)
23	515.12	21.06	353.0689, 191.0546, 179.0335, 135.0438	$C_{25}H_{24}O_{12}$	3,5-Dicaffeoylquinic acid
24	447.09	21.7	284.0305, 255.0279, 151.0021	$C_{21}H_{20}O_{11}$	Kaempferol hexose
25	593.15	21.73	327.0502, 285.0381, 284.0306, 255.0296	$C_{27}H_{30}O_{15}$	Kaempferol 3-O-rutinoside/robinobioside
26	463.1	21.97	301.0331.	$C_{21}H_{20}O_{12}$	Quercetin hexose
27	515.12	22.81	353.0852, 191.0545, 179.0335, 173.0439, 135.0434	$C_{25}H_{24}O_{12}$	3,4-di-O-Caffeoylquinic acid
28	519.11	26.88	314.0404, 299.0174, 271.0227	$C_{24}H_{24}O_{13}$	Isorhamnetin 3-(6-acetylgalactoside)
29	639.13	27.78	477.1031, 463.0862, 300.0259, 301.0338, 255.0289	$C_{31}H_{28}O_{15}$	Quercetin 3-(6-ferulylgalactoside) (Isomer 1)
30	639.13	27.79	463.0885, 300.0263, 301.0327	$C_{31}H_{28}O_{15}$	Quercetin 3-(6-ferulylglucoside) (Isomer 2)

Characterization of Caffeoyl derivatives

In the Amaranthus leaf extracts, five caffeoyl derivative molecules (**1**, **2**, **9, 23**, and **27)** were identified respectively. Molecules **1** and **2** at Rt 1.24 and 3.37 min had similar precursor ions at *m/z* 371.06 ($C_{15}H_{16}O_{11}$). These molecules yielded fragment ions at *m/z* 209 and *m/z* 191 that indicated the presence of ([aldaric acid-H]$^-$) and [(aldaric acid-H-H_2O]$^-$) that defines the presence of glucaric acid or galactaroyl acid (Cao *et al*. 2016; Oliveres-Caro *et al*. 2020). The precursor ion at *m/z* 371.06 yielded a caffeoyl cleavage as fragment ion at *m/z* 209 (Oliveres-Caro *et al*. 2020). Hence, these molecules are annotated as 5-O-trans-Caffeoylgalactaric acid isomers (Cao *et al*. 2016) (Refer to supplementary **Fig S1 and S2**). This molecule was also reported in a citrus plant, *Citri retuculatae* Folium (Cao *et al*. 2016).

Molecule **9** at retention time 8.81 min produced parental ions at *m/z* 353. 09 ($C_{16}H_{18}O_9$) (Refer to supplementary **Fig S9**). This molecule is fragmented to produce prominent daughter ions at *m/z* 191 that represent a free quinic acid due to the loss of caffeoyl moiety (Cao *et al*., 2016; Ncube *et al*. 2017; Clifford *et al*. 2003). This molecule was annotated as 5-O-caffeoylquinic acid in agreement with Ncube *et al*. (2017) who detected a similar molecule in *Centella asiatica* herb that can be utilized as a culinary vegetable or a medicinal herb.

Molecules **23** and **27** at retention times 21.06 min and 22.81 min were found to share a precursor ion at *m/z* 515.12 ($C_{25}H_{24}O_{12}$) respectively. Molecule **23** exhibited product ions at *m/z* 353.0689, at *m/z* 191.0546, at *m/z* 179.0335, and at *m/z* 135.0438. Similarly, molecule **27** has an extra peak at *m/z* 173 that differentiates it from molecule **23**. The absence of 4-acyl substitution that resulted in the loss of one caffeoyl residue (*m/z* 353) was the difference between the two molecules and the presence of caffeic acids (at *m/z* 173 and at *m/z* 179) and quinic acid (at *m/z* 191) (Nengovhela *et al*. 2021; Liang and Xu., 2016). The molecules were annotated as positional -isomers namely 3,5-Dicaffeoylquinic acid and 3,4-di-O-Caffeoylquinic acid respectively (Ramabulana *et al*. 2020; Nengovhela *et al*. 2021).

Characterization of Feruloylquinic derivatives

A total of seven molecules derived from the feruloyl group (**3**, **4**, **5, 6**, **8**, **9**, and **13**) were identified and annotated based on the fragmentation data. Molecules **3**, **4**, **5, 6**, and **8** at Rt 4.18 min, 4.86 min, 8.12 min, 8.61 min, and 8.79 min showed a parent ion at *m/z* 385 ($C_{16}H_{18}O_{11}$), and the daughter ions were at *m/z* 209 (due to the loss of aldaric acid), at *m/z*

193 that lost a ferulic acid moiety, at *m/z* 191 that represent a galactaroyl acid due to loss of water in the galactaroyl group, and at *m/z* 147 as glucuronide group moiety that lost both carbon dioxide and water (Olivares-caro *et al*. 2020; Apea-Bah *et al*. 2021; Cao *et al*. 2016) (**Fig 4** and Refer to supplementary **Fig S3**, **S4**, **S6**, and **S8**). Contrary to this study, Oliveres-Caro *et al*. (2020) annotated *m/z* 385 molecule as an O-feruloyl galactaric acid found in Calafate (*Berberis mycrophylla* G. Forst) extracts. Apea-Bah *et al*. (2021) annotated it as Feruloylaldaric acid in rice (*Oryza sativa* L.) extracts. In agreement with Cao *et al*. (2016) who annotated *m/z* 385 molecules as Feruloyl glucarate or galactarate isomers. In this study, these molecules are annotated as feruloyl glucaric acid isomers.

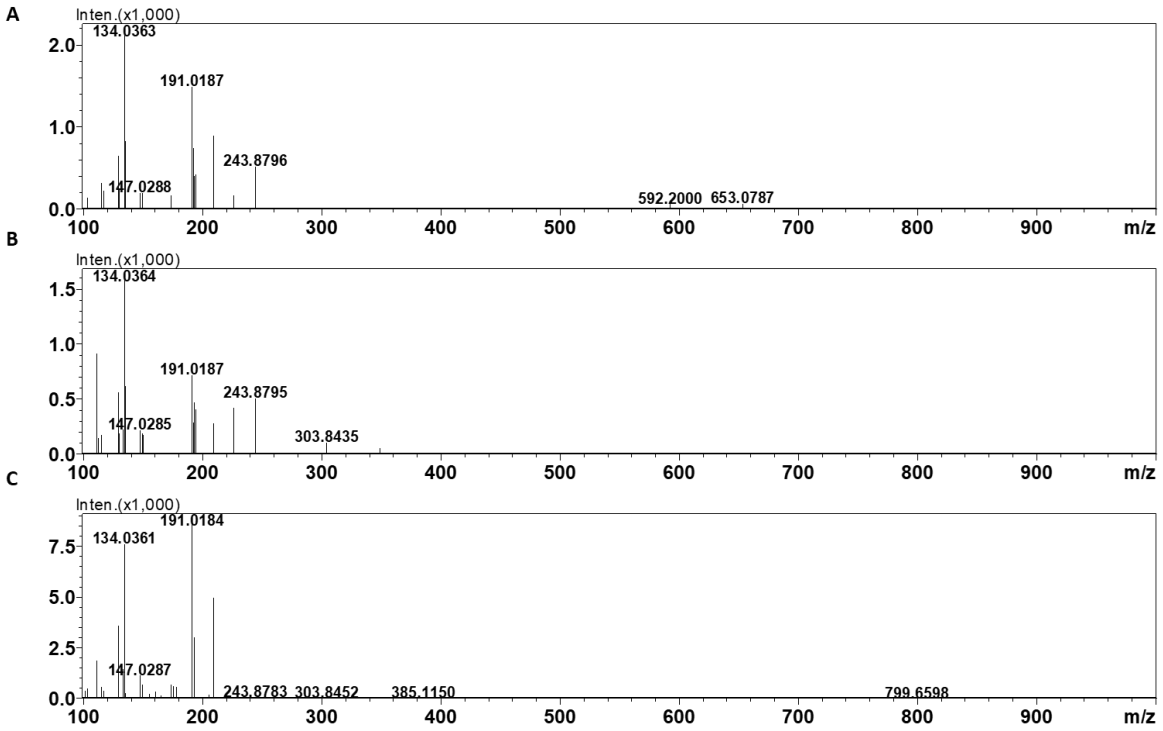

Figure 4: Representative mass spectra showing fragmentation pattern of (a) feruloyl glucaric acid (Isomer 1), (b) feruloyl glucaric acid (Isomer 2), and (c) feruloyl glucaric acid (Isomer 3).

Molecule **13** at Rt= 14.60 min showed a parent ion at *m/z* 367.10 ($C_{17}H_{20}O_9$) and daughter ions at *m/z* 193 that represents a feruloyl group, at *m/z* 191 representing a quinic acid group, and at *m/z* 134 representing ferulic acid that underwent decarboxylation (Cao *et al*. 2016; Ncube *et al*., 2017).In the spectrum, the base peak at *m/z* 193 was intense and it signals the positioning of the feruloyl residue as it was attached in position three (Ramabulana *et al*. 2020). This molecule was therefore annotated as 3-O-Feruloylquinic acid. The characterization of the molecule was justified by literature (Cao *et al*. 2016).

Characterization of kaempferol derivatives

Molecules **10**, **21**, **24**, and **25** were tentatively identified as kaempferol glycosides. Molecule **10** at Rt= 13.79 min showed a parent ion at *m/z* 609.14 ($C_{27}H_{30}O_{16}$) (**Fig S10**). The parent ion produced daughter ions at *m/z* 447 represents the loss of glucoronyl, at *m/z* 446 and at *m/z* 285 due to a loss of acetyl-hexose moiety (Li *et al*. 2016; Gbashi *et al*. 2016). Therefore, this molecule was respectively annotated as Kaempferol 3,7-diglucoside. Interestingly, Kaempferol 3,7-diglucoside was found to exist with isobars, which have quercetin aglycone (See Table 1). However, these molecules yielded different fragment ions at different retention times. Furthermore, molecule **10** according to the literature was not recorded in any Amaranthus plant species before, but it was reported in eggplant fruit skins (*Solanum melongena*) and cauliflower by-products (*Brassica oleracea* L. *var. botrytis*) (Li *et al*. 2016; Singh *et al*. 2017). Kaempferol 3,7-diglucoside is part of the flavonoid group and they are well known for health benefits in fruits and vegetables (Singh *et al*. 2017). Detecting this molecule in the *Amaranthus hybridus* can add more knowledge and understanding on their bioactivities (Singh *et al*. 2017).

Molecule **21** and **25** at Rt= 19.69 min and 21.73 min showed a similar prominent peak at *m/z* 593 ($C_{27}H_{30}O_{15}$). Molecule **21** and **25** yielded daughter ions at *m/z* 285, and at *m/z* 284 while molecule **25** yielded extra daughter ions at *m/z* 327, and at *m/z* 255 respectively. Molecule **21** [produced a fragment ion at *m/z* 285 corresponds to kaempferol aglycone that lost an acetyl-hexose moiety and at *m/z* 284 that denotes a kaempferol moiety that lost hydrogen (Gbashi *et al*., 2016; Singh *et al*., 2017). In this study, the molecule was annotated as Kaempferol 3-O-rutinoside/robinobioside.

As stated above, molecule **25** produced extra fragment ions at *m/z* 327.0502, and at *m/z* 285.0381 indicating the loss of C_2H_2O molecule (Aouey *et al*. 2016). Fragment ion at *m/z* 255.0296 resulted from the base peak at *m/z* 285.0381 after losing hexosyl moiety (Aouey *et al*. 2016). Hence this molecule was annotated as Kaempferol 3-O-rutinoside/Robinoside. The annotation of these molecules was in line with Makita *et al*. (2016) who identified a similar molecule due to the fragment ions that eliminated a rutinoside moiety. Having similar molecular formulas but different structures, they can be annotated as isomers (Makola *et al*. 2016). Kaempferol 3-O-rutinoside is regarded as one of the major flavonoids that are found in *Tetrastigma hemsleyanum* plants. This metabolite is associated with anti-cancer properties

that provide strategies to cure lung cancer or even prevent cancer, and therapy as some flavonoids are regarded as pre-infection inhibitors of diseases (Li *et al*. 2021; Vagiri *et al*. 2017).

Molecule **24** at Rt= 21.70 min was observed to have a precursor ion at *m/z* 447 ($C_{21}H_{20}O_{11}$). This molecule produced a fragment ion *m/z* 285 that can be used as a determinant of annotation either as luteolin or kaempferol (Li *et al*. 2016). However, the molecule produced product ions at *m/z* 285.0803, at *m/z* 284.0305, and at *m/z* 255.0278 respectively. Daughter ions at *m/z* 285, and at *m/z* 284 are indicative of kaempferol units that lost a hexose moiety (Li *et al*. 2016; Makita *et al*. 2016; Aouey *et al*. 2016; Madala *et al*. 2016). Hence this molecule was tentatively identified as kaempferol hexose.

Characterization of quercetin derivatives.

A total of nine quercetin derivative molecules (**15**, **16**, **17**, **18**, **19**, **22**, **26**, **29**, and **30**) were detected herein. Molecule **15** at Rt= 15.99 min with a precursor ion at *m/z* 625.13 ($C_{27}H_{30}O_{17}$) was found to produce product ions at *m/z* 301.0335 and 300.0255, showing the presence of quercetin aglycone (Madala *et al*. 2016; Dabeek *et al*. 2019). Moreover, product ion at *m/z* 463 shows the loss of one hexose unit (-162 Da) (Apea-Bah *et al*. 2021; Dabeek *et al*. 2019). Consequently, this molecule was annotated as Quercetin 3-glucosyl-(1->6)-galactoside.

Molecules **16**, **17**, **18,** and **19** at Rt= 18.28 min, 18.72 min, 18.79 min, 19.38 min, and 19.40 min respectively appeared at precursor ion at *m/z* 609 ($C_{27}H_{30}O_{16}$) fragmenting to by-product ions at *m/z* 301.0339, at *m/z* 300.0264. Molecules at *m/z* 301.0339, and at *m/z* 300.0264 are quercetin aglycone units that lost a rutinoside sugar (Gbashi *et al*. 2016; Madala *et al*. 2016). Therefore, molecules **16**, **17**, **18,** and **19** in this study were annotated as Quercetin-3-rhamnosyl -glucoside isomers because of a similar fragmentation pattern. The annotation of these metabolites is in agreement with Gbashi *et al*. (2016). Molecules such as quercetin-3-rhamnoside together with other phenolic molecules in green fruits have a protective function against UV radiation where they can strongly absorb UV radiation within 280-315 nm (Marteska and Perucka 2005). Such high absorbance of UV radiation can shield photosynthesizing cells that are located deeper from the surface (Marteska and Perucka 2005). Interestingly, these molecules that act as protectors for photosynthesis apparatus can later become transitional compounds to further help in the transformation of secondary metabolism (Marteska and Perucka 2005).

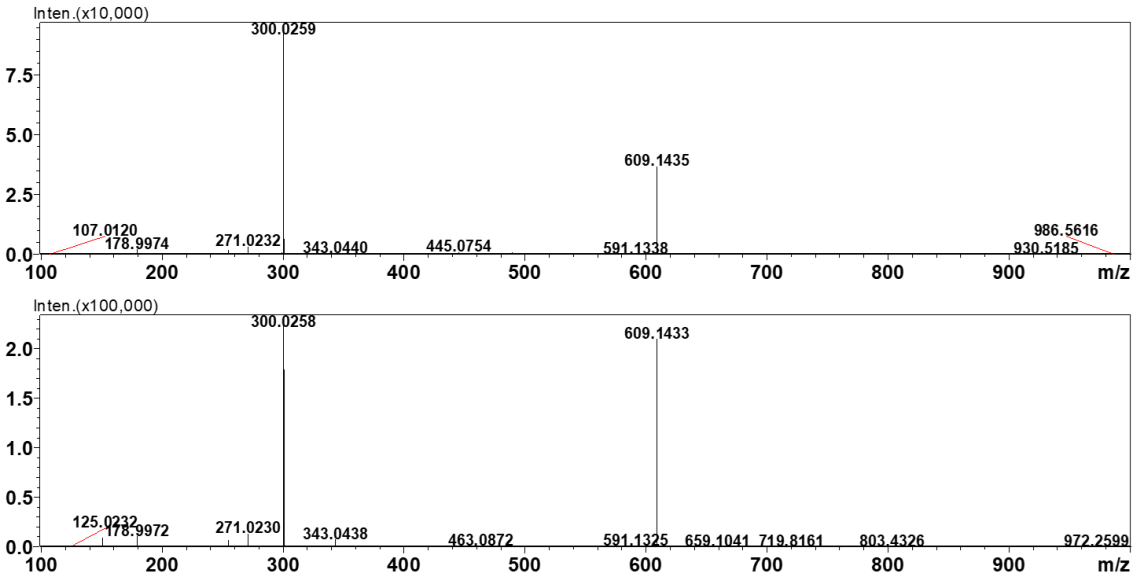

Figure 5: Representative mass spectra of isomers showing fragmentation pattern of (a) Quercetin-3-rhamnosyl-glucoside (Isomer 1), and (b) Quercetin-3rhamnosyl-glucoside (Isomer 2).

Molecule **26** at Rt= 21.97 min with a precursor at *m/z* 463.10 ($C_{21}H_{20}O_{12}$) with a product ion at *m/z* 300.0332 was detected herein. The daughter ion showed a neutral loss of hexose moiety (Liang and Xu. 2016; Li *et al*. 2016). This molecule has been reported in different plant species extracts such as cowpeas and *Bidens Pilosa* L. (Liang and Xu. 2016; Gbashi *et al*. 2017). No literature has reported this molecule in Amaranthus spp, and herein it was annotated as a Quercetin hexose (Gbashi *et al*. 2016; Liang and Xu. 2016).

Molecule **22** at Rt= 20.55 min identified as Quercetin -acetylgalactoside with precursor ion at *m/z* 505.10 ($C_{23}H_{22}O_{13}$) yielded product ion at *m/z* 300 and at *m/z* 301 respectively. Fragment ion at *m/z* 300, and at *m/z* 301 from the parent ion indicates quercetin aglycone and the loss of acetyl hexose moiety respectively (Makita *et al*. 2016; Madala *et al*. 2016). This molecule was previously identified in *Moringa oleifera* and *Moringa ovalifolia* (Makita *et al*. 2016). It was also found in two related species *Crataegus monogyna* and *Crataegus laevigata* (Hawthorn) in different parts of the plants including leaves, fruits, and their herbal derived drops (Makita *et al*. 2016; Karar and Kuhnert 2015).

Molecule **29** and **30** at Rt= 27.78 min and Rt= 27.79 min respectively showed precursor ions at *m/z* 639.13 ($C_{31}H_{28}O_{15}$). The product ion at *m/z* 463 was due to the loss of feruloyl unit which further loses a hexose moiety to produce a product ion at *m/z* 301 (Liang and Xu. 2016, Nobela 2018). The presence of fragment ion at *m/z* 300.0259 and at *m/z* 255.0289 in the fragmentation spectra strongly support the annotation of this molecule as isomers of Quercetin 3-(6-ferulylgalactoside) (Liang and Xu 2016)

Characterization of *p*-Coumaric derivatives

Two molecules (**11** and **12**) were respectively identified as coumaric acid derivatives. These molecules at Rt= 14.19 min and Rt= 14.20 min were authenticated to be isomers based on the fragmentation patterns from the parent ion at *m/z* 337.09 ($C_{16}H_{18}O_8$). These molecules yielded similar product ion at *m/z* 191 that indicates the loss of a coumaroyl unit (Nengovhela *et al*., 2021). These molecules were distinguished according to literature, whereby molecule **11** had a base peak at *m/z* 163, and molecule **12** showed a product ion at *m/z* 191 that was visible enough (Clifford *et al* 2003). Therefore, the molecules were annotated as 3-O-p-Coumaroylquinic acid and 5-O-p-Coumaroylquinic acid respectively. Both molecules have been reported to be present in *Bidens pilosa* leaves extracts (Ramabulana *et al* 2020).

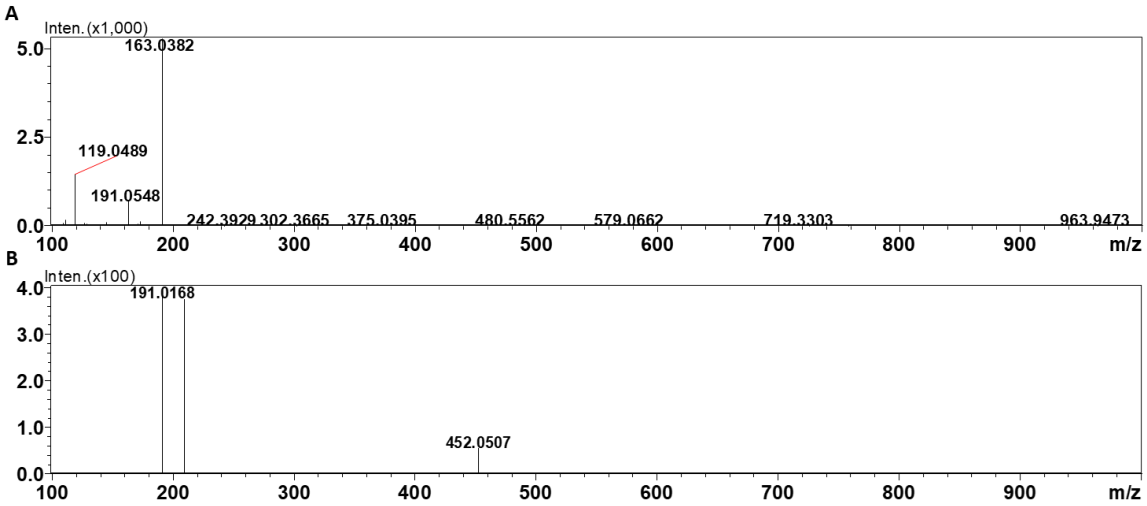

Figure 6: Representative mass spectra of isomers showing fragmentation pattern of (a) 3-O-p-Coumaroliquinic acid, and (a) 5-O-p-Coumaroliquinic acid respectively.

Unknown metabolites

Molecule **14** at Rt= 15.58 min with precursor ion at *m/z* 403.16 ($C_{18}H_{28}O_{10}$) was annotated as 6-Deoxylamioside that yielded product ion at *m/z* 197.1168 (Liang and Xu., 2016). Molecule

20 at Rt= 19.54 yielded a precursor ion at *m/z* 289.00 ($C_{22}H_{10}O$) did not produce any product ions and it was tentatively characterized as Catechin derivative (Karar and Kuhnert., 2015). This molecule has also been reported in grapevines (*Vitis vinifera* L.) (Escobar-Avello *et al*. 2019). Molecule **28** at Rt=26.88 min was characterized as Isorhamnetin 3-(6-acetylgalactoside) because it showed fragment ions at *m/z* 314.0404, at *m/z* 299.0174 and at *m/z* 271.0227 that were achieved from precursor ion at *m/z* 519.11 ($C_{24}H_{24}O_{13}$) (Escobar-Avello *et al*. 2019).

The effect of Frass treatment on Metabolite composition in Amaranthus

Using UHPLC-QTOF-MS based metabolite fingerprinting, the current study was conducted in an attempt to establish metabolite perturbation caused by frass application or treatment. The differences in metabolite distribution pattern could not be established through visual inspection and, as such, a chemometric-based model, principal component analysis was applied. The large dataset in this study was analyzed using a PCA score plot (Tagizimana *et al*., 2013). PCA is a mathematical method that visualizes similarities or dissimilarities between and within samples by clustering them according to their metabolite distribution patterns (Ramabulana *et al*. 2020; Taguzimana *et al*. 2013). Herein, PCA was computed using XC-MS online software and further visualized through Metabo-analyst (Ramabulana *et al*. 2020).

Thereafter, the resulting spreadsheet containing metabolite information was further exported to Metabo-analyst software for advanced statistical modeling. In the PCA score plot, samples are grouped according to frass treatment thereby suggesting a differential metabolite distribution pattern (**Fig 7**). The score plot represents the metabolites distribution patterns in various frass treatments, NPK treatment samples, and control (untreated). The score plot explained a total variation of 43.4 % where the highest variation of 25.9 % was explained by PC1 and 17.5 % was accounted for by PC2. Interestingly, some groups were found to form a tight cluster such as Frass 5g, Frass 20g, and NPK and some were found to form widely dispersed groups such as Frass 10g and the control sample. Therefore, it can be concluded that frass treatment such as Frass 5g is sufficient to induce a common metabolite perturbation between plants. Whereas treatment with 10g frass resulted in an uncommon effect on plants, hence sample dispersion on a PCA score plot.

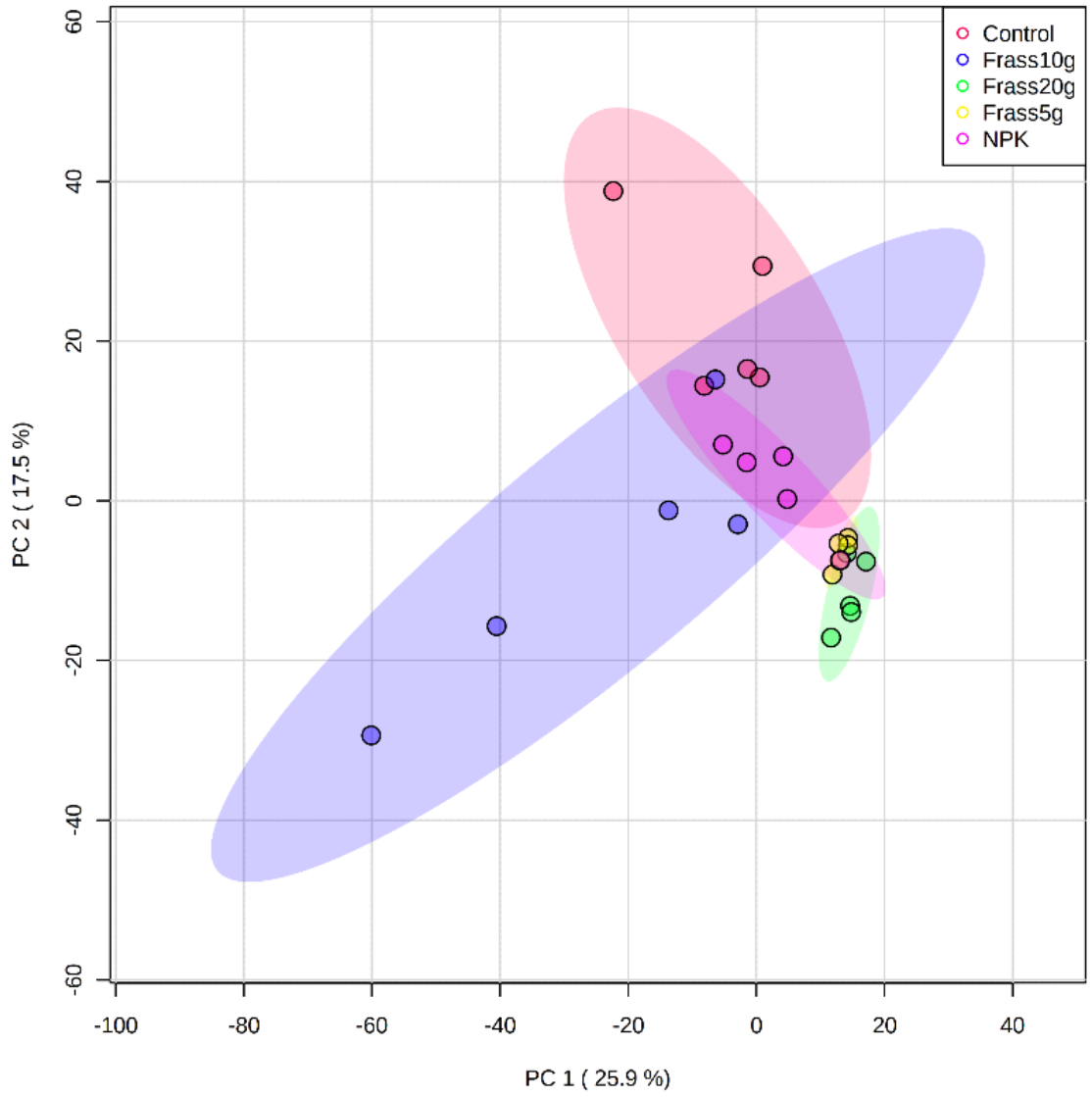

Figure 7: PCA score plot of different frass application rates compared to the commercial fertilizer (NPK). The PCA is based on the UHPLC-QTOF-MS chromatograms from negative ionization data.

As such, it was important to unearth all the metabolites of which the levels were affected by the treatments as depicted by the PCA score plot. Thereafter, the peak intensity of each molecule representing metabolite distribution per treatment level was shown in box and whisker plots (**Fig 8** and **9**). Similarly, metabolites that were perturbed were also annotated as shown above. The use of the UHPLC-QTOF-MS approach synergistically worked with other

chemometric analysis software (R-Software, and PCA) responsible for processing the data that resulted in key findings in this study.

Statistically, Analysis of Variance (ANOVA) was used to evaluate the significant effect on metabolite distribution in different treatment levels and controls. Different effects on metabolite distribution in peak intensity (thus, $p < 0.05$) were observed amongst the treatments (**Figure 8** and **9**). Significant effect on metabolite distribution was observed in different treatment levels of frass, control, and inorganic fertilizer (NPK). Molecules **1**, **3**, and **4** in **Fig. 8** and molecules **12, 13**, **14**, and **15** in Fig. **9** are examples of the molecules that showed significant effects because of different fertilizer treatments levels. As previously stated, Molecules **1** and **2** (5-O-Caffeoylgalactaric acid) in Table 1, were annotated as isomers due to them eluting at close retention times and similar molecular formula. Interestingly, Mol. **1** was found to be significantly different between 10 g Frass treatment and Frass 20g treatment. Mol. **2** did not show any significant differences statistically.

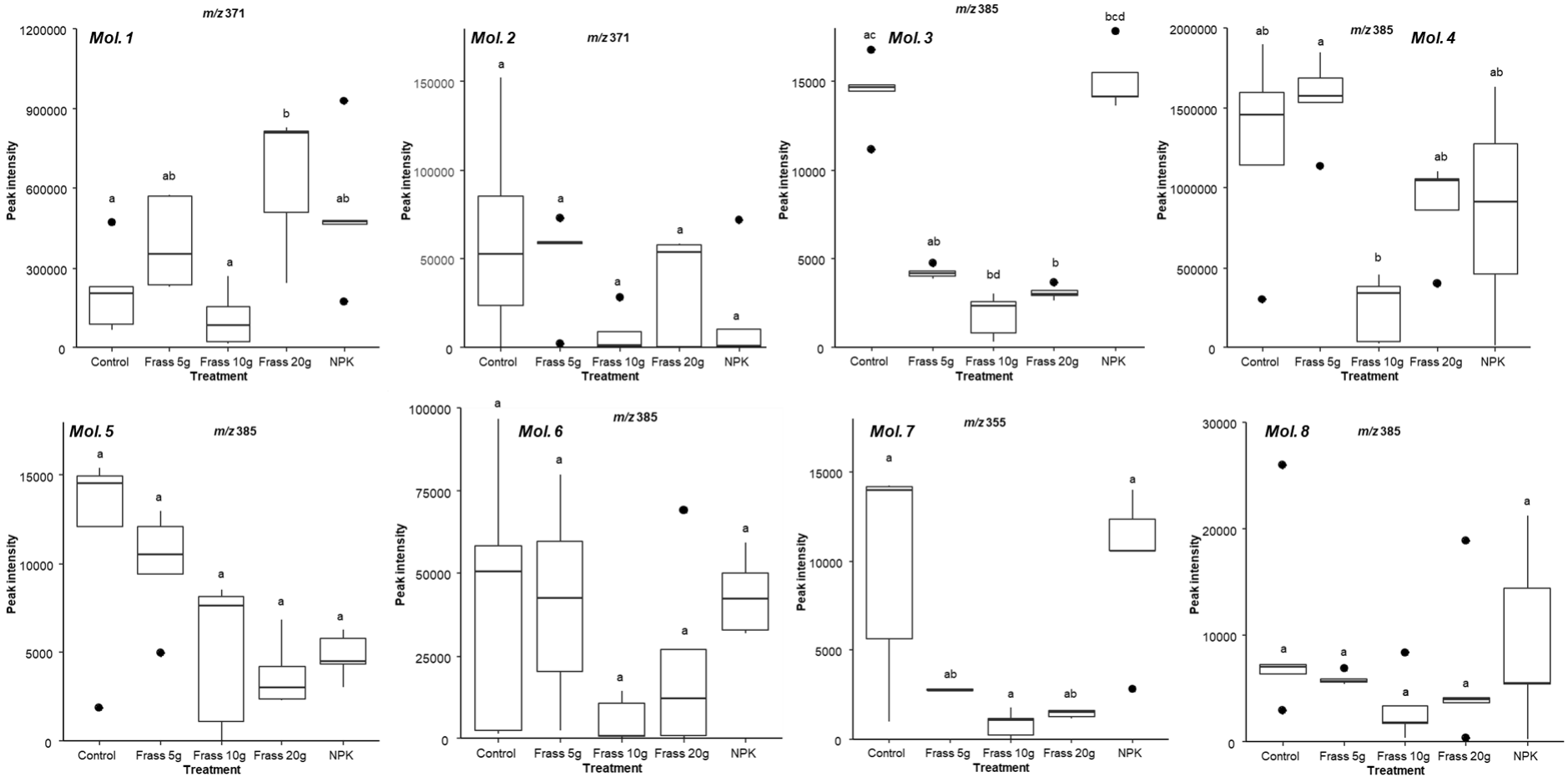

Figure 8: Differences in metabolites composition detected in *Amaranthus hybridus* leaves extracts. Different letters represent a significant difference in pair-wise comparisons and bold circles represent outliers (molecule 1-8).

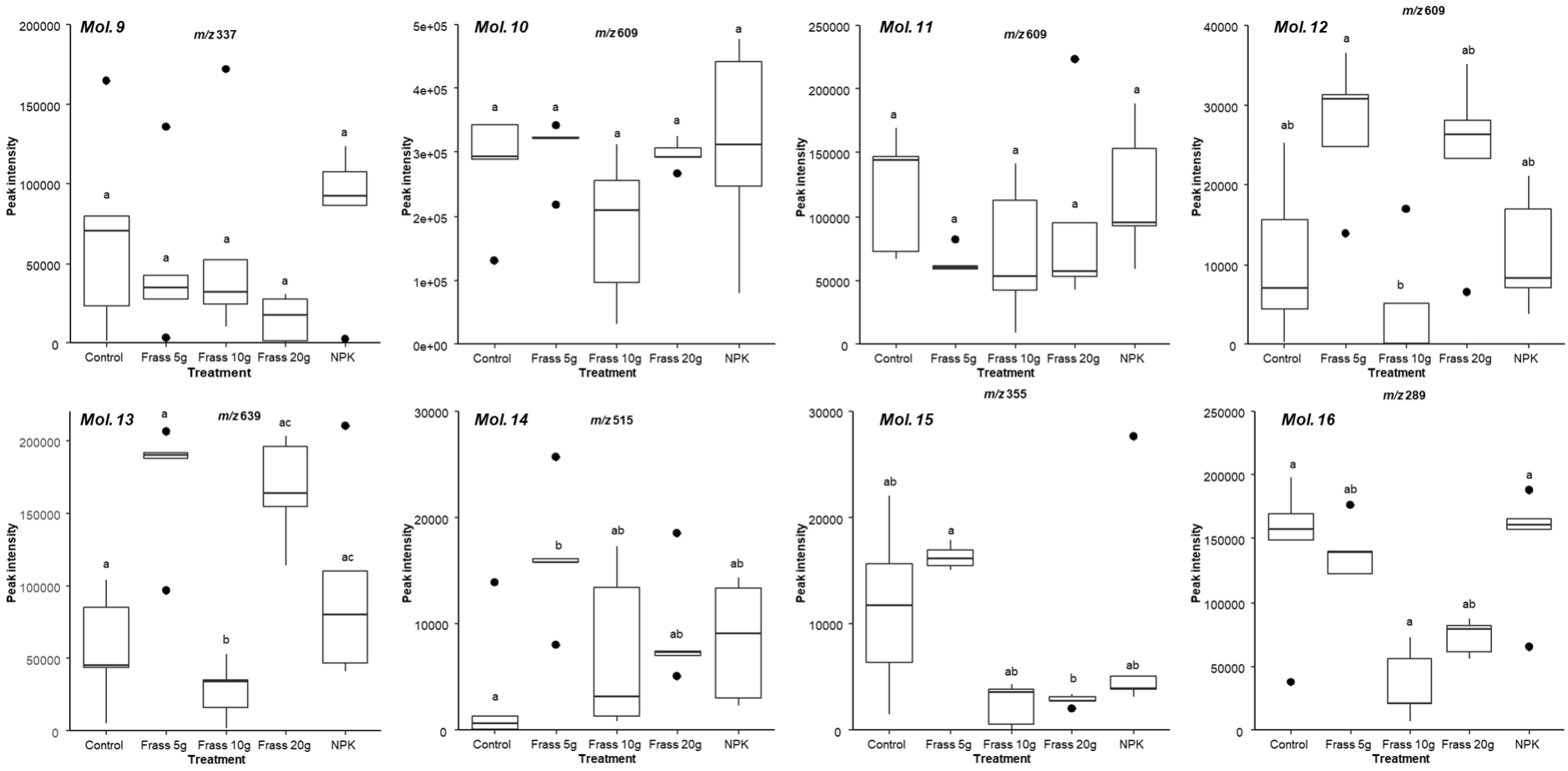

Figure 9: Differences metabolite composition detected in *Amaranthus hybridus* leaves extracts. Different letters represent a significant difference in pair-wise comparisons and bold circles represent outliers (Molecules 9-16).

In **Fig. 8**, Mol. **3** and **4 (**Feruloyl glucaric acid) are isomers. Mol. **3**, Frass 20g, and Control significantly differed in metabolite distribution. Mol. **4**, Frass 5g, and Frass 10g were significantly different from each other on metabolite distribution respectively. Figure **9,** Mol. **12** (Quercetin-3-rhamnosyl-glucoside) revealed significant differences between Frass 5g and Frass 10g respectively. Mol. **13** followed the trend observed in Mol. **12**. Mol. **14** (3,4-di-O-Caffeoylquinic acid) uncovered significant differences between Frass 5g and control. Mol. **15** (1-O-Feruloyl-beta-D-glucopyranose) disclosed significant differences between Frass 5g and Frass 20g

.

The result obtained in the chemometric models can be used to conclude that the distribution of metabolites in *Amaranthus hybridus* can be affected by different frass treatment application rates and NPK treatment in comparison to the controls. Therefore, the use of robust and sensitive UHPLC-QTOF-MS instruments has assisted greatly to detect the distribution and content of metabolites in the leaves of *Amaranthus hybridus*. Although the objectives were achieved, it would be great if the data was processed to enable the exact amount of the metabolites that the plant contains within the respective application rates of frass using the commercially available authentic chemical standard. Frass remains the best option to sustain future needs of organic food demand to the ever-growing population that is expected to double by 2050. The use of these organic fertilizers is not only good for sustaining growing populations, but they are also efficient to reduce global warming challenges and improve soil quality as well as contributing to biodiversity and its wellbeing.

Chapter 4

Conclusion and recommendations

Frass is the by-product of bioconversion where insect exuvia, feces, and uneaten waste are combined. This study focused on frass as an organic fertilizer and biopesticides. The human population is growing exponentially, and people need organic food. However, about 75 % of the food produced globally ends as waste. Using black soldier fly larvae (BSFL) is more advantageous as the fly can convert organic waste and turn it into frass, the larvae feeding on waste can be used as animal feed. This fly is good for the economy considering that big insect companies such as Ynsect, a French insect farming company have raised approximately $125 million that aims to improve livelihood by creating job opportunities as they produce insects for feed and frass as a soil improver.

In the first chapter of this study, a systematic review of published literature on BSFL frass was done. The literature was then divided into two sections for comparison between developed and developing countries. More studies have been done in developed countries (82 %) as compared to developing countries (18 %). In Sub-Saharan Africa, there are six countries that published literature on BSFL frass. The farming of the BSFL is recommended at low scale farming in rural landscapes because there is no need for special equipment. Thus can alleviate financial stress to local people in developing countries as they can produce animal feed and frass to improve poor soils (soil nutrients).

In the second chapter, the main investigation focused on frass from the black soldier fly as a potential organic fertilizer and biopesticide for crops, and the evaluation of the effects of frass treatments on *Amaranthus hybridus***.** Comparatively, all the dependent variables (i.e crop height, number of leaves, chlorophyll content, and dry biomass) differed significantly in response to the frass treatments. *Amaranthus hybridus* leafy vegetables were transplanted into pots for eight weeks. In literature it is reported that frass effectiveness is determined by the waste stream used to feed the BSFL. In this study, organic waste that was converted into frass included tomatoes, onions, cabbage, lettuce, and carrots. Plants grown in frass 20g and commercial fertilizers did not differ significantly, but plants grown in frass 5g and the control differed significantly to double the optimal rate of the frass (Frass 20g). We recommend that

frass studies must be practiced in long term studies in the field (seasons) as more literature focused on greenhouse studies.

Frass maturity was tested through a phytotoxicity test. The relative root growth was less than the phytotoxicity threshold (<50 %) indicating that the frass was not fully matured. Seed germination was above 94 % in all the frass aliquot replicates and the controls. However, after germination, plants in the frass aliquot did not have healthy signs of growth as the mean root length was 5 mm. This could be explained by the fact that the frass used in this study could have had high electroconductivity (EC) and ammonium concentration that hinder plant growth. These two components were not measured in this study. We recommend that the above-mentioned key features must be recorded before experiments to give insight into the frass.

Boiled and Brewed Frass teas were used as a biopesticide solution in this study. Leaves of *Amaranthus hybridus* were sprayed on both sides until they started dripping the tea. Leaf damage was quantified using the proportion of the number of leaves per replicate and the number of leaves within the replicate. More leaf damage was observed in Boiled frass compared to the Brewed frass and the control. This is explained by the fact that microorganisms get killed when frass is boiled as compared to brewed frass which allows microorganisms to pass through into the liquid. We recommend that frass teas and vermicompost teas can be improved through the preparation methods, and it also depends on the add-ins such as molasses.

In Chapter 3, the focus was on the distribution and effect of frass on metabolites at different application rates. Green leafy vegetables such as *Amaranthus hybridus* contain secondary metabolites that are necessary to protect plants against pests, and other functions in humans that exhibit various pharmacological activities such as protection against cardiovascular diseases, cancer, and diabetes. In this study, a total of 30 metabolites were recorded and annotated. The characterized metabolites include derivatives of coumaric acid, caffeic acid, ferulic acid, and flavonoids. Some of these noticeable derivatives that were annotated are 5-O-trans-Caffeoylgalactaric acid, Feruloyl glucaric acid, Kaempferol 3-O-rutinoside/robinobioside, and Quercetin 3-(6-ferulylgalactoside). These metabolites were

characterized using ultra-high-performance liquid chromatography- quadrupole time-of-flight mass spectrometry (UHPLC-QTOF-MS). Moreover, multivariate statistics (XC-MS, Metabo-analyst, R-software) were utilized to analyze and put the data together.

Different treatment levels of frass affected the distribution of metabolites that are found in the *Amaranthus hybridus*. When we look at some of the isomers, throughout the different treatment levels they do not differ significantly, and some differ amongst treatments (Figure 8, Mol 1 and 2). A large group of metabolites was grouped through PCA score plots according to frass treatment levels. In summary of the PCA score plot, it can be said that some treatments including Frass 5g, Frass 20g, and NPK were clustered. Control and Frass 10g were dispersed. To this point, it would be great if the data was processed to enable the exact amount of the metabolites that the plant contains within the respective application rates of frass using commercially available authentic chemical standards.

In conclusion. Frass can be utilized as an effective organic fertilizer for crops. It can also be used as a biopesticide for crops when applied in a liquid form. The frass biopesticide properties are the metabolites found in plants as they serve different functions. Plants themselves contain secondary metabolites, and frass have the ability improve those metabolites. To this point, frass remains the sustainable way to improve soil without harming the environment and cater the ever-growing population with organic food.

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Supplementary materials

Chapter 1

Table S1: Stem and roots dry biomass, wet leaves, stem, and roots of each applied treatment (mean ± se). The experiment was terminated after eight weeks. Different letters represent a significant difference in pair-wise comparisons according to the Kruskal Wallis Test. Asterisks `*` represent significant impact on all the response within each column.

		Dry weight						Wet weight			
		Stem		Roots		Leaves		Stem		Roots	
Treatment	**N**	**mean**	**se**	**mean**	**se**	**mean**	**se**	**mean**	**se**	**mean**	**se**
P-value		***		***		***		***		***	
Control	8	0.54^{a}	0.06	0.38^{a}	0.05	2.81^{a}	0.18	2.42^{a}	0.2	0.92^{a}	0.12
Frass 5g	10	0.68^{b}	0.05	0.49^{b}	0.04	5.48^{b}	0.36	3.68^{b}	0.28	1.55^{b}	0.15
Frass 10g	10	0.97^{bc}	0.09	0.71^{c}	0.06	7.50^{c}	0.56	5.29^{c}	0.39	2.25^{c}	0.23
Frass 20g	10	1.50^{c}	0.1	1.44^{c}	0.14	15.36^{c}	0.48	9.05^{c}	0.52	5.33^{c}	0.52
NPK	10	3.35^{c}	0.35	2.17^{c}	0.22	22.35^{c}	2.53	17.04^{c}	1.69	11.51^{c}	1.08

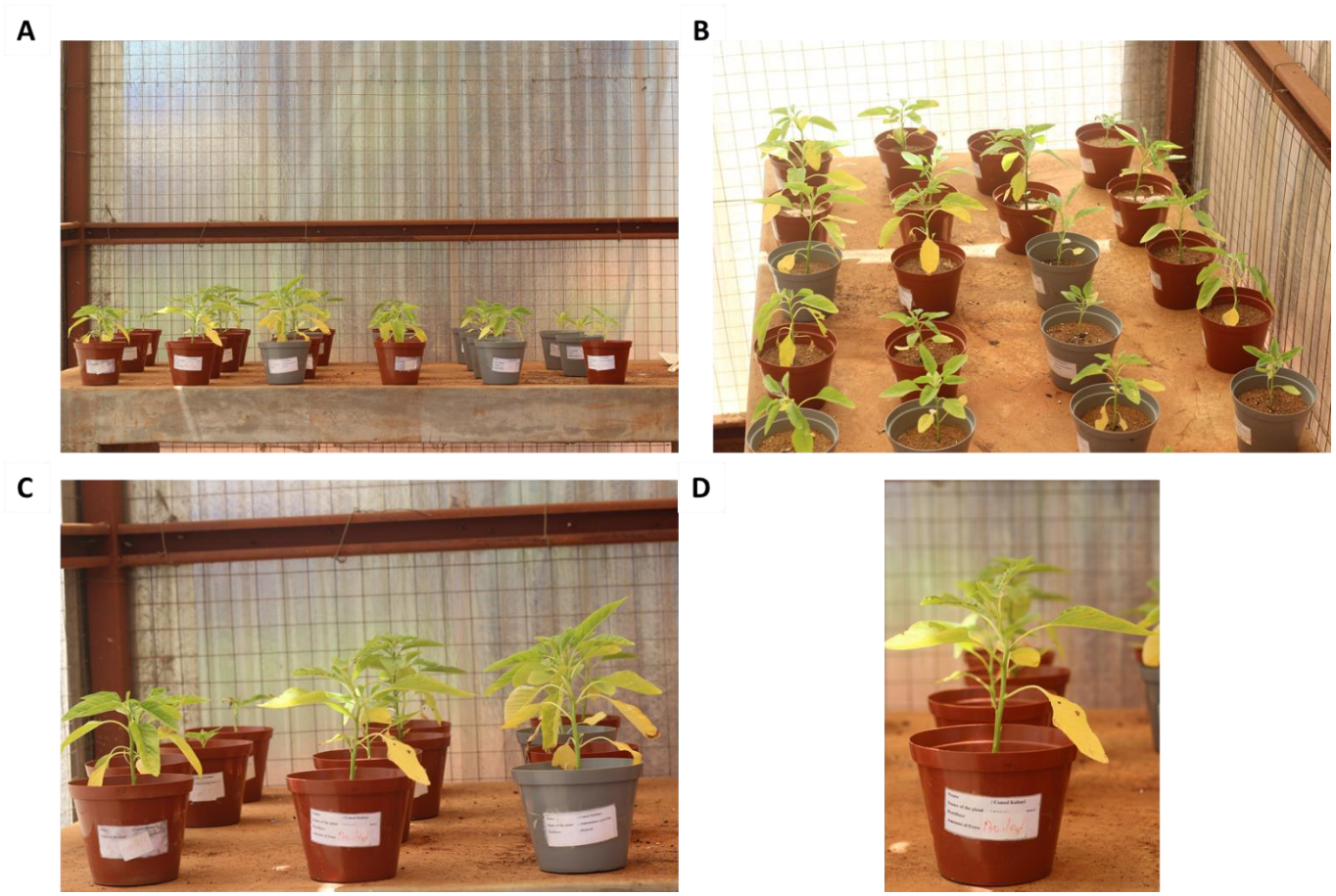

Figure S1: *Amarhantus hybridus* plants in a greenhouse nursery for frass tea experiment that was terminated after five weeks.

Figure S2: Pest damage from the control treatments in the frass tea experiments (A and B). The experiment was terminated after five weeks.

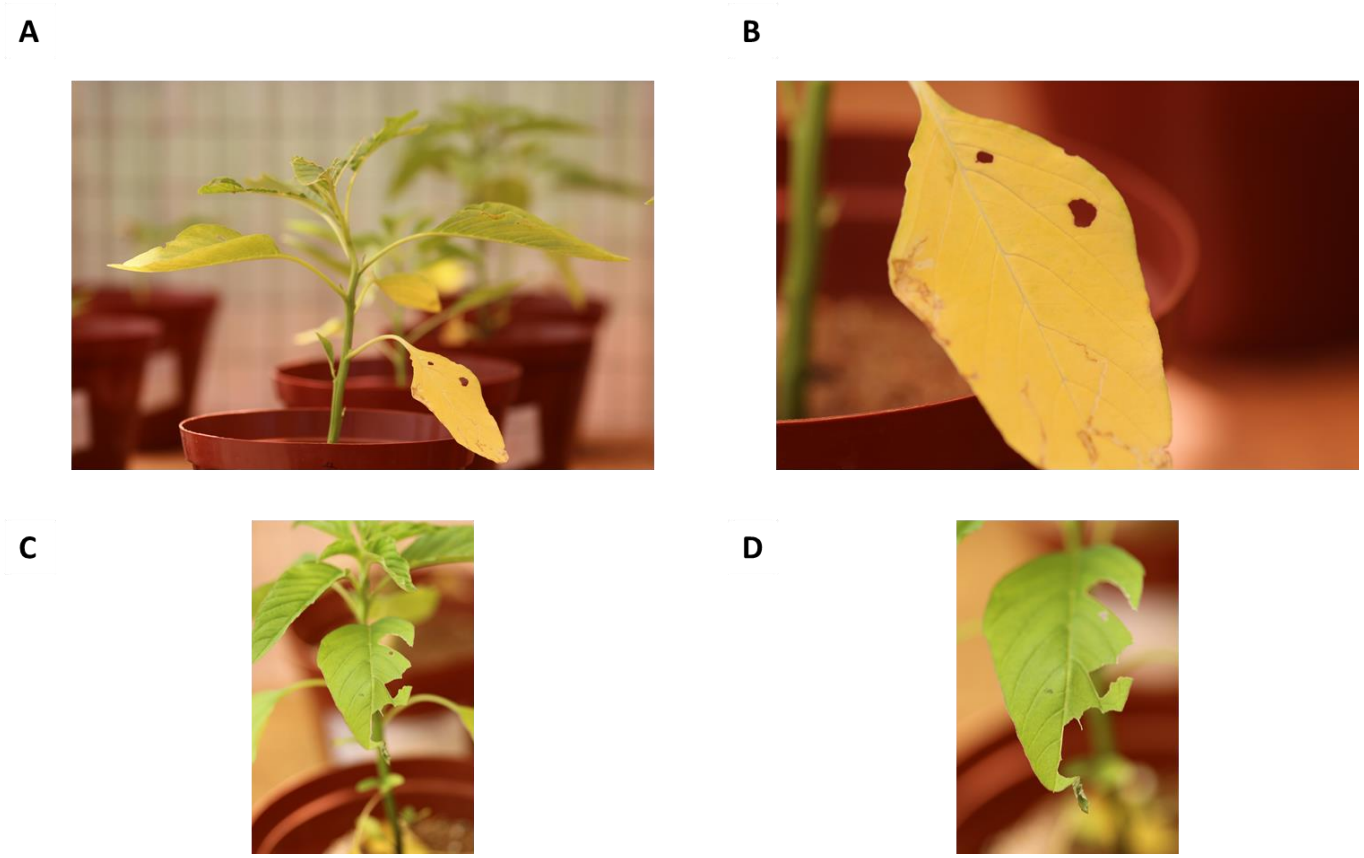

Figure S3: Pest damage in the boiled frass tea (A and B) and damage from in the plants grown in the control (C and D). The experiment was terminated after five weeks.

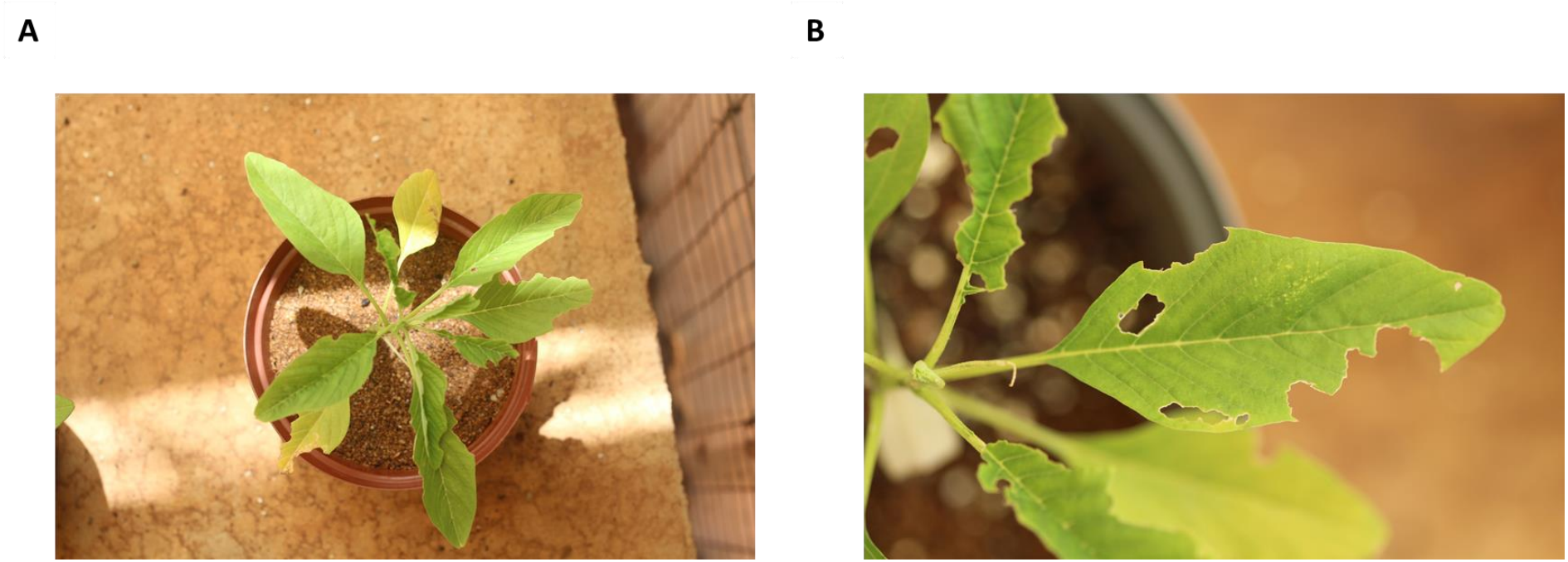

Figure S4: Pest damage in the brewed frass tea (A and B). The experiment was terminated after five weeks.

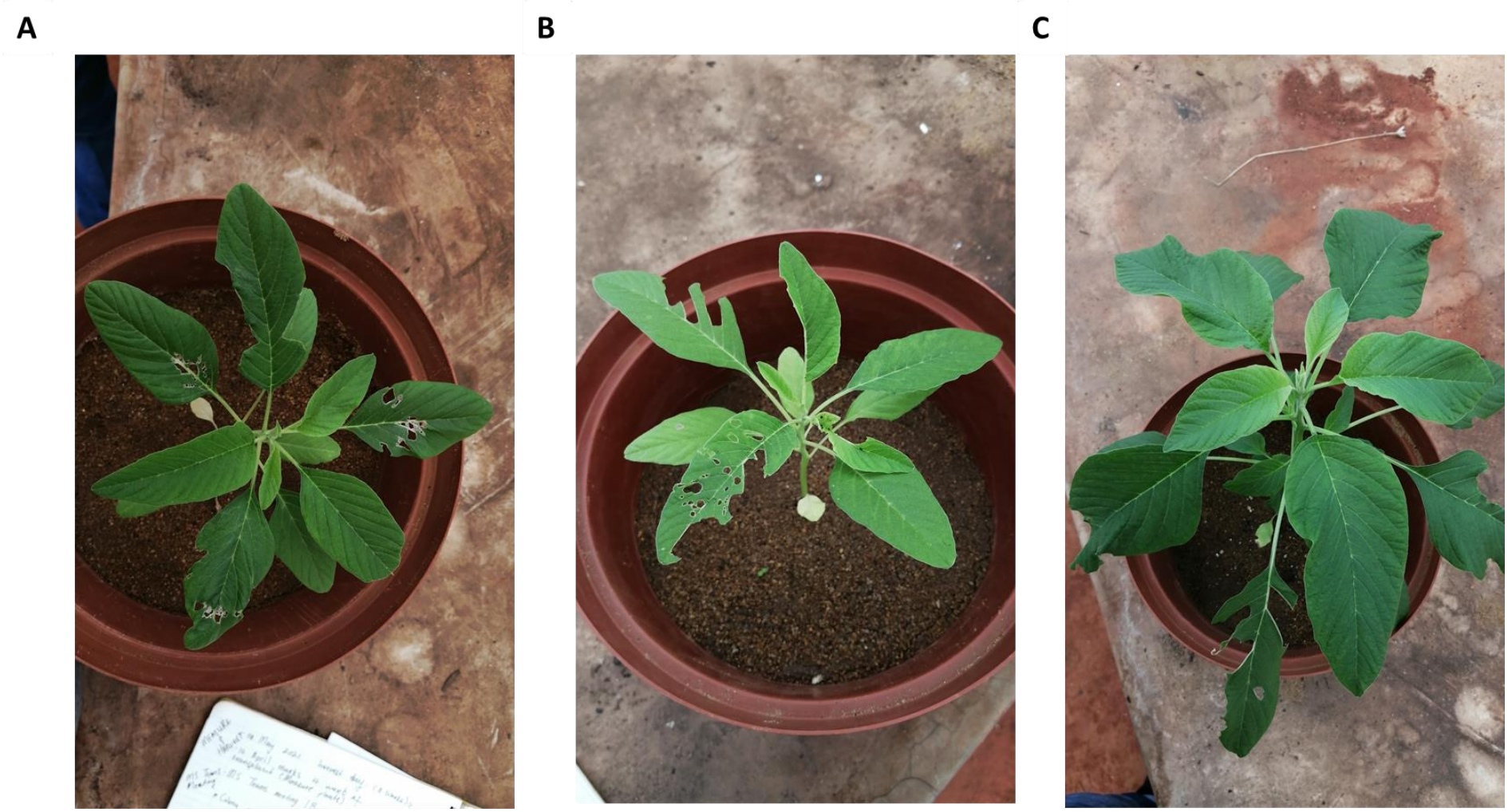

Figure S5: Pest damage in the solid frass experiment for evaluating growth and effectiveness of frass as an organic fertilizer. Experiment was terminated after eight weeks.

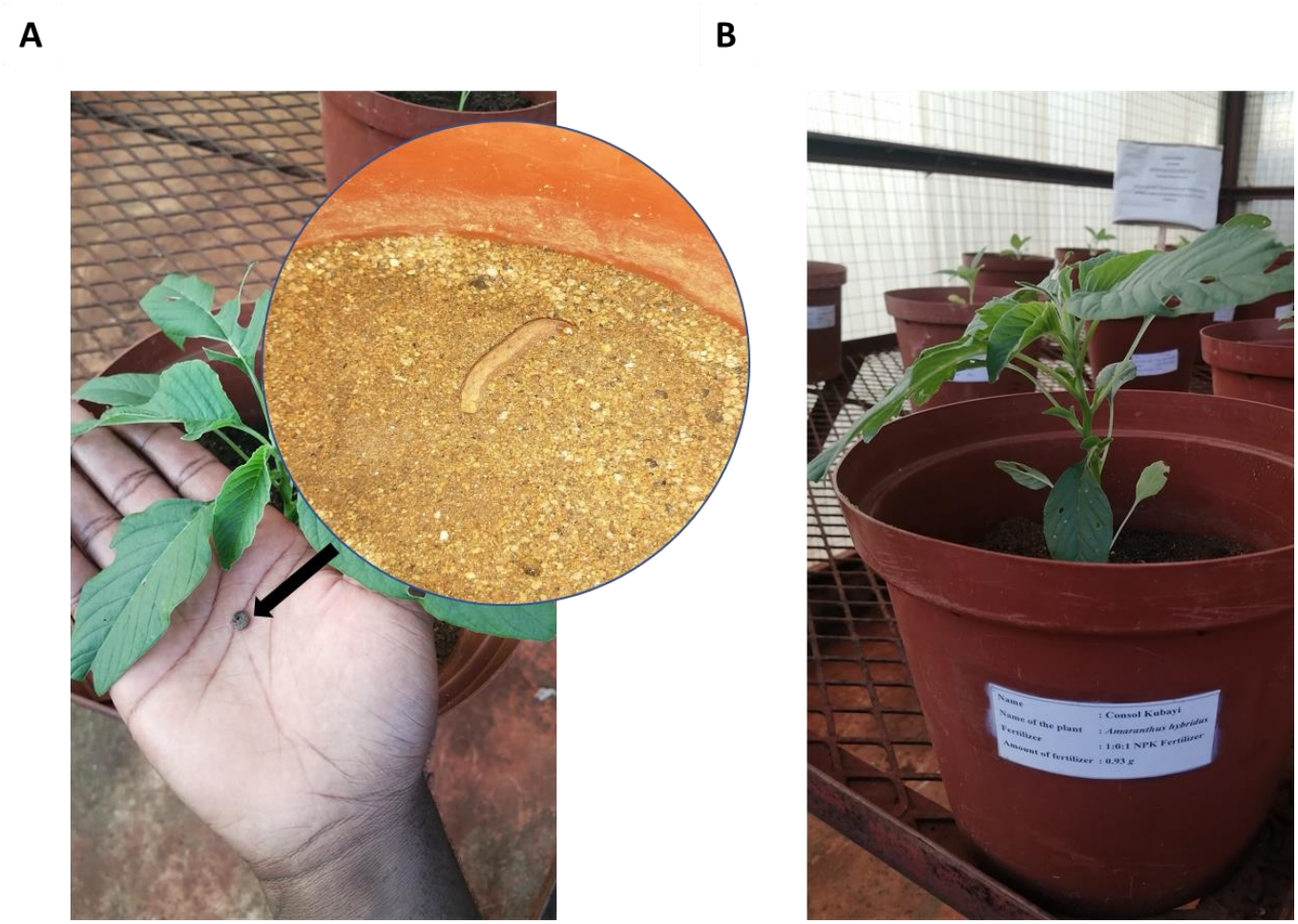

Figure S6: One of the pests that were feeding on *Amarhantus hybridus*. The insect was not identified and they were not removed in the eight weeks period of the experiment.

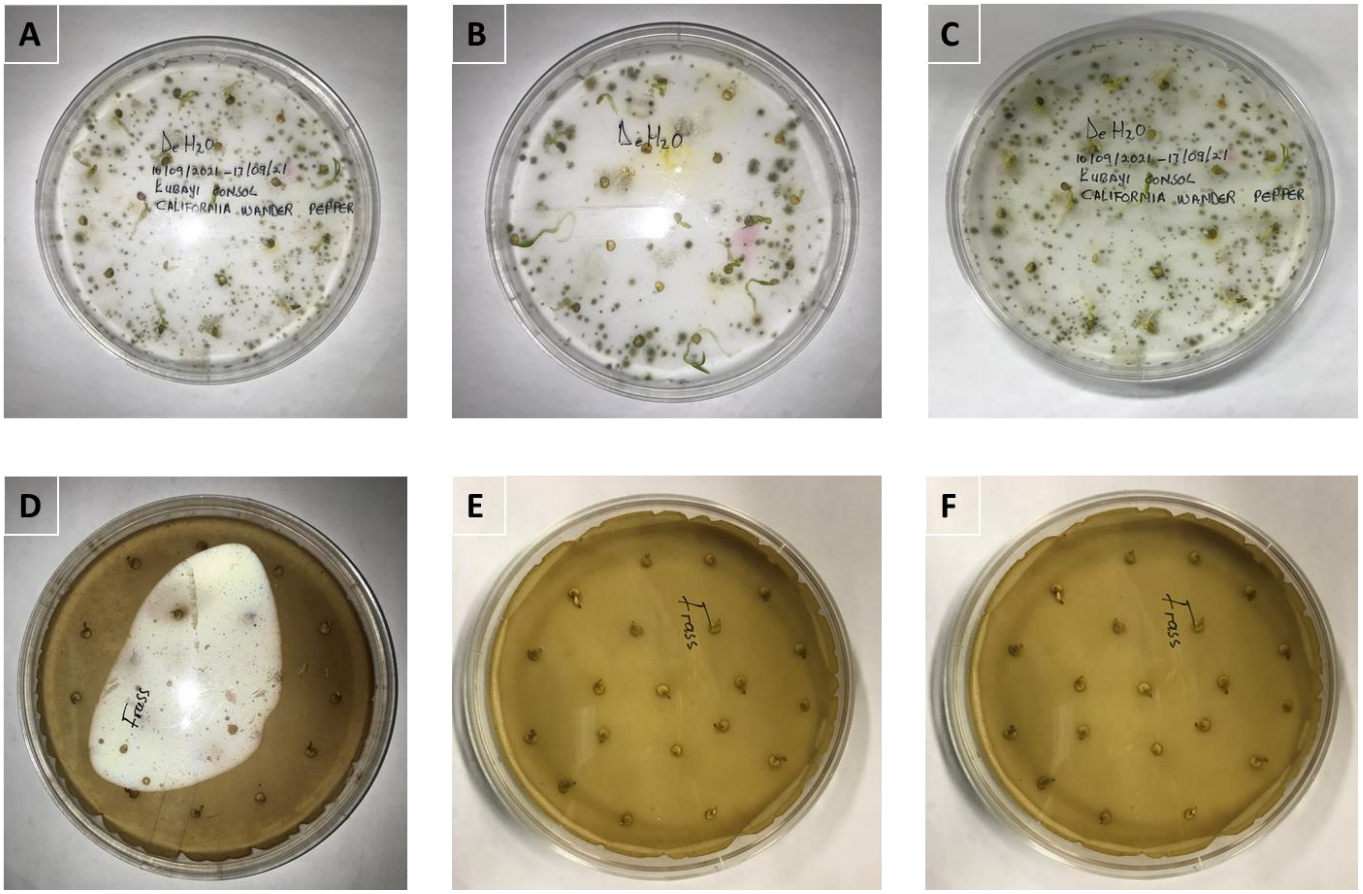

Figure S8: Phytotoxicity experiment to test frass maturity using *Capsicum L.* seeds to perfotm the trials (n=3).

Chapter 3

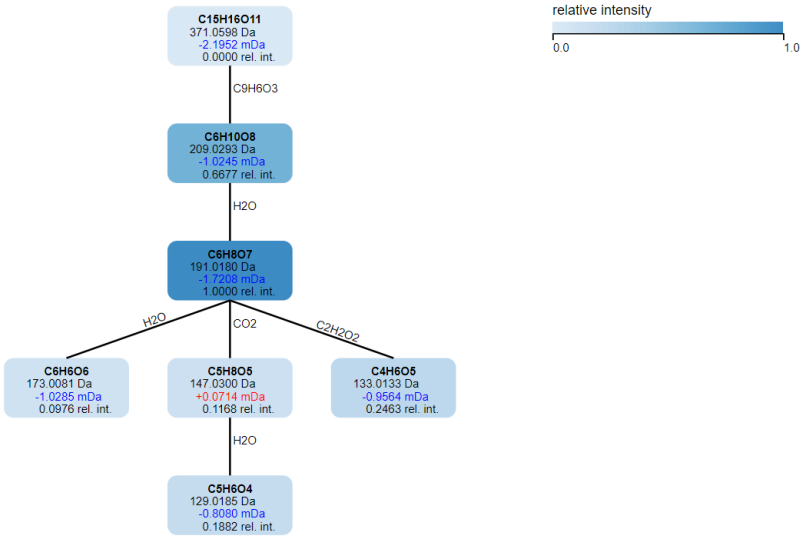

Figure S1: Detailed fragmentation tree of 5-O-trans-Caffeoylgalactaric acid (Isomer 1).

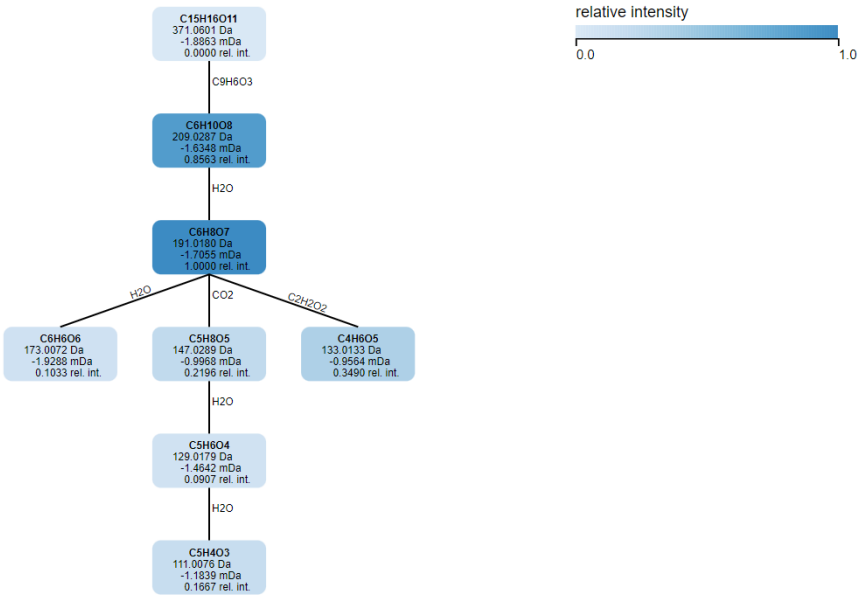

Figure S2: Detailed fragmentation tree of 5-O-trans-Caffeoylgalactaric acid (Isomer 2).

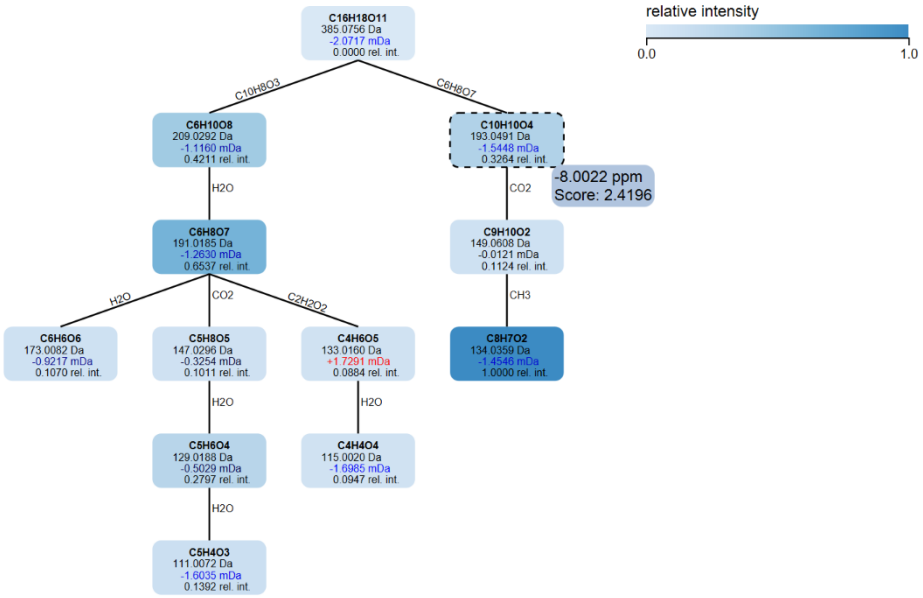

Figure S3: Detailed fragmentation tree of feruloyl glucaric acid (Isomer 1).

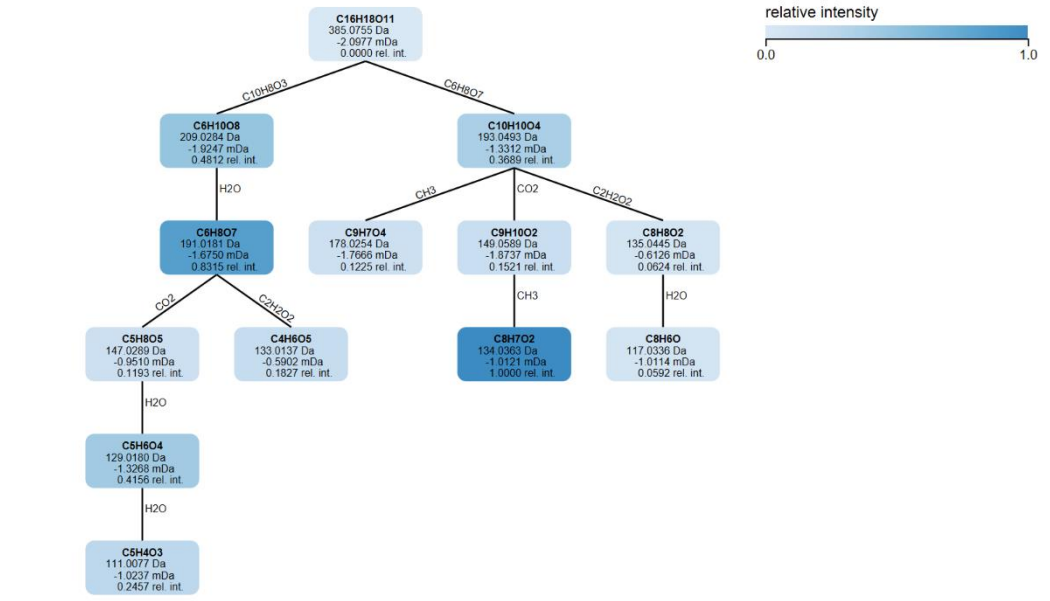

Figure S4: Detailed fragmentation tree of feruloyl glucaric acid (Isomer 2).

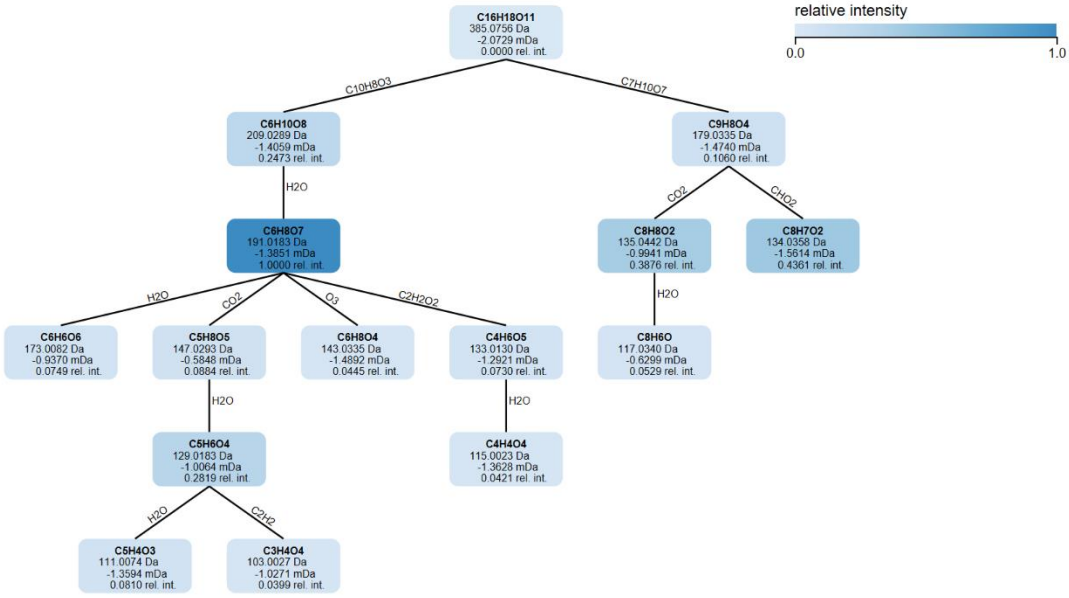

Figure S5: Detailed fragmentation tree of feruloyl glucaric acid (Isomer 3).

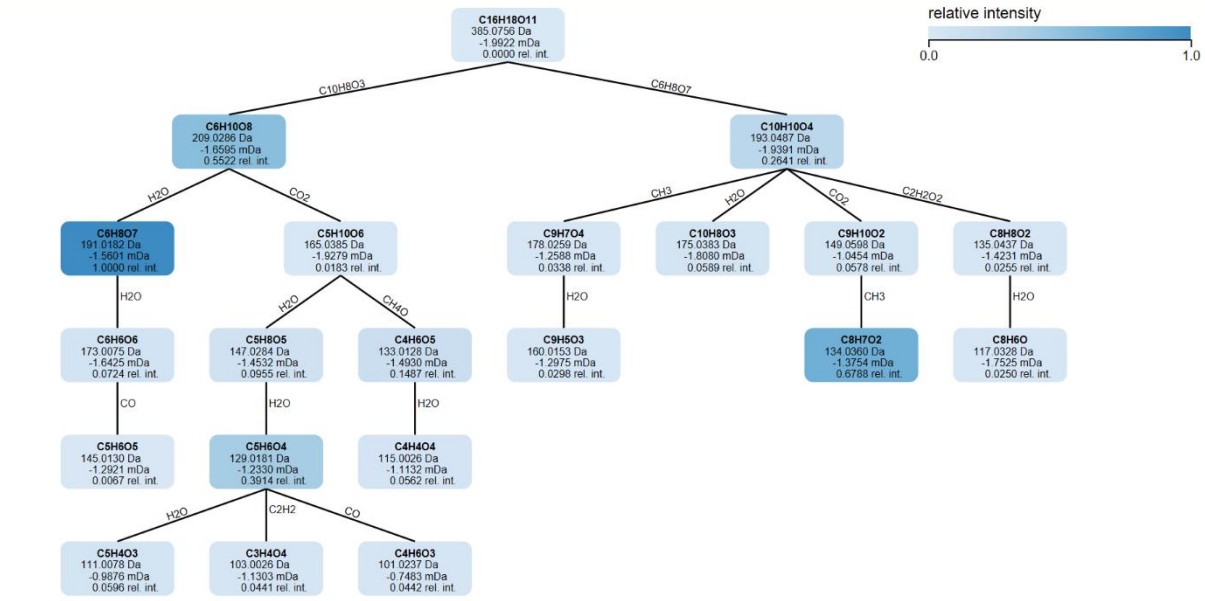

Figure S6: Detailed fragmentation tree of feruloyl glucaric acid (Isomer 4).

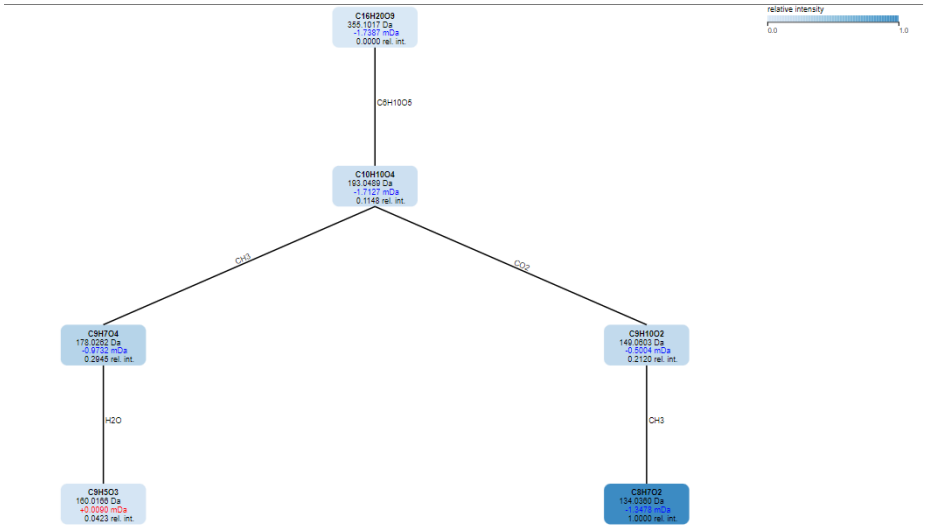

Figure S7: Detailed fragmentation tree of 1-O-Feruloyl-beta-D-glucopyranose.

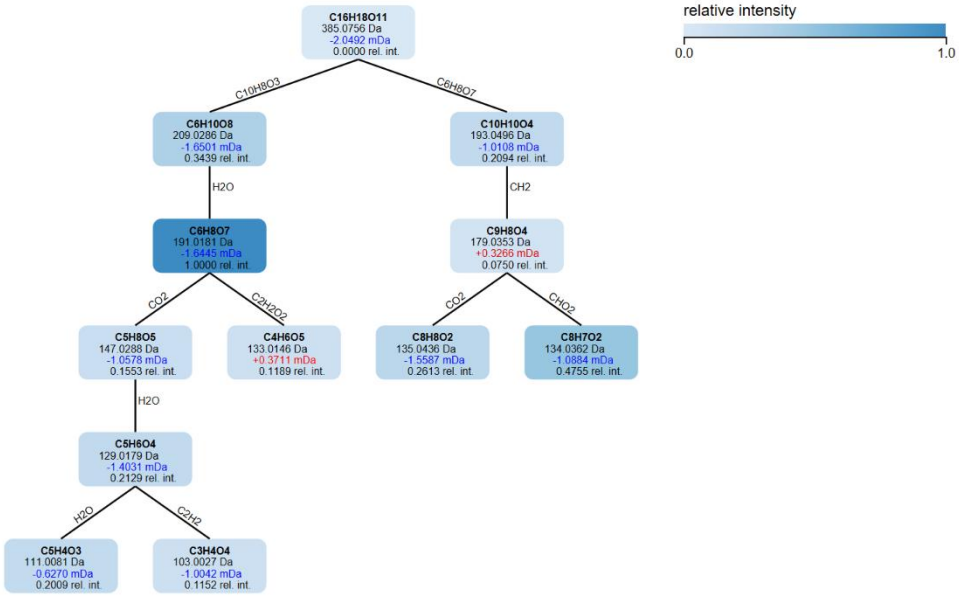

Figure S8: Detailed fragmentation tree of feruloyl glucaric acid (Isomer 5).

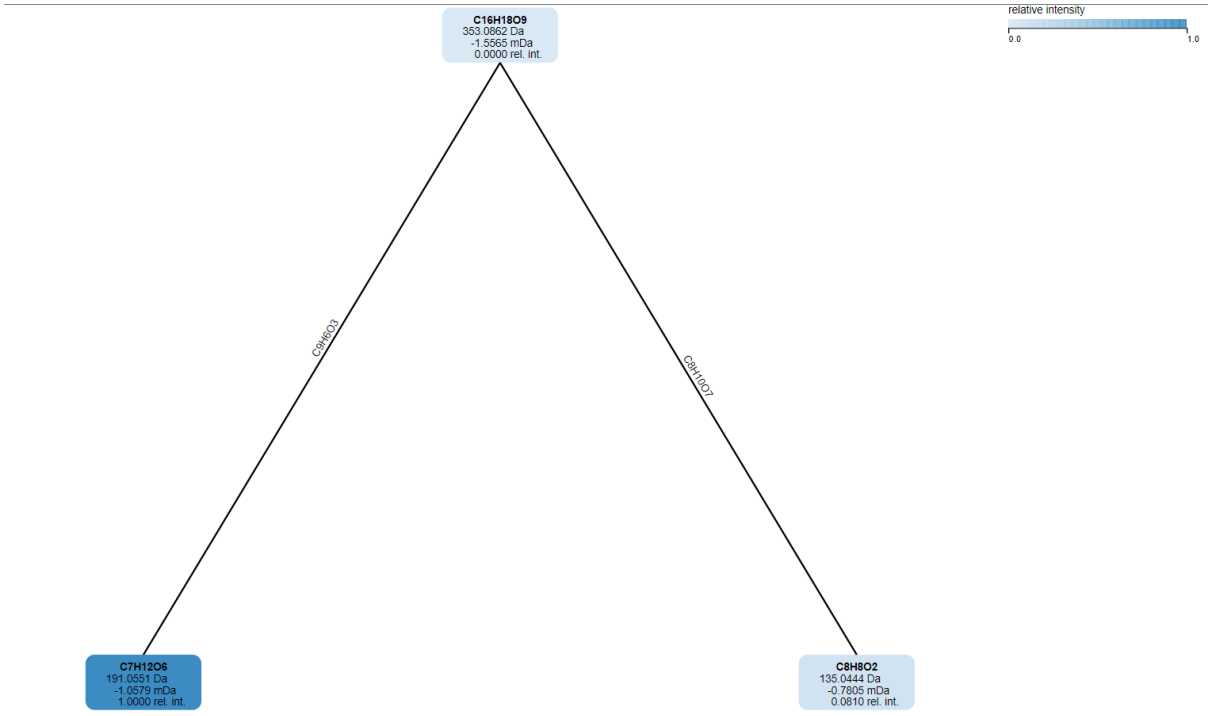

Figure S9: Detailed fragmentation tree of 5-O-caffeoylquinic acid.

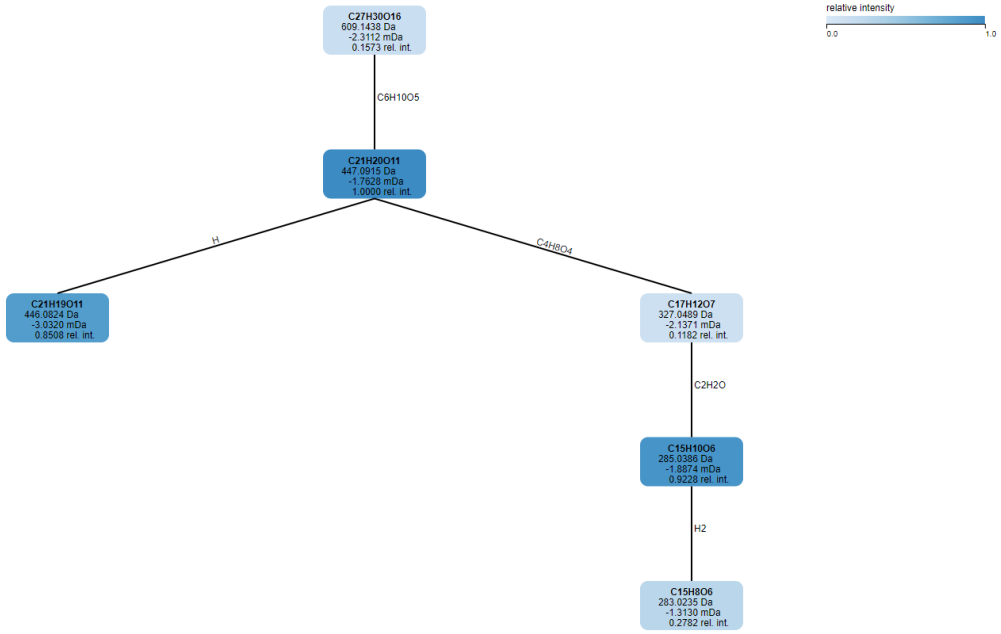

Figure S10: Detailed fragmentation tree of Kaempferol 3,7-digalactoside.