The effect of false codling moth (*Thaumatotibia leucotreta*) larval growth stages on 'Midknight' valencia citrus fruit biochemical properties, selected secondary metabolites and antioxidant activities

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DECLARATION

I, Shilombe Tess Mathonsi, declare that this is my original research work and that it has never been submitted before by anyone for examination for any degree at any other institution. All the material and information used from other sources has been indicated and acknowledged by means of complete references.

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Signature	**Date**
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Signature (Supervisor)	**Date**

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DEDICATION

I dedicate this dissertation to my parents, George and Olvah Mathonsi and my siblings Nsovo, Lulekani and Khanani Mathonsi.

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ABSTRACT

Worldwide, citrus is the most produced crop and accounts for the biggest export in South Africa. Citrus is a tropical crop; therefore, it is a major host of several phytosanitary pests, including false codling moth (*Thaumatotibia leucotreta*) (FCM). False codling moth is known for fruit damage; and therefore, restricted on certain lucrative international markets. However, there is an information gap on how FCM larvae affect citrus fruit biochemical attributes, secondary metabolites as well as antioxidant activities. Therefore, the study evaluated the effect of false codling moth (*Thaumatotibia leucotreta*) larval growth stages on 'Midknight' valencia citrus fruit biochemical properties, selected secondary metabolites and antioxidant activities. In this experiment, matured 'Midknight' valencia citrus fruit were harvested from Joubert en Seuns farm in Schoemanskloof and transported to the University of Mpumalanga, Nelspruit, South Africa for treatment and quality evaluation. The 'Midknight' valencia citrus fruit were exposed to the following treatments: T_0 = no FCM larvae, T_1 = fruit were exposed to first instar FCM larvae and T_2 = exposed until second instar FCM larvae. For the first and second instar treatment, fruit were exposed to larvae for 3 and 12 days, respectively, then squeezed and tested for total soluble solids (TSS), titratable acids (TA), total phenolic contents (TPC) as well as total flavonoid contents (TFC), and total antioxidants using the ferric reducing power (FRAP), 2,2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays. The results showed that false codling moth first and second instar did not affect ($p > 0.05$) the TSS, TA as well as the antioxidant activity measured by ABTS of fruit. However, FCM larval growth stages had a significant influence ($p < 0.05$) on 'Midknight' valencia citrus fruit TPC, TFC and antioxidant activity quantified by FRAP and DPPH. False codling moth first instar had a great influence on phenolic compounds (Figure 3.2 and 3.3) and antioxidant activities quantified by DPPH (Figure 4.3) compared to the second instar. Meanwhile the second instar greatly affected antioxidant activities quantified by FRAP (Figure 4.1). In conclusion, false codling moth (*Thaumatotibia leucotreta*) had no influence on biochemical attributes but affected the selected secondary metabolites and antioxidant activity of 'Midknight' valencia fruit.

CHAPTER 1

GENERAL INTRODUCTION

1.1. Background

The biggest exported subtropical fruit in South Africa (SA) is citrus with around R20 billion export revenue even when exposed to stress, such as, drought (Citrus Growers' Association [CGASA], 2019; FreshFruitPortal.com, 2019). In terms of South Africa's gross domestic products (GDP), the citrus industry is one of the most significant contributing agricultural sectors (Dlikilili, 2018). Furthermore, citrus industry is mainly producing in Mpumalanga, Western Cape, Eastern Cape, Limpopo, KwaZulu-Natal, as well as the Northern Cape province in South Africa (Chisoro-Dube and Roberts, 2021; Sikuka and Caldwell, 2021). Climate is the difference among these producing provinces and best suitable for different types of cultivars in South Africa (SA DAFF, 2018). Additionally, the total produced citrus fruit types include soft citrus (12 %), grapefruit (13 %), lemon and lime (15 %) and oranges (59 %) in South Africa (Gautom et al., 2021).

Amongst the oranges, there are two sweet orange types produced mainly in South Africa, namely the navel and valencia oranges (Brodie, 2020). Navel ripens before the valencia oranges and are known for their miniature fruit developing by the fruit base (Barry et al., 2020; Brodie, 2020). According to Bonsu (2017), navel was named after the human navel and is mainly produced in the Eastern and Western Cape. Meanwhile valencia oranges were named after the city in Spain called Valencia, however, that is not their true origin (Collins, 2018). Valencia oranges are large, round with a bright thin skin and easy to peel; sweeter than navels and are mainly used for juice production (Collins, 2018). South African valencia orange production is the biggest type of citrus produced. Valencia oranges produces roughly twice the volume of navels and is exported from the country (FPEF, 2019). In 2017, the South African valencia oranges planted area was higher at 37% compared to other citrus types and about 59% of production was in Limpopo province (SA DAFF, 2018). About $1.7 billion were exported to the European Union (Nyambura and Bonorchis, 2022). Therefore, citrus is also of great value to global markets.

According to Dlikilili (2018), the global annual citrus production weighed up to 140 million tons in 2014. In 2015, the world citrus export added up to $12.5 million (CGA, 2019). Spain is the biggest fresh citrus exporter while the South African citrus industry is the second (Gaulier and Zignago, 2010; Chisoro-Dube and Roberts, 2021). In 2015, 67% of South African produced citrus were exported to various international markets, with 29% of the citrus exported to Europe (Grout, 2016). According to Grout, (2016), the countries importing South African citrus include the United Kingdom, Russia, Asia and Canada. Importing countries require South African citrus fruits to meet the quarantine needs to avoid phytosanitary pest attacks in the citrus industry (Tshikhudo et al., 2021).

Phytosanitary pests are of high economic significance to the endangered production areas (Moore, 2021). According to Mommsen and Bester (2018), citrus phytosanitary pests include carob moth, thrips, red scale mealy bug and false codling moth in South Africa. These pests are of great concern to export markets like America, the European Union and China (PPECB, 2007; Mommsen and Bester, 2018). Moreover, citrus fruit with these phytosanitary pests cannot be exported to markets with phytosanitary restrictions, as this will lead to consignment rejection in markets; and thereby, endangering the economy (Li et al., 2022).

1.1.1. Problem Statement

False codling moth is a major pest of peach, cotton, plum, citrus, macadamias, and avocados causing fruit loss through larval burrowing to feed (Maniania et al., 2017). False codling moth adults lay eggs on citrus rind, and upon hatching the larvae begins to feed on the fruit causing discolouration (Grout and Moore, 2015). In the southern African citrus industry, false codling moth larvae incidences cause an estimation of more than R100 million annual loss (Moore et al., 2004; Moore and Kirkman, 2008). Exporting countries impose quarantine restrictions against FCM infested fruits due to its great damage. According to Moore (2012), FCM larvae detection in one fruit may cause the entire consignment to be rejected, which leads to a direct financial loss. Although South Africa is the second biggest citrus exporter, and FCM being a native pest in some parts of the sub-Saharan Africa (Malan et al., 2018), there is little information on how phytosanitary pests such as FCM affect citrus phytochemicals.

1.1.2. Motivation of study

Numerous studies have been conducted on citrus fruits and phytosanitary pests. However, there is an information gap related to the effect of these phytosanitary pests on biochemical attributes, selected secondary metabolites and antioxidant activities of these fruits. According to Mommsen and Bester (2018), phytosanitary pests cause high fruit damage, hence are of great concern to international trade market. In addition, there is an increase in the demand for exporting fruits that are phytosanitary pests free. International markets require fruits to be cleared from all quarantine pests before being exported (Ndhlovu, 2022). Therefore, this study aims to address the effects of FCM as a phytosanitary pest on biochemical attributes, certain secondary metabolites and antioxidant activities quantified by selected spectrophotometric methods. This will bridge the information gap and generate new information for citrus producers and customers. The information will bring understanding on how much effect insects have particularly on fruit juice. Furthermore, the study will introduce the need for non-destructive instruments such as near infrared spectroscopy to determine internal fruit quality in a cheap and easy way.

1.2. Aim

The aim of the research was to evaluate whether there will be changes in ‘Midknight’ valencia citrus fruit biochemical attributes, secondary metabolites and antioxidant activities when infested with false codling moth (FCM) first and second larval instar.

1.3. Objectives

i. To evaluate if there were changes in ‘Midknight’ valencia citrus fruit biochemicals and selected secondary metabolites when exposed to false codling moth (FCM) first and second larval instar.
ii. To evaluate changes in antioxidant activity assayed using selected spectrophotometric methods of ‘Midknight’ valencia citrus fruit infested with false codling moth (FCM) first and second larval instar.

1.4. Hypotheses

i. There will be changes in ‘Midknight’ valencia citrus fruit biochemicals and selected secondary metabolites when exposed to false codling moth (FCM) first and second larval instar.
ii. There will be changes in antioxidant activity assayed using selected spectrophotometric methods of ‘Midknight’ valencia citrus fruit infested with false codling moth (FCM) first and second larval instar.

1.5. Dissertation structure

Chapter 1: Provides background information of the study and the importance of citrus fruit. Furthermore, the chapter presents the problem statement, motivation, aim and objective of the research study.

Chapter 2: Presents the review of past and relevant work to the study. Factors affecting sugar levels, acidity, secondary metabolites and antioxidant activities of citrus fruits are discussed.

Chapter 3: Investigates the effect of false codling moth (FCM) larval growth stages on ‘Midknight’ valencia citrus fruit biochemical attributes and secondary metabolites.

Chapter 4: Evaluates the effect of false codling moth (FCM) larval growth stages on the antioxidant activity of ‘Midknight’ valencia citrus fruit.

Chapter 5: Presents the overall summary, limitations and conclusion of the study mainly from Chapters 3 to 4 as well as future research recommendations.

CHAPTER 2

LITERATURE REVIEW

2.1. Citrus fruit

The genus *Citrus* (Rutaceae) is known to be one of the old, most famous, and traded crops in the world (Dosoky and Setzer, 2018). The origin of citrus is still debatable; however, it is said to have originated from Southeast Asia and spread across the world, specifically tropical and subtropical regions with favourable soil for growth (Magwaza et al., 2011; Zubair et al., 2015; Dosoky and Setzer, 2018). In international trade, citrus fruits are the most valued crops and produced worldwide, however, there is an unclear number of types (Magwaza et al., 2011). The well-known citrus fruits include lemon and limes, sweet oranges, soft citrus, and grapefruits (Liu et al., 2012; Ollitrault and Navarro, 2012). According to El-Otmani et al. (2011), citrus fruits are preferred for their taste, flavour, and human health properties.

Citrus fruits are known to prevent chronic diseases, such as, cancer, diabetes, neurological deficits, asthma, and cardiovascular diseases thus are important to human health (Liu et al., 2012; Aslin, 2014). In the human diet, citrus fruits provide nutritional value for the human body (Reddy et al., 2016). Furthermore, citrus fruits provide macronutrients, including, dietary fibre, vitamin C and B, minerals, and simple sugars, as well as micronutrients including thiamine, calcium, magnesium, and copper (Liu et al., 2012; Dosoky and Setzer, 2018).

Globally, citrus fruits are mostly consumed as fresh produce and a third processed for juice or jam (Liu et al., 2012; Dosoky and Setzer, 2018). According to Baldwin et al. (2014), fruit quality is used to categorise the use of the citrus. Some categories include allowable tolerance to change, juice percentage, injuries and defects in fruit minimum contents of TSS, TA and consumer demand (Suszek et al., 2018). Citrus fruit quality such as high juice content, elevated TSS, low TA are processed into oils, cleaning products, medicine, perfumes, cosmetics, several foods, and beverages (Abouzari and Nezhad, 2016; Dosoky and Setzer, 2018). Fruit quality combines all important features used to satisfy consumer needs including colour, size, shape, defects, brightness, sweetness, bitterness and acidity (Magwaza et al., 2011; Cayuela, 2012; Phalane, 2017).

2.1.1. Components affecting the production of citrus fruits

The southern African citrus enterprise and its fruit quality is impacted by many abiotic and biotic parameters, which affect plant growth, fruit quantity and quality (Zubair et al., 2015). Both the abiotic and biotic parameters affect the internal and the external appearance of the fruits, thus, reducing production. Abiotic factors include temperature, with pests and diseases as the major biotic factors (Zekri, 2011).

2.1.1.1. *Influence of temperature on citrus fruit quality*

During high temperatures and drought, there is splitting of fruit also known as creasing which leads to yield loss (Sato, 2015). Increased temperature may result in the rising of fruit drop leading to yield loss (Sato, 2015; Waleed, 2019). Furthermore, temperature also affects the citrus fruit quality, however, valencia citrus fruits are adapted to a various temperature range and produce great quality due to high adaptability (Zekri, 2011). Contrary to navel oranges which produce optimum quality in the Mediterranean region, while in the cool temperatures, valencia oranges grow big fruits with acidic juice and low TSS (Waleed, 2019). In addition, high temperature increases respiration rate, allowing the fruit to mature fast (Tano et al., 2005). Therefore, there is low TSS accumulated and reduced acidity, which cause the fruit to dry quickly (Zekri, 2011).

2.1.1.2. *Influence of pests and diseases on citrus fruit quality*

In citrus production, pests and diseases play a crucial role and are known to be the largest threat to production and exporting of citrus fruits (Moore, 2002). Furthermore, both pests and diseases highly impact production due to tree damage, leading to reduced yields and poor fruit quality (Jaouad et al., 2020). According to Moore, (2012), pests and diseases result in damage to fruits and trees, and may never recover, thus, leading to high productive orchard loss. Furthermore, pests and diseases may cause high damage to produced fruits (Hattingh, 2006). According to Gautom et al., (2021), about 65% of produced citrus fruit in SA are sold to above sixty countries worldwide. Markets require good fruit quality with no blemishes; thus, pest and disease infested fruits cannot be sold (Moore, 2012).

2.1.2. Factors affecting sugar and acidity levels in citrus fruits

Fruit ripening is usually described by the breaking down of stored carbohydrates to sugars, concomitantly, acidity reduction, increase in aromatic volatile and flavour (Klee and Giovannoni, 2011; Cherian et al., 2014). In citrus fruit, acidity parameters include pH and/or TA and a significant aspect of fruit quality (Esti et al., 2002; Harker et al., 2002; Bugaud et al., 2011). According to Etienne et al. (2013), organic acids, citric and malic acids present determine fruit acidity. In different fruit species, organic acid types vary; for example, apples and pear have malic acid while citrus fruits mainly have citric acid (Yamaki, 1984; Lu et al., 2011; Etienne et al., 2013). Moreover, other factors affecting fruit acidity include water supply, production practice, source: sink ratio and pests (Etienne et al., 2013).

2.1.2.1. *Influence of water supply on fruit quality*

Several studies have proven that water supply may have positive or negative correlation with fruit titratable acidity. According to Etienne et al. (2013), water stress reduces titratable acidity in ripe ‘Spring Bright’ and ‘Summer Bright’ nectarines due to dehydration. Meanwhile, osmotic adjustment is another water status mechanism affecting fruit acidity (Hou et al., 2020). According to Hummel et al. (2010), during water stress, plant tissues collect organic acids and sugars to reduce osmotic potential and inhibit a reduction in cell turgor pressure. When water stress rises, there is an accumulation of organic acid in the xylem fluid, leaves and fruits, thus, increasing fruit acidity (Hummel et al. 2010; Etienne et al., 2013).

2.1.2.2. *Influence of source: sink ratio on fruit quality*

Plant source: sink ratio is affected by thinning, defoliation, pruning which result in dispersed sugar supply and fruit growth; thereby influencing fruit acidity (Etienne et al., 2013). In fruits, source: sink ratio is responsible for sugar production needed for growth metabolic requirements (Thakur, and Singh, 2012; Smith et al., 2018). According to Lechaudel et al. (2005), a rise in source: sink ratio causes an early rise in citrate content during fruit growth and reduces when almost matured in mango (*Mangifera indica*) and peaches (*Prunus persica*). Moreover, fruit acidity decreases, concomitantly, sugar levels increase during fruit development (Ninio et al., 2003).

2.1.2.3. *Influence of other production practices on fruit quality*

In fruit production, other factors influencing sugar levels include pruning, irrigation and nitrogen level (Beckles, 2012). According to Fake (2012), pruning is important to internal fruit quality as it exposes the fruit to sunlight, thereby increasing TSS during fruit development. Furthermore, proper irrigation and nitrogen application rises the fruit size, TSS, weight and juice content due to increased photosynthesis rate in plants (Zekri and Obreza, 2013). According to Zhang et al. (2021), nitrogen in plants assist with the synthesis of active substances such as hormones, nucleic acid, proteins, thus influencing photosynthesis process in the plant.

2.1.2.4. *Influence of pests and diseases on fruit quality*

In plants, sugars make up the main substrate giving energy and structural material for defence and response (Morkunas, and Ratajczak, 2014). According to Bolouri-Moghaddam and Van den Ende (2013), sugars can be considered a signalling molecule as they participate in different mechanisms such as defence and immune responses. Furthermore, sugar regulates stress inducible genes to protect fruit against herbivory attack (Rolland et al., 2002). During insect and pathogen attack, sugars in plants increase as they play a role as a signalling molecule responding to the insect infestation (Wingler and Roitsch, 2008).

2.1.3. Factors affecting secondary metabolites in citrus fruits

Secondary metabolites are molecules produced by plants, allowing competitive effects on the plant and other organisms (Teoh, 2015). In plants, secondary metabolites induce development, growth and signalling (Pang et al., 2021). Furthermore, secondary metabolites attract or repel insects, meanwhile, medicinal plants use secondary metabolites as their mode of action (Teoh, 2015). According to Seca and Pinto (2018), secondary metabolites demonstrate certain bioactivities that are significant to human's wellbeing, including, anti-cancer, anti-oxidative features, and protection from cardiovascular consequences.

Secondary metabolites are active even in small amounts; however, quality and quantity differ according to external and internal factors (Massenti, 2013; Li et al., 2020). Factors including cultivar, taxon, soil composition and growing conditions,

ripeness, diseases, management practices of citrus fruit (Li et al., 2020). Citrus fruits have numerous secondary metabolites including phenol acids, carotenoids, alkaloids, flavonoids, limonoids, coumarins and essential oils (Lv et al., 2015). According to Gattuso et al. (2007), various fruits have different types of flavonoids which contribute to juice and fruit quality. Flavonoids also influence the nutritional value and taste of the fruits (Lv et al., 2015). For example, lemon and orange have hesperidin which contribute to the fruit's sediments while naringin brings about the bitterness to grapefruit juice (Gattuso et al., 2007). However, secondary metabolites quality and quantity in fruits are affected by abiotic and biotic factors (Ochoa-Velasco et al., 2017). Biotic factors include insects and pathogen microorganisms, meanwhile, abiotic are natural ecosystem, postharvest conditions (Jones et al., 2014; Ochoa-Velasco et al., 2017).

2.1.3.1. *Influence of abiotic stress on fruit quality*

In a natural ecosystem, crops face various abiotic stress combinations, aligning to specific physiological and molecular responses to deal with the introduced stress (Mittler and Blumwald, 2010). Abiotic stress includes environmental factors such as wounding, high light, nutrient deficiencies, heavy metals, drought, salt stress and temperature (Ramakrishna and Ravishankar, 2011). These factors increase phenylpropanoids, meanwhile, cold stress influences phenolic compound production and accumulation of anthocyanins in fruits (Dixon and Paiva, 1995; Griffith and Yaish, 2004). In addition, heavy metals, drought and salt stress also increase secondary metabolite production in fruits (Ramakrishna and Ravishankar, 2011). According to Sharma et al. (2022), abiotic stress induces secondary metabolites as a response for plants to keep their physiology in fruits.

2.1.3.2. *Influence of pests and diseases on fruit quality*

According to Bonaventure (2018), insect pests release saliva and depolarise plasma membrane, followed by a calcium increase, thereafter, reactive oxygen species (ROS) production occurs in plant cells during herbivory feeding. These ROS are produced as a plants' immune response to induce defences (War et al., 2015). High ROS concentration degrades DNA, protein structure and organelles of the plant (Juan et al., 2021). The ROS are then reduced to water through the release of the peroxidase

(POD) enzyme (War et al., 2012; Koch et al., 2016). Thereafter, peroxidase enzyme catalyses lignin production as well as oxidative phenols to assist with cell wall structure strengthening (Zhao et al., 2016), thereby, reducing secondary metabolites in the plants through oxidation (Duffey and Stout, 1996). Alternatively, phenylalanine ammonia lyase (PAL) is synthesized to increase plant resistance through the production of secondary metabolites during fruit attack by insect pests (Bhonwong et al., 2009).

2.1.4. Factors affecting antioxidant activities in citrus fruits

Citrus fruits are main sources of functional phytochemicals including flavonoids, carotenoids, mineral elements, vitamin A, C and E (Zhou, 2012). According to (Zou et al., 2016), these antioxidants are consumed through fresh fruits and their by-products and reported to have different biological uses such as anti-aging, antiinflammation, antioxidant to human's well-being. Antioxidants in citrus fruits signify the ability of compounds to sustain the cell structure as well as responsible for hindering lipid peroxide reactions, clearing of free radicals and oxidative damage prevention (Bravo, 1998). However, antioxidants are influenced by parameters including environmental conditions, management practices, pests and diseases (Zou et al., 2016).

2.1.4.1. *Influence of environmental factors on fruit quality*

Some environmental factors influencing antioxidants in citrus fruits include storage conditions, temperature and gas composition (Moretti et al., 2010; Zou et al., 2016; Carmona et al., 2022). Studies have shown that the correct storage conditions increase bioactive compounds in fruits thus improving their antioxidant activities (Zou et al., 2016). According to Carmona et al. (2022), during cold storage citrus fruits have a raise in the potential to influence flavonoid accumulation. For example, during cold storage 'Moro' blood oranges increases in anthocyanins, flavanones and a slight reduction in Vitamin C (Rapisarda et al., 2008). Furthermore, gas composition in storage has an impact on antioxidant activities of citrus fruits, thus the need to appropriately control carbon dioxide (CO_2) and oxygen (O_2) (Moretti et al., 2010). According to Yang et al. (2008), high oxygen in storage delivers an increase in DPPH scavenging capacity.

2.1.4.2. *Influence of management practices on fruit quality*

Post-harvest management practices in citrus fruits include the application of ethylene, exposure to ultraviolet (UV) radiation (Zou et al., 2016). Ethylene is a significant agricultural plant hormone responsible for inducing fruit ripening, it is the main enzyme in the producing of phenolic compounds and phenolic constituent accumulation (Iqbal et al., 2017). Additionally, ethylene also takes part in phenylalanine ammonia lyase activity stimulation in citrus fruit post-harvest treatments (Hyodo and Yang, 1971). According to Huang et al. (2010), citrus fruits treated with 1.5% of oligochitosan post-harvest tend to increase antioxidant enzymes including polyphenol oxidase (PPO), peroxidase (POD), ascorbate peroxidase (APX), superoxide dismutase (SOD) activities. Additionally, UV radiation in post-harvest treatment is used to sanitize fruits resulting in the stimulation of phenylpropanoid metabolism and induction of biological stress leading to the production of phytoalexin compounds such as flavonoids in citrus (Zou et al., 2016).

2.1.4.3. *Influence of pest and diseases on fruit quality*

Generally, in the absence of stress, plants show adequate growth and development using the present oxygen to photosynthesise (Kaur et al., 2022). However, during plant stress caused by factors such as pest and disease attack, the present oxygen is rather used to produce reactive oxygen species (ROS) to protect plant tissues (Singla et al., 2019). As a result, causing photo-oxidative damage to the cellular structures and biomolecules in the plant (Xie et al., 2016). According to (Kaur et al., 2022), these microbes interactions cause the plant to induce an excess number of biochemical changes related to stress signalling and consequently activating defense pathways. Some of the defense mechanism induced include different enzymatic components, phenol metabolism enzymes as well as antioxidant enzymes (Akter et al., 2015). Furthermore, other defense mechanism such as lipophilic organic compound and carotenoid are also used to detoxify produced reactive oxygen species (Das and Roychoudhury, 2014).

2.2. Citrus pests

2.2.1. False codling moth (FCM), *Thaumatotibia leucotreta* (Lepidoptera: Tortricidae)

Worldwide, false codling moth (FCM) is a significant phytosanitary pest that causes economic impairment on citrus and other subtropical, deciduous fruits and vegetables (Prinsloo and Uys 2015). False codling moth pest status is significant due to its large adaptability range; and has been recorded in all South African citrus-producing areas (de Jager, 2013). False codling moth feeds on the inside of the fruit, its larvae burrow into the fruit, causing internal damage; and thereby, reducing market suitability (Malan et al., 2018).

2.2.1.1. Pest distribution

False codling moth is an Ethiopian native pest and present in most parts of sub-Saharan Africa, near India, Atlantic Ocean islands and Israel (Malan, et al., 2018; Love, 2015). In SA, the pest has been noted in all seven provinces that produce citrus and reported as a harmful citrus pest, however, they have different impacts due to differences in climate and biomes per region (Moore and Hattingh, 2012). According to Love (2015), the FCM pest is associated with deserts, grasslands, savannahs, tropical and subtropical areas with moist broadleaf forest.

2.2.1.2. Host range

In South Africa, false codling moth has been noted in about 24 cultivated plants including macadamia nuts, litchis, guavas, avocados and citrus fruits (Grout and Moore, 2015). False codling moth is the greatest problem in citrus fruits such as soft oranges and grapefruit (Moore, 2012; Love, 2015). False codling moth also has been recorded to damage maize stems and cobs in Central Africa as well as the heads of sorghum and cotton in Zimbabwe (Love, 2015).

2.2.1.3. Life cycle

The false codling moth is a holometabolous insect, meaning the adult and juvenile do not look alike; therefore, do not consume similar food types (Bernays, 1986; Gullan and Cranston, 2000). The FCM juvenile is the most important stage as it feeds on fruits (Moore, 2012). The development and feeding of larvae have an impact on fruit

at any developmental stage; and thereby, resulting in premature fruit ripening and dropping (Daniel, 2016). False codling moth life cycle may take from 30 to about 174 days, with about 2 to 10 generations yearly (Daniel, 2016). However, this may depend on certain factors involving food availability and quality, temperature, and humidity to survive (Stibick et al., 2010).

2.2.1.3.1. Adult stage

False codling moth adults are small and unnoticeable (Moore, 2022). The FCM adult's front wings have black, grey, and orange-brown marks with a triangular mark outside (Daniel, 2016). The female FCM adults are 19 to 20 mm long and their lifespan is 16 to 70 days; meanwhile the males are 15 to 16 mm long and live for about 14 to 57 days (de Jager, 2013; Daniel, 2016). During the day, false codling moth adults hide in the host plant's shaded portions as they are only active at night (de Jager, 2013; Daniel, 2016). In the dark, FCM adults communicate using pheromones produced by females (Stibick, 2010; de Jager, 2013). In about five hours after sunset, there is a peak in the release of pheromones, which reduces until the sun rises (Stibick, 2010; de Jager, 2013; Daniel, 2016). During the peak, mating and egg laying occurs (de Jager, 2013).

2.2.1.3.2. Egg development

The FCM females randomly lay tiny eggs singly on the fruit's rind, fallen fruits or foliage during the night (Love, 2015). As the tree height increases, eggs laid also raises, especially on the sides where more sunlight is received (Love, 2015). According to de Jager (2013), the insects lay an average of 3 to 8 eggs on each fruit at 25°C. However, in heavy infestations more females lay eggs on one fruit, and not all of them will live because of cannibalism (Stibick et al., 2010). The female can lay up to 800 flat, oval, and about 1 mm long eggs in her lifetime (Grout and Moore, 2015). The eggs are clear in colour and pale when just laid (Daniel, 2016). As the organism inside develops, the egg changes from a pink-red colour to grey-black with the grey-black part being the developing larva's head capsule (Moore, 2012; Love, 2015; Daniel, 2016). According to de Jager (2013), temperature is an important influence on the duration of egg development before hatching. As temperature increases, development rate also

increases (Malan et al.¸ 2018). In favourable temperatures, eggs may take 9 to 14 days to hatch into the first instar (Daniel, 2016).

2.2.1.3.3. Larval stage

The false codling moth (FCM) has five instars, which take about two weeks to a month during hot conditions and five weeks to two months to complete its larval development when the conditions are cool (de Jager, 2013). The first, second and third instars are small and cream in colour with dark spots and brown blackheads while the fourth and fifth instars transform in body colour to pink red (Love, 2015). Furthermore, the first instar enters the host fruit through a hole that it creates in the rind, thus causing a discoloured wound (Stibick et al. 2010; Love, 2015; Daniel, 2016). According to Stibick et al (2010) and Moore (2022), any larvae may enter the fruit, however, only one larva survives due to cannibalism. The larvae begin to eat and develop as they are inside the pulp, thereafter, move to the centre of the fruit and continue growing even after the fruit drops (de Jager, 2013). For hanging fruits, the matured juvenile utilizes a silk thread to fall to the soil (Daniel, 2016). Matured larvae (fifth instar) exit the fruit using holes filled with insect frass, which are the excrements of larvae left on the fruit (Zahn, 2017). The damage leads to fruit drop with the larvae ready for pupation in the soil or debris inside a silky cocoon (Love, 2015; Daniel, 2016).

2.2.1.3.4. Pupal stage

During the pre-pupal stage, a new cocoon is formed on the surface of the soil, pre-pupae are immobile and do not feed, and last for 2 to 27 days (Love, 2015). Pre-pupae have a sex ratio of 1:1, and this may be because of temperatures in the surrounding (Daniel, 2016). According to van den Berg (2001) and (Daniel, 2016), the pupa is cream in colour and soft at first, however, they become dark and hard as they mature. Pupae are sensitive to rainfall and low temperatures, therefore, higher mortality rate as temperatures decrease to below 10.5°C (de Jager, 2013). Before adult emergence, the pupae come out of the cocoon, meanwhile, the pupal case and cocoon remain attached (Daniel, 2016). The females begin to emerge out of the pupal case from 11 to 39 days, while males begin to emerge from 13 to 49 days (de Jager, 2013; Daniel, 2016).

2.2.1.4. Fruit damage

In citrus fruits, FCM occurs in any developmental stage and causes direct and indirect damages (Malan et al., 2018). According to de Jager, (2013), young FCM larvae penetrate under the surface of fruits; and bore into the skin causing fruits to ripen prematurely. Fruits that have just been penetrated by FCM may be a threat to production as they cannot be easily detected until post-harvest, thus the need for careful fruit inspection (Grout and Moore, 2015; Malan et al., 2018). As the larvae develop in the fruit, and attempt to leave the fruit for pupation, FCM larvae uses available previous penetrated sites increasing the hole size resulting to insect frass (Malan et al., 2018). In addition, penetration in immature green peel turns to yellow while matured fruits with orange peels show decaying and become brown and sunken (de Jager, 2013). Damaged fruits are vulnerable to fungal infections and scavengers and take about three to five weeks to drop (Daniel, 2016).

2.2.1.5. Economic importance

False codling moth is an economically important pest because most of its host plants are valuable crops and potential economic damage on most of the crop commodities (de Jager, 2013; Moore, 2021). In the southern African citrus industry, FCM accounts for an annual loss estimated to R100 million due to post harvest damage and decay as well as rejection of export consignment as fruits show signs of false codling moth infestation (Moore and Kirkman, 2008). According to Moore (2012), if a consignment destined to phytosanitary markets show any sign of FCM infestation, the whole shipment is rejected leading to significantly high economic loss. In addition, false codling moth (FCM) larvae found in the fruits usually results in premature ripening causing economic loss (Mkiga et al., 2019).

2.3. Total antioxidant assay

Total antioxidant assay evaluates the antioxidant activity of plant extracts, beverages (Bibi-Sadeer et al., 2020). Antioxidant assaying methods have been grouped according to the estimation of inhibition low density lipoprotein oxidation and their dependence on the free radical scavenging capacity quantification (Pisoschi et al., 2016). Due to the oxidant/antioxidant reaction, the techniques are also grouped into single electron transfer (SET) and hydrogen atom transfer (HAT) groups, whereby,

SET based technique depends on a single electron transfer reductive ability of a radical species versus an antioxidant compound (Kasote et al., 2015). An example of SET technique includes cupric reducing antioxidant capacity (CUPRAC) and ferric reducing antioxidant power FRAP. Meanwhile, HAT techniques evaluate the antioxidant capacity to trap free radicals through donating a hydrogen atom, these includes techniques such as (Oxygen Radical Antioxidant Capacity) ORAC and total radical-trapping antioxidant parameter (TRAP) (Huang et al., 2005). Other techniques such as (2,2-Diphenyl-1-picrylhydrazyl) (DPPH) and ({2,2' - azinobis-(3-ethyl-benzothiazoline-6-sulphonic acid)}) (ABTS) are known for using both SET and HAT because the radicals can be scavenged using either electron reduction or radical quenching involving hydrogen transfer (Pisoschi et al., 2016).

2.3.1. Total phenolic content by Folin-Ciocalteu

The Folin-Ciocalteu assay is a technique used to determine TPC (Krishnaiah et al., 2011). According to Huang et al. (2005), the technique was used to evaluate proteins and later adopted to analyse phenolic contents in wine. Currently, Folin-Ciocalteu assay is a routine test for plant and food antioxidant assay (Karadag et al., 2009). Furthermore, Folin-Ciocalteu extract nature contains phosphomolybdic/phosphotungstic acid complex that is reduced to produce a blue chromophore when assayed spectrophotometrically at 765 nm maximum absorption (Munteanu and Apetrei, 2021). According to Kasote et al. (2015), the Folin-Ciocalteu technique is SET based and involves phenolic antioxidant the reducing power. For this test, gallic acid is used as a standard and the outcomes are reported as Gallic acid equivalents (GAE) (Krishnaiah et al., 2011). However, other standards such as caffeic acid, catechins, chlorogenic acid or ferulic acid equivalents can also be used to express total phenolic contents (Munteanu and Apetrei, 2021).

The Folin-Ciocalteu is simple to use, robust and reproducible, however, for reliable results the reaction state should be accurately selected as the method is sensitive to reaction time, pH, and temperature (Prior et al., 2005; Munteanu and Apetrei, 2021). Additionally, for the Folin-Ciolcalteu method, total phenolic content overestimation is of concern, contributing to the non-phenolic reducing agents in the system during Folin-Ciocalteu reagent reduction (Blasco et al., 20005). Therefore, when compared to the high-performance liquid chromatography technique, the total phenolic content

results may be overestimated by one size (Munteanu and Apetrei, 2021). The Folin-Ciocalteu test is done in aqueous systems with limited lipophilic phenol application besides when solvent system modifications are applied (Prior et al., 2005).

2.3.2. Ferric reducing antioxidant power (FRAP) assay

The FRAP test evaluates ferric ion ligand complex reduction to the blue ferrous complex by antioxidants at a low pH condition (Alam et al., 2013). Antioxidant activity is assayed using a spectrophotometer at 593 nm absorbance with the results reported in connection to a standard antioxidant in the FRAP test (Antolovich et al., 2002). Furthermore, FRAP is used in low pH conditions where pH = 3.6, so iron solubility is maintained (Munteanu and Apetrei, 2021). When carried out in a low pH, the ionisation potential that controls electron transfer decreases while, redox potential increases, resulting in a shift in the main reaction mechanism (Hegerman et al., 1998). According to Berker et al. (2007), the FRAP assay utilises tripyridyltriazine (TPTZ) to bind iron ion to the ligand and the final product is spectrophotometrically assayed to show the tested antioxidant reducing power. In this case, the antioxidant reduces the ferric ion complex in the solution to ferrous complex, linking the free ferric ions to produce a Prussian blue colour (Munteanu and Apetrei, 2021).

The FRAP method is fast, cheap, and easy to use, however, time is essential as the Prussian blue has the tendency of precipitating to make a suspension as well as stain the measurement vat (Prior et al., 2005; Munteanu and Apetrei, 2021). To prevent Prussian blue against precipitation, it was suggested by Berker et al. (2010) to add a tensioactive compound, sodium dodecyl sulphate and optimum pH to maintain ferric ion redox activity and inhibit hydrolysis (Berker et al., 2010). Furthermore, the FRAP method was continuously improved by choosing acetone/water as a solvent in the absence of the randomly methylated β-cyclodextrine solubility potentiator allowing the measurements of lipophilic and hydrophilic antioxidants at the same time (Berker et al., 2012).

2.3.3. ABTS ({2,2' - azinobis-(3-ethyl-benzothiazoline-6-sulphonic acid)}) assay

The ABTS assay is also called Trolox equivalent antioxidant capacity (TEAC) and evaluates the antioxidants' capacity to neutralize ABTS stable radical cation ($ABTS^{\bullet+}$) (Munteanu and Apetrei, 2021). Furthermore, the $ABTS^{\bullet+}$ is made using ABTS

oxidation with potassium persulfate, it is a blue green chromophore, and its reduction is spectrophotometrically measured at 734 nm maximum absorption (Krishnaiah et al, 2011; Munteanu and Apetrei, 2021). The ABTS technique is used to determine colour loss when an antioxidant is added to $ABTS^{\bullet+}$ and during the process, antioxidant reduces $ABTS^{\bullet+}$ to ABTS (Alam et al., 2013). According to Krishnaiah et al. (2011), antioxidant activity assayed by ABTS can be calculated using the Trolox standard curve and expressed as Trolox equivalent antioxidant capacity of the extract (TEAC/mg).

Furthermore, $ABTS^{\bullet+}$ is water soluble and other organic solvents including, ethanol, methanol, allowing antioxidant activity determination in hydrophilic and lipophilic compounds, such as carotenoids (Munteanu and Apetrei, 2021). The TEAC test permits the evaluation of different antioxidant substances because ABTS radical reacts with natural and synthetic substances in food such as peptides, phenols, amino acids, vitamin C and E (Walker and Everette, 2009). However, according to Tian and Schaich (2013), several phenolic compounds contain low redox potential, thus can react with ABTS radical. In addition, TEAC reaction may differ for slow reactions thus taking time to reach the endpoint. Using a short duration endpoint (6 minutes) may result to antioxidant capacity underestimation as the reading was done before reaction was completed (Munteanu and Apetrei, 2021).

2.3.4. (2,2-Diphenyl-1-picrylhydrazyl) DPPH assay

DPPH is defined as a stable free radical due to the delocalisation of the extra electron over the molecule so that there is no molecule dimerized (Huang et al., 2005; Krishnaiah et al., 2011). According to Alam et al. (2013), electron delocalisation increases a violet colour defined by an absorption band in an organic solvent except for water. This reaction is measured spectrophotometrically at the maximum absorbance of 517 nm with discolouration as an antioxidant activity indicator (Munteanu and Apetrei, 2021). According to Alam et al. (2013), to analyse the antioxidant activity using free radical scavenging, DPPH radical change in optical density is monitored. The DPPH radical scavenging percentage equation is as follows:

$$\% \text{ inhibition of DPPH radical} = ([A_{br} - A_{ar}]/A_{br}) \times 100$$

Where A_{br} is the absorbance before reaction and A_{ar} is the sample absorbance (Alam et al., 2013).

The DPPH radical usually soluble in organic solvents such as ethanol, methanol, or their aqueous mixture but it is not soluble in water. When using aqueous mixture, the water content should not be over 60% so the radical is more readily soluble (Staško et al., 2007). According to Munteanu and Apetrei (2021), when the water content is high, there is a conversion of the dissolved DPPH quintet spectrum to singlet. Furthermore, the DPPH method, is cheaper and easy, reproducible, has automation possibilities, can be used at room temperature however, the free radical used is stable and not present in vivo (Prior et al., 2005).

CHAPTER 3

EFFECT OF FALSE CODLING MOTH (*THAUMATOTIBIA LEUCOTRETA*) LARVAL GROWTH STAGES ON 'MIDKNIGHT' VALENCIA CITRUS FRUIT BIOCHEMICALS AND SELECTED SECONDARY METABOLITES

Abstract

The false codling moth (FCM) poses a significant threat to citrus fruit production and marketing in South Africa. Infestation by the FCM can cause changes in fruit biochemical components, subsequently affecting the fruit quality for consumption and commercial sale. In this study, we associated false codling moth (FCM) infestation and two developmental larval instar growth stages with chemical property change in 'Midknight' valencia citrus fruit. 'Midknight' valencia citrus fruit were infected with FCM first and second larval developmental stages for 3 and 12 days, respectively. Thereafter, fruit juice was used for the determination of TSS, TA, TPC, and TFC. The results showed that TSS, TA, and TSS/TA ratio were not significantly different ($p > 0.05$) between FCM larvae infected fruit and control fruit. However, the TSS level and TA% were higher for the FCM larvae infected fruit, irrespective of the instar larvae growth stage. Furthermore, fruit infected with FCM first and second instar larvae exhibited significantly higher TPC and TFC when compared with control fruit. This study found that the larval growth stages of the false codling moth infestation led to slight changes in chemical properties but a significant increase in the phytochemical concentration of 'Midknight' valencia citrus fruit.

Keywords: Total soluble solids, titratable acids, total phenolic contents, total flavonoid contents

3.1. INTRODUCTION

South Africa is the world's second largest citrus exporting country and is responsible for 10% of global exports (Chisoro-Dube and Roberts, 2021). The South African citrus industry exports 65% of the produce and processes about 25% (Gautom et al., 2021). Common citrus cultivars that South Africa exports include grapefruit, lemons, soft citrus, and oranges (Ntombela and Moobi, 2013). However, the South African citrus industry faces significant constraints such as pest and disease attack, as well as environmental challenges. Constraints such as pest attack can lead to high losses of harvestable and marketable citrus fruit due to zero phytosanitary pest tolerance imposed by trading nations, such as the FCM (Moore, 2021).

False codling moth *Thaumatotibia leucotreta* is a South African export phytosanitary pest as it is native to sub-Saharan Africa (Moore, 2012). According to SA DAFF (2015) false codling moth has a phytosanitary status in South African international markets such as the United States of America, Europe. In South Africa, FCM is an economically significant pest that impacts citrus fruit marketability because these markets have zero tolerance for this pest (DAFF, 2015; Grout and Moore, 2015). According to Li et al. (2022), when FCM larvae have penetrated citrus fruit, it is no longer marketable to international markets as one FCM seen can result to the rejection of the whole consignment.

Insects such as FCM feeding induces an increase in the synthesis of ROS and hydrogen peroxide in the plant (Goggin and Fischer, 2022). According to Rashid and Chung (2017) the increase in ROS and hydrogen peroxide triggers oxidative stress response to the damaged plant as a defense mechanism against the attacking insect. Additionally, the increase in ROS and hydrogen peroxide (H_2O_2) can directly kill insect pests through intestinal disruption or indirectly induce pathogen elimination (Paiva and Bozza, 2014). However, an increase in ROS may also cause oxidative damage, a loss in enzyme activity as well as programmed cell death to the plant due to their high oxidative features thus the need to detoxify it (Xie et al., 2014; Huang et al., 2019; Kovalikova et al., 2019). To detoxify reactive oxygen species phenols and phenylalanine ammonia-lyase (PAL) are synthesized by the plant using the shikimic acid pathway (Kovalikova et al., 2019).

The shikimic acid pathway brings about carbon flow from carbohydrate metabolism in plants to aromatic compound biosynthesis (Wu et al., 2022). According to Lin et al. (2016) in plants, phosphoenolpyruvate and erythrose 4-phosphate are converted to chorismate through seven shikimate pathway steps. Chorismate serves as the main precursor to produce aromatic amino acids, namely tryptophan, tyrosin, phenylalanine (Zhang et al., 2012). Phenylalanine is a substrate for various secondary metabolites including flavonoids, phenylpropanoids, anthocyanin (Feduraev et al., 2020). According to Zhang et al. (2012) these secondary metabolites are responsible for plant growth and development, structural element lignin and phenolic compounds. Meanwhile phenolic compounds are responsible for plant defense mechanisms against stress caused by various factors including pests such as FCM (Zhang, 2022).

When FCM larvae bore through fruit peels, the damage can appear discoloured and blemished, but can only be detected in a thorough inspection (Grout and Moore 2015). In unripe fruit, FCM larvae infested in peels cause yellowing, whereas ripe fruit are initially orange before becoming brown and sunken later in the decay process (Moore, 2021). Therefore, to guarantee that fruit is not infested with this pest's eggs or larvae during export, many export markets often require postharvest treatment (Pryke and Pringle, 2008). In this study, we associated false codling moth infestation and two developmental larval instar growth stages with biochemical properties and secondary metabolite change in ‘Midknight’ valencia citrus fruit. The purpose of this study is to fill a knowledge gap about the effects of FCM on the biochemical properties and secondary metabolite of 'Midknight' valencia citrus fruit.

3.2. MATERIALS AND METHODS

3.2.1. Study area

Matured ‘Midknight’ valencia citrus fruit were collected from Joubert en Seuns farm, Schoemansfkloof in Mbombela, Mpumalanga, South Africa (25°27′16″ S, 30°25′18″ E). The ‘Midknight’ valencia citrus fruit were transported to the University of Mpumalanga (25°43’64’’ S, 30°98’17’’ E), in Mbombela, Mpumalanga, South Africa for laboratory treatment and analysis.

Figure 3.2. 1. Matured 'Midknight' valencia citrus fruit used

3.2.2. Sample preparation

False codling moth (FCM) eggs were provided by Citrus Research International (CRI), Eastern Cape (25°47'90" S, 30°99'28" E). The eggs were couriered to the University of Mpumalanga. At the laboratory, the eggs were left to hatch into larvae overnight at room temperature (± 25°C). Thereafter, twenty (20) 'Midknight' Valencia citrus fruit were exposed to the larvae using a thin paintbrush. Approximately 60 insect larvae were collected and brushed onto each 'Midknight' Valencia citrus fruit. The larvae were left to burrow into the fruit at room temperature. After three days, 10 infested fruit were randomly selected to test the effect of FCM first instar on quality features such as TSS and TA. Ten (10) fruit that were not exposed to FCM larvae were tested as a control. The remaining fruit were stored for 12 days at room temperature to test the effect of FCM second instar larvae on the fruit quality features. All the fruit were dissected into two parts and juice was squeezed into separate beakers for each 'Midknight' Valencia citrus fruit to analyse the quality features at room temperature (± 25°C). The excess fruit juice was stored in centrifuge tubes at -80°C for further evaluation of TPC and TFC.

3.2.3. Determining total soluble solids (TSS) and titratable acidity (TA)

Total soluble solids was determined using the method previously prescribed by Pila et al. (2010). 'Midknight' valencia fruit total soluble solids was evaluated by use of a

handheld refractometer (Model 121, Yagami International Ltd, Japan) at 25°C and results were reported as °Brix. The refractometer calibration was done using distilled water prior to each reading. Furthermore, TA was tested by use of the revised method prescribed by Wills and Ku (2001). A total of 10 ml of juice was poured into an Erlenmeyer flask. The sample was added 5 drops of phenolphthalein as an indicator and titrated with 1 M sodium hydroxide (NAOH) until pink and data were expressed in citric acid percentage that was calculated as described by Sadler and Murphy (2010):

$$\% \text{ Acid (wt/vol)} = \frac{\mathrm{N} \times \mathrm{V1} \times \mathrm{Eq\ wt}}{\mathrm{V2} \times 1000} \times 100$$

Where: N = normality of titrant, usually NaOH (mEq/ml); V_1 = volume of titrant (ml); Eq. wt. = Equivalent weight of predominant acid (mg/mEq) = 0.06404; V_2 = volume of sample (ml); 1000 = factor relating mg to grams (mg/g) (1/10 = 100/1000)

3.2.4. Determining total phenolic content (TPC)

Total phenolic content was evaluated by the use of a modified Folin-Ciocalteu method (Abeysinghe et al., 2007). A juice sample of 0.5 ml was poured into a glass test tube with 6 ml of distilled water. In the solution, 0.5 ml of Folin-Ciocalteu reagent was added then left to react for 3 minutes. After the 3 minutes incubation, 1 ml of 20% sodium carbonate was added and the solution was mixed and placed in a hot water bath for 1 minute. To prepare a blank, 0.25 ml of methanol was used instead of juice. In determination of the absorbance, a Spectrophotometer (Model: UV-1900i, Shimadzu Scientific Instruments, Inc., USA) was used and set at 650 nm. To prepare a calibration curve, gallic acid standard was used. The results were reported as mg gallic acid equivalent (mg GAE)/ 100 g DM.

3.2.5. Determining total flavonoid content (TFC)

Total flavonoid content was tested as previously prescribed by Abeysinghe et al. (2007). 'Midknight' valencia citrus juice (0.5 ml) was poured into a glass test tube with 3.5 ml of ethanol. Thereafter, 4 ml of 90% diethylene glycol was added while thoroughly mixing then 0.1 ml of 4 M NaOH was added to initiate the reaction. The solution was vortexed, and after incubating for 10 minutes before reading the absorbance at 420 nm in room temperature using a Spectrophotometer (Model: UV-1900i, Shimadzu Scientific Instruments, Inc., USA). For a standard, Rutin was used,

and the total flavonoid concentration was expressed as mg rutin equivalent (mg RUE)/100 g DM).

3.2.6. Statistical analysis

All statistical analyses were carried out using Statistix-10 software. Data analysis was subjected to Analysis of Variance (ANOVA). Means were separated using the Least Significant Difference (LSD) at a 5% level. All results were presented as means.

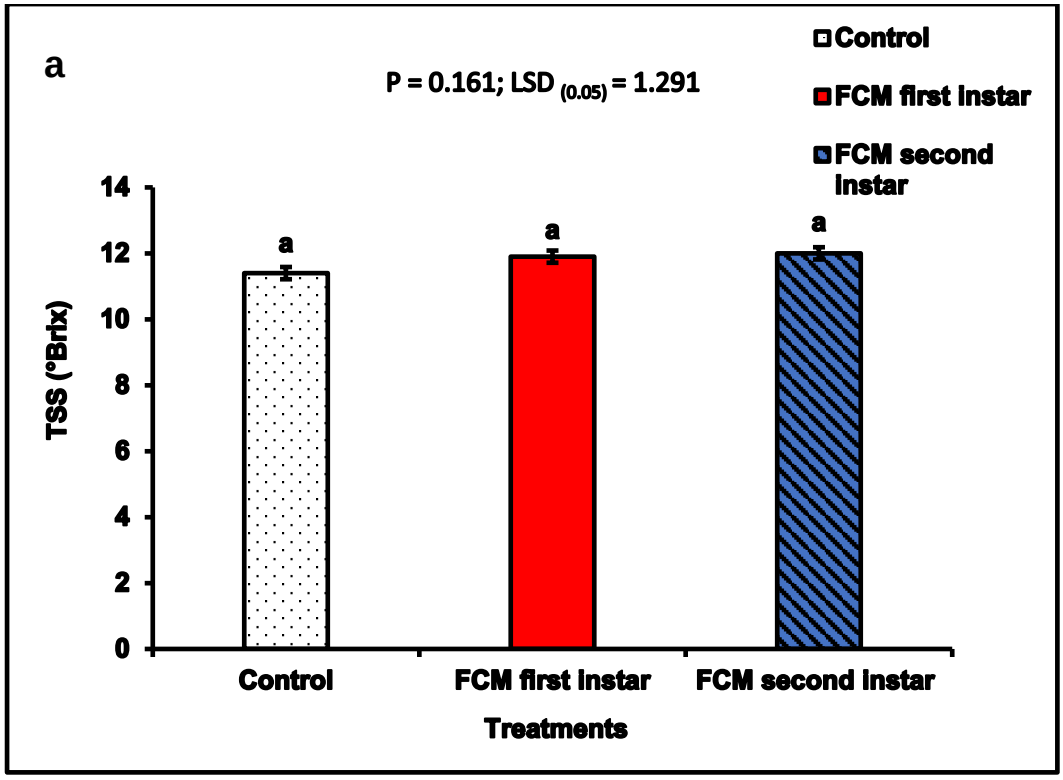
3.3. RESULTS AND DISCUSSION

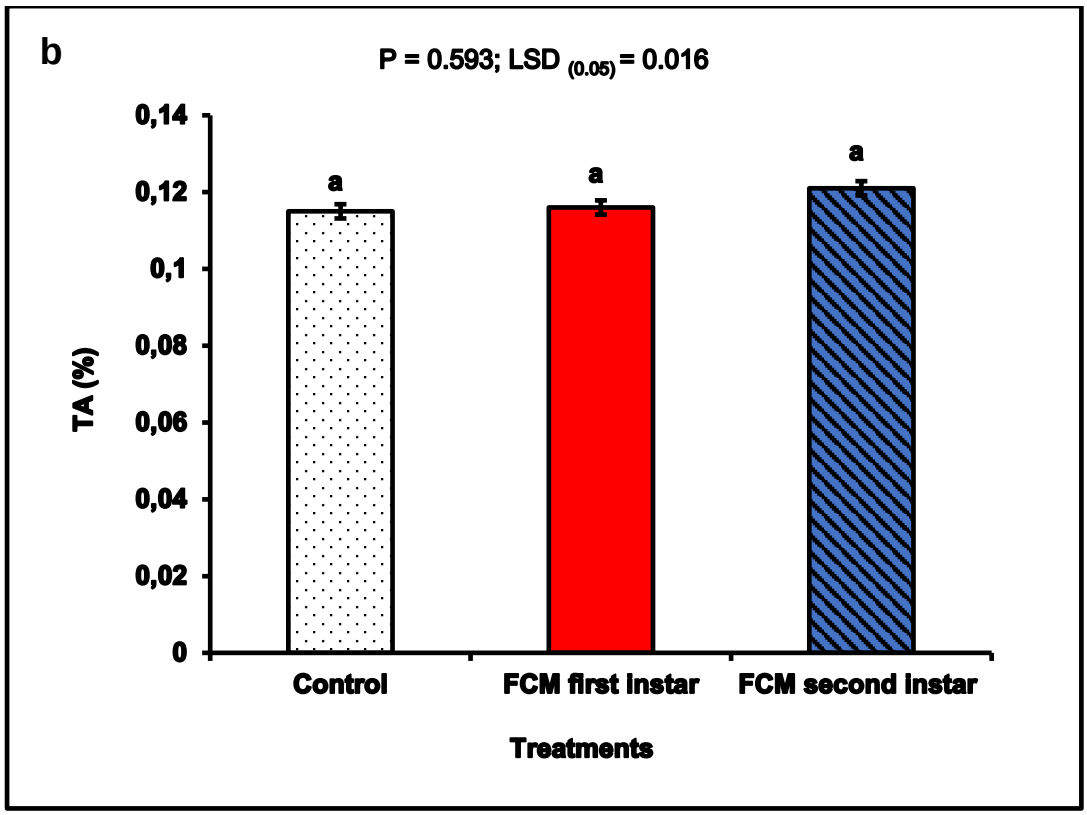
3.3.1. Changes in total soluble solids, titratable acid and TSS/TA ratio after false codling moth (FCM) larvae infestation

In this study, there were no statistically significant differences ($p > 0.05$) in TSS between FCM larvae infected when compared with control fruit (Figure 3.1a). According to Adom et al. (2021), the first instar is small and responsible for cutting open a very tiny wound in the rind, and this could explain the non-statistical difference between the treatments. However, the TSS level was higher for the FCM larvae infected fruit when compared with the control. Similarly, Léo et al. (2020) found altered TSS after Mediterranean fruit fly (*Ceratitis capitata*) infestations in guava ('Tailandesa'). The FCM larvae infest fruit through wounds and cracks, causing secondary infestations by fungi or bacteria, further reducing fruit quality (Adom et al., 2021). In this study, the increase in TSS level could be attributed to larva feeding damage induced by puncturing. Thus, the punctured opening allowed the larvae to colonize inside the fruit which promoted an enzymatic reaction that altered its chemical composition. It has been demonstrated that eggs and larvae deposited in the fruit can induce cell stresses, leading to unexpected metabolic reactions (Omoloye et al., 2016). Moreover, Formela-Luboińska et al (2020) found that cubense (*Fusarium oxysporum*) infection resulted in a substantial abscisic acid (ABA) accumulation in tissues containing high soluble sugar levels, implying soluble solids not only function as respiratory substrates but also trigger metabolic signals that affect plant defence genes. Moreover, sugar metabolism and signalling play a significant role in promoting plant defence responses and led to the definition of the term "sweet immunity" (Wang et al., 2016; Bezrutczyk et al., 2018). A study conducted by Svara et al. (2020) investigated the effect of exogenous levan polysaccharide treatments on the susceptibility of 'Borkh' apple to the apple scab fungus (*Venturia inaequalis*). In this

research work, it was reported that sugar concentrations in the infected samples were significantly higher than those in control samples, regardless of the treatments (Svara et al., 2020). In this study, the observed slight increase in TSS level may also be attributed to signalling functions of soluble sugars caused by feeding and extracellular digestion of fungi or bacteria associated with FCM infestation and stress responses after FCM larvae infestation (Figure 3.1a).

In general, TSS and TA are the main ripe citrus fruit soluble components and highly affect taste and take part in fruit sourness, thus contributing to flavour (Malundo et al., 2001). Omoloye et al. (2016) reported that Mediterranean fruit fly (*Ceratitis capitata Wiedemann*) infestation results in a reduced shelf-life and a bitter taste in sweet citrus ('Agege 1') juice because it affected the sweet-to-acid (TSS/TA) ratio. This was observed in this study as the highest titratable acids (TA) percentage was recorded in FCM second instar followed by the first instar larvae stage of infestation, with the lowest percentage recorded for the control fruit (Figure 3.1b). This evidence suggested that FCM larvae puncturing the fruit and feeding resulted in the fruit losing quality, thereby becoming bitter due to an increase in TA percentage. Moreover, the change in TA after FCM larvae infestation followed the same trend as TSS, therefore 'Midknight' valencia citrus fruit infected with FCM had a higher TA percentage when compared with the control. It has been reported that a decrease in organic acid (TA) concentration is associated with an increase in soluble sugars (TSS) in mangoes ('Ataulfo') (Palafox-Carlos et al., 2014; Quirós-Sauceda et al., 2019). In studies, most organic acids in fruit flesh are synthesized through the tricarboxylic cycle, not imported from foreign sources (Famiani et al., 2015). These studies have also found that fruit organic acid content can be altered by biotic and abiotic factors (Famiani et al., 2015; Li et al., 2017; Solomon et al., 2018). This may explain the observed decreasing trend in TSS/TA ratio (Figure 3.1c) when FCM larvae infested the fruit when compared with the control.


a
P = 0.161; LSD (0.05) = 1.291
Control
FCM first instar
FCM second instar
TSS (°Brix)
14
12
10
8
6
4
2
0
a
a
a
Control
FCM first instar
FCM second instar
Treatments


b
P = 0.593; LSD (0.05) = 0.016
TA (%)
0,14
0,12
0,1
0,08
0,06
0,04
0,02
0
a
a
a
Control
FCM first instar
FCM second instar
Treatments

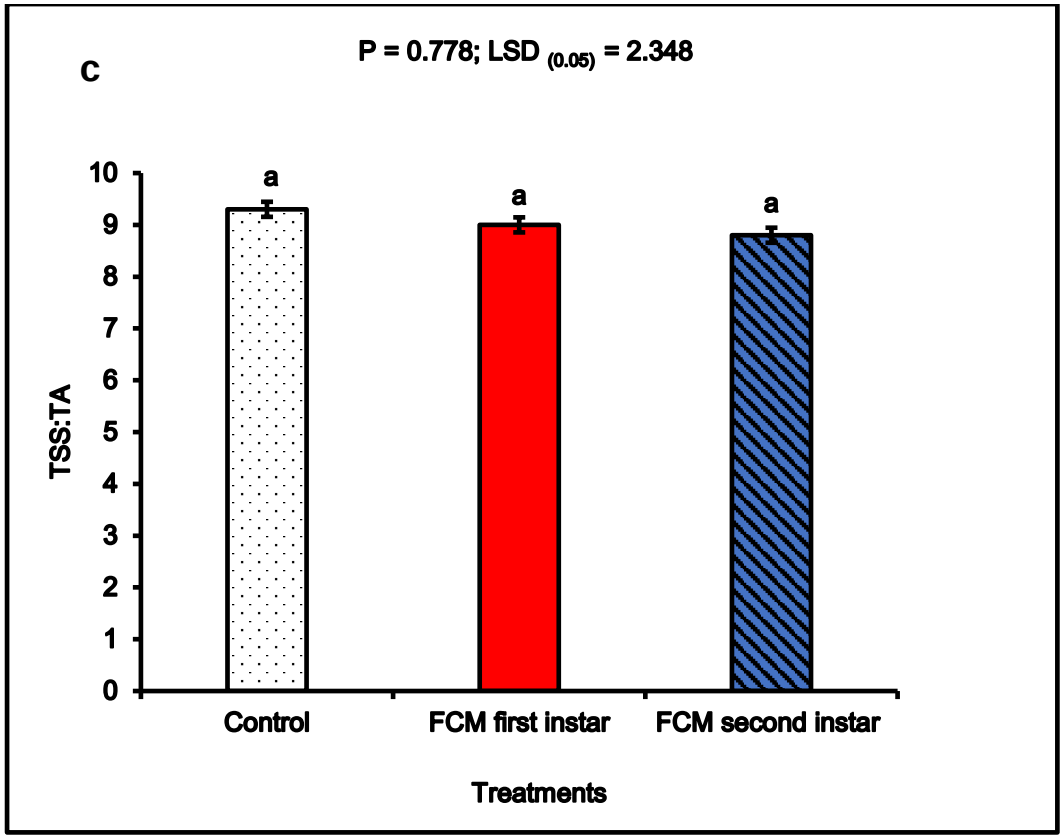


Figure 3. 1. Effects of false codling moth larval growth stages on 'Midknight' valencia citrus fruit (a) total soluble solids (TSS) (b) titratable acids (TA) (c) total soluble solids/ titratable acids ratio (TSS/TA). Vertical bars with similar letters and overlapping error bars represent non-significant difference between treatments according to LSD test at p = 0.05

3.3.2. Changes in total phenolic content after FCM first and second instar infestation

In this study, the inoculation of false codling moth (FCM) was associated with significantly ($p < 0.05$) higher total phenolic contents (TPC) when compared with those of the control fruit (Figure 3.2). In general, the presence of phenolic compounds in host plants play a great role to their resistance to herbivores, including insects, and also provides protection against ROS (War et al., 2012). Phytochemically, phenols are synthesized by plants using shikimic acid, and PAL the enzyme responsible for catalyzing the phenylpropanoid pathway that leads to polyphenols synthesis (Kovalikova et al., 2019). Studies found that PAL activity in mums ('Keiun', 'Han6' and 'Jinba') was strongly enhanced after aphid (*Aphidoidea*) infestation as well as in kale ('Khanyari' and 'Kawdar') after cabbage butterfly (*Pieris brassicae*) herbivory and correlated with higher phenol concentrations (He et al., 2011; Ibrahim et al., 2018). In

this current study, FCM larvae feeding induced oxidative stress in the fruit, leading to the production of ROS, and leading to increased total phenolic content. It may be for this reason that FCM larvae infected fruit had the highest TPC when compared with controls (Figure 3.2). Physiological stress caused by insect infestation can also trigger plant defence mechanisms by activating the jasmonic acid (JA) signalling pathway (War et al., 2012). Furthermore, JA activates antioxidative enzymes, such as peroxidase (POD), lipoxygenase (LOX) and polyphenol oxidase (PPO), and proteinase inhibitor (PI) production (War et al., 2012). In plants, jasmonic acid is a signaling molecule associated with secondary metabolism, resistance to stress and defense against pathogen (Koo et al., 2009; Wang et al., 2016). Yan et al. (2009) and Rahimi et al. (2014) reported that JA then regulates genes that encode secondary metabolites such as phenolic biosynthesis in wounded fruits.

In wounded grape tomato (‘Burpee’) fruit the induction of stink bug's (*Halyomorpha halys*) saliva genes was caused by jasmonate and the jasmonate pathway induces the production of phenolics (Devoto and Turner, 2003; Peiffer and Felton, 2014). Zhou et al. (2016) concluded that stink bug (*Halyomorpha halys)* saliva may stimulate phenolic production in blueberry fruits (‘Bluecrop’ and ‘Elliott’) by activating the jasmonate pathway. Kaur et al. (2017) found that aphid (*Aphidoidea*) infestation on wheat (‘PBW 658’) led to a significant increase in total phenol. The increase in phenols may be a defense mechanism since phenolic compounds have been found to prevent larval growth in fruits (Kaur et al., 2017). In the present study, 'Midknight' valencia citrus fruit inoculated with FCM larvae had higher TPC due to the phenolic compound synthesis as a defense mechanism against larvae infestation and feeding.

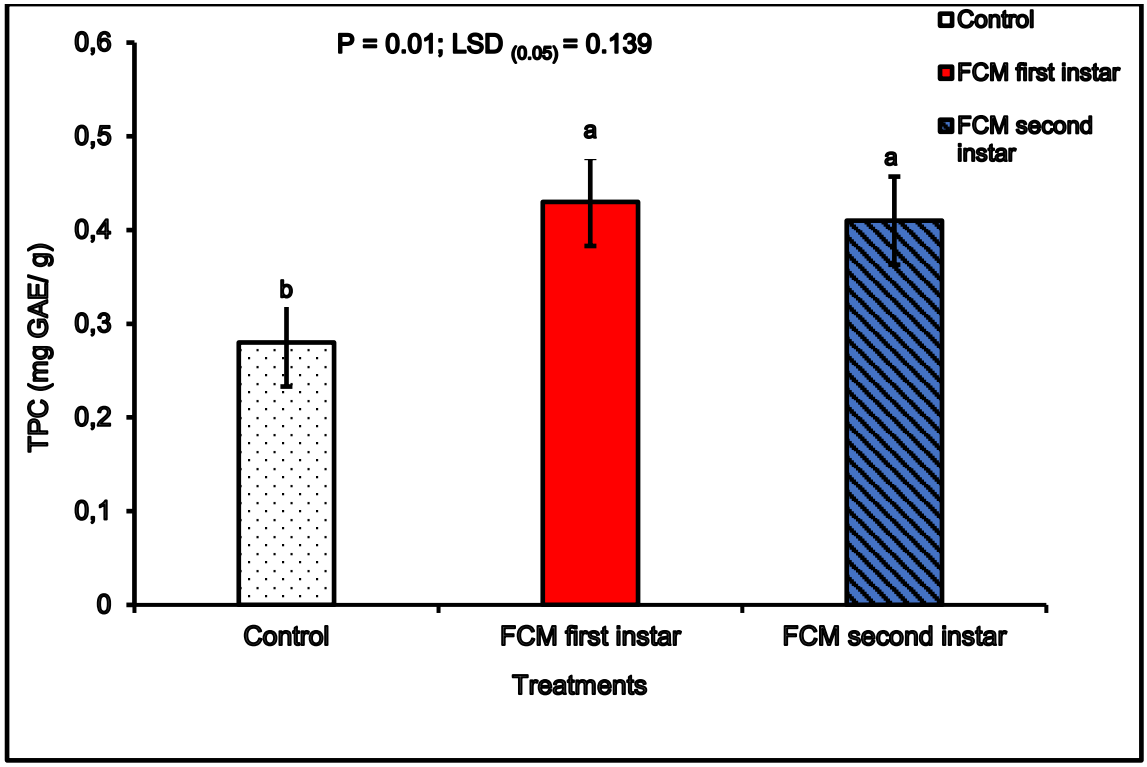


Figure 3. 2. Effects of false codling moth larval growth stages on 'Midknight' valencia citrus fruit total phenolic content (TPC). Vertical bars with different letters and no overlapping error bars represent the statistical differences between treatments according to the LSD test at p= 0.05

3.3.3. Changes in total flavonoid content (TFC) after FCM first and second instar infestation

There was a significant effect ($p < 0.05$) of false codling moth inoculation on the TFC of 'Midknight' valencia citrus fruit (Figure 3.3). A significant increase in TFC was observed with FCM larvae inoculated fruit when compared with control fruit. The total flavonoid content of 'Midknight' valencia citrus fruit infected with FCM first and second instar larvae was higher when compared with the control (Figure 3.3). This study was comparable to the one conducted by Ivancic et al. (2022), whereby, the effects of brown marmorated stink bug (*Halyomorpha halys)* on phenolic compounds in damaged and controlled different olive fruit cultivars ('Istrska belica' and 'Pendolino'). The authors found that 'Pendolino' olive fruit damaged by the stink bug (*Halyomorpha halys)* had a significant increase in flavones and flavanols when compared to the control and the undamaged fruit that had been exposed to the stink bug.

Infested fruit, the increase in flavanols and flavones was linked to flavonoids acting as plant defence mechanism, thereby deterring insects, changing plant palatability, decreasing digestibility, reducing nutritional content, or having toxic effects on pests (Ivancic et al., 2022). According to other studies, flavonoids play a crucial role in insect-plant interactions and protection against pests and can be divided into six categories, such as isoflavones, anthocyanins, flavones, flavanones, flavanols, and flavan-3-ols, (Mierziak et al., 2014; Yamagata et al., 2019). According to Kahramanoglu and Usanmaz (2021), flavonoids such as quercetin, Kaempferol, tangeretin, rutin and naringin deter insect pests and provide tolerance against biotic stresses in citrus fruit. During feeding, insect pests release saliva and increase calcium in fruit, thereafter, producing reactive oxygen species (ROS) (Bonaventure, 2018). Flavonoids are then synthesized by the injury sight to quench ROS in the fruit (Mierziak et al., 2014; Kahramanoglu and Usanmaz 2021). In this study, the TFC in fruit inoculated with FCM larvae were significantly higher when compared with the controls (Figures 3.3). It may also imply that phenolic acid, which is also a substrate for other secondary metabolites, participated in the biosynthesis of flavonoids during larvae feeding. As a result, flavonoids produced increased, perhaps functioning as a defence mechanism against FCM larvae infections (Mierziak et al., 2014).

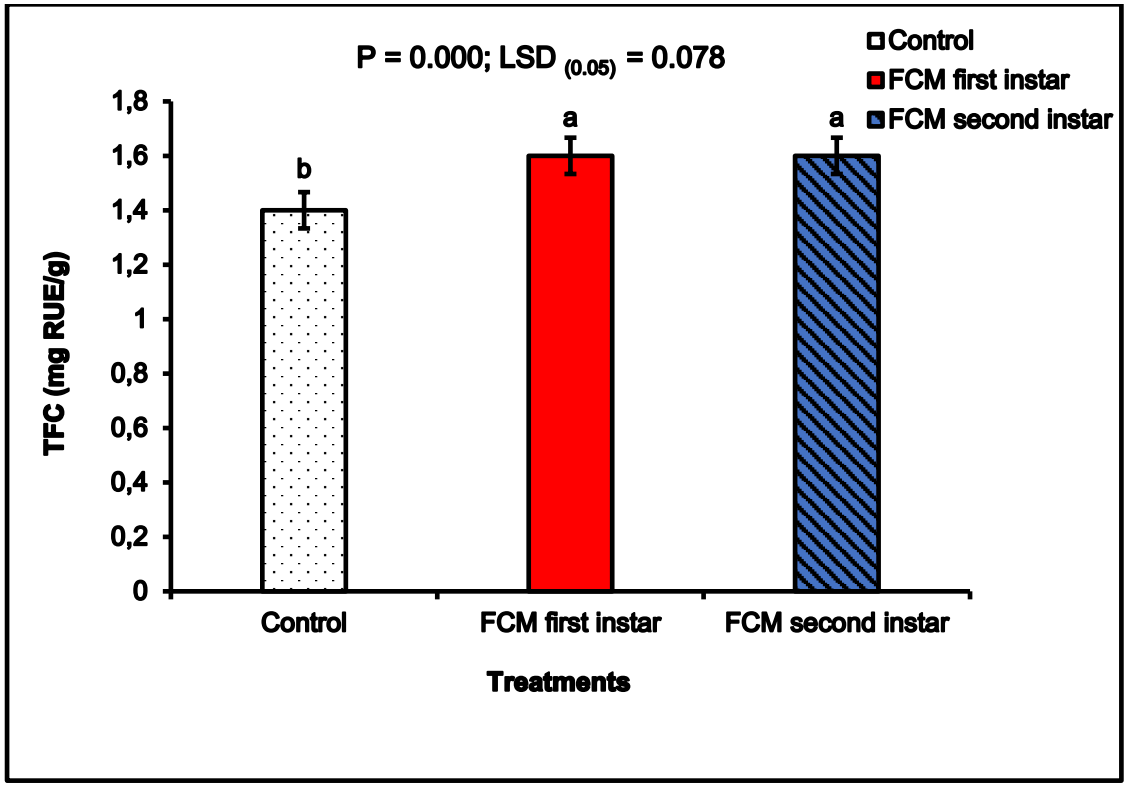


Figure 3. 3. Effects of false codling moth larval growth stages on ‘Midknight’ Valencia citrus fruit total flavonoid content (TFC) in FCM larval growth stages. Vertical bars with different letters and no overlapping error bars represent the statistical differences between treatments according to the LSD test at p = 0.05

3.4. CONCLUSION

This study found that the larval growth stages of the false codling moth (FCM) infestation led to moderate changes in chemical properties but a significant increase in phytochemical concentration. The changes considerably affected the quality of ‘Midknight’ valencia citrus fruit, causing fruit to be unsuitable for consumption and processing. Based on the results of this study, it is recommended that field infestation of FCM should be considered a serious problem, and efforts should be focused on controlling this pest and preventing larval development after harvest.

CHAPTER 4

THE EFFECT OF FALSE CODLING MOTH (*THAUMATOTIBIA LEUCOTRETA*) LARVAL GROWTH STAGES IN 'MIDKNIGHT' VALENCIA CITRUS FRUIT TOTAL ANTIOXIDANT ACTIVITIES USING SELECTED SPECTROPHOTOMETRIC METHODS

ABSTRACT

Globally, citrus is one of the most produced fruits and significantly contributes to the country's economy. However, fruit production is affected by factors such as insect pest including FCM. False codling moth (FCM) affect fruit physical and chemical properties, thus leading to the host being unmarketable. The main objective for this study was to investigate the effect of false codling moth (FCM) on antioxidant activities quantified by the ferric reducing power (FRAP), 2,2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays in 'Midknight' valencia citrus fruit. To achieve this objective, 'Midknight' valencia citrus fruit were infested with FCM larvae until the first and second instar larvae developmental stages for 3 and 12 days, respectively. Thereafter, squeezed fruit juice was used to determine antioxidant activities by FRAP, ABTS and DPPH assays. In this study, FCM larvae did not significantly ($p > 0.05$) affect 'Midknight' valencia citrus fruit antioxidant activity assayed by ABTS. However, 'Midknight' valencia citrus fruit antioxidant activity assayed by FRAP and DPPH assay was significantly ($p < 0.05$) affected by FCM larvae growth stage. In addition, 'Midkight' valencia antioxidant activity of fruit exposed to first and second FCM larval instar was higher compared to the control. In conclusion, FCM larval stages had a significant influence on fruit antioxidant activity when assayed by FRAP and DPPH, meanwhile ABTS recorded an insignificant impact in 'Midknight' valencia citrus fruit.

Key words: ferric reducing power (FRAP), 2,2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH)

4.1. INTRODUCTION

On a global scale, citrus has a largest production culture in the fruit sector (Primo-Capella et al., 2021). In South Africa, two thirds of produced citrus fruit is exported fresh and yearly generates 95% earnings (Chisoro-Dube and Roberts, 2021). Citrus fruit are known to be great sources of phytochemicals that have different of biological functions including antiinflammation, anti-aging, antioxidants to human health (Zhang et al., 2018). Due to these human health benefits, citrus fruit demand has elevated, thus, the need to increase production (Zou et al., 2016). However, citrus fruit production is faced with several constraints such as phytosanitary pests attack which hinder its development and reduces production (Jaouad et al., 2020). Phytosanitary pests such as mealybugs, mites, fruit fly and FCM also have negative impact on citrus quality and yield (Moore, 2021).

According to de Jager (2013) false codling moth (FCM) is an economically significant pest as it causes damage to various agricultural crop such as citrus fruits. In South Africa, FCM infested orchards may result in 80% yield loss within five months, diminishing the marketability (Malan et al., 2018; Adom et al., 2021). According to Moore (2002) and Hattingh (2006), FCM present in a country has immediate export market effects; one larva detected in fruit marked for export could result to the rejection of the whole consignment. Export market consequences could be in a form of a ban from exporting host fruit traded by the country where FCM pest is established (Adom et al., 2021). Several authors have reported that FCM larva is the most destructive stage as it is a chewing insect and is a threat to the market.

In fruits, chewing insects such as FCM larvae make holes, surface discolouration and trigger plant hormones, protein phosphorylation increasing reactive oxygen species and transient Ca^{2+} influxes into the cytoplasm (Barbercheck, 2010; Takahashi et al., 2011). According to Poljsak (2013), ROS production results in plant oxidative stress which balances oxidant/antioxidant. Naturally, plants respond to oxidative stress through the development of antioxidant defense mechanism to neutralize ROS (Ali et al., 2019). Studies have reported that antioxidants also take part as defense mechanisms during insect such as false codling moth attack.

Antioxidants are substances that inhibit oxidizable substrates from oxidizing when present in low concentration than the substrate (Kasote et al., 2015; Ravimannan and Nisansala, 2017). Antioxidants are produced in the cytosol and chloroplast stroma and Nicotinamide adenine dinucleotide phosphate (NADPH) is used as the electron donor (Alscher et al., 1997). According to Foyer and Noctor (2005) these antioxidants are low in molecular weight and used as redox buffers that communicates with different cellular components as well as induce plant growth and development. Furthermore, antioxidants also take part in gene expression in relation to abiotic and biotic stress response to protect the plant (Kasote et al., 2015). During abiotic and biotic factors, there is an increase in plant ROS thus induces oxidative stress (Krishnamurthy and Rathinasabapathi, 2013). As a result, there is an increase in plant antioxidants accumulation and production such as phenolic acids, vitamin C to scavenge free radicals and reduce agents (Lobo et al., 2010; Kasote et al., 2015). According to Bibi-Sadeer et al. (2020), these antioxidants are assayed using total antioxidant capacity. Total antioxidant capacity is tested using the concept of how many free radicals were quenched by the use of a test solution to assay the antioxidant activity of a given sample (Munteanu and Apetrei, 2021). Furthermore, in these assays, plants are evaluated for their responsibility as hydrogen atom donors, reducing agents, metal chelators, singlet oxygen quenchers (Kasote et al., 2015). These assays can also be used to determine antioxidant activity in plants when attacked by pest such as FCM.

According to Malan et al. (2018) and Adom et al. (2021), false codling moth larvae burrows into the fruit feeding on seeds and the pulp. False codling moth entry point is left with frass on the fruit surface and rind discoloration (Stibick, 2006; de Jager, 2013). However, there is currently lack of information on how false codling moth affects fruit antioxidant activities. Thus, the aim of this current study was to evaluate the effect of FCM on ‘Midknight’ valencia citrus fruit antioxidant activity assayed by FRAP, ABTS and DPPH.

4.2. MATERIALS AND METHODS

4.2.1. Sample preparation

Sample preparations were conducted according to chapter 3.

4.2.2. Determining antioxidant activity by Ferric Reducing Power (FRAP)

The FRAP quantification was conducted using an adjusted method of Li et al. (2006). The technique was based on ferric 2,4,6-trripyridyl-s-triazine complex (Fe^{3+}- TPTZ) reduction to the ferrous form (Fe^{2+}- TPTZ) using a reductant, afterwards, evaluating the mixed antioxidant power of antioxidant molecules found in the tissue. The FRAP reagent was prepared by adding 300 Mm sodium acetate buffer of pH 3.6, 10 mM Fe^{2+}- TPTZ in 40 mM HCL and mM $FeCL_3 \bullet 6H_2O$ (10:11); 1000 µL FRAP reagent was combined with 30 µL sample and left for 10 minutes to react and the absorbance read at 593 nm by use of Spectrophotometer (Model: UV-1900i, Shimadzu Scientific Instruments, Inc., USA).

4.2.3. Determining antioxidant activity by 2,2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS)

The antioxidant activity was tested using the extract's potential to scavenge ($ABTS^{\bullet+}$) radicals (Liyana-Pathirana and Shahidi, 2006). A 7 mM ABTS solution in water was prepared and potassium persulphate (2.45 mM) was added to the solution to form $ABTS^{\bullet+}$. The ABTS radical was incubated for 16 hours, thereafter, ethanol was added to the stock solution until the absorbance reached 0.7 ± 0.02 at 734 nm. The $ABTS^{\bullet+}$ diluted solution (200 µL) was mixed with10 µl sample and incubated for 5 minutes at 30°C. The absorbance decreased reflecting the $ABTS^{\bullet+}$ radical scavenging capacity of the antioxidant. In the control, the absorbance of $ABTS^{\bullet+}$ without sample was tested by use of Spectrophotometer (Model: UV-1900i, Shimadzu Scientific Instruments, Inc., USA). The following equation was used to determine the inhibition percentage: Inhibition % = [(AC – AS)/AC] ×100, where AC is the absorbance of the control and AS is the absorbance of the sample plus ABTS radical at t = 5 minutes.

4.2.4. Determining antioxidant activity by DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity

Total antioxidants were assayed using antioxidant assay according to the method prescribed by Karioti et al. (2004). Two (2) millilitres of 'Midknight' valencia citrus juice was poured into centrifuge tubes (Model: 309, Hermile labortechnik, Germany). Thereafter, 10 ml of 100% methanol was added and mixed at room temperature for 30 seconds. Afterwards, 15 µl of the sample was added into the Eppendorf tubes with

735 µl of 100% methanol in each sample. Then 750 µl of DPPH solution was added to each sample. The test tubes were closed and placed in the dark for 30 minutes incubation. Methanol (100%) was used to prepare a blank and the absorbance was read at 517 nm by use of Spectrophotometer (Model: UV-1900i, Shimadzu Scientific Instruments, Inc., USA). Radical scavenging activity was calculated using the DPPH percentage that was scavenged using the following formula:

Radical scavenging activities (%) = (1 - AE / AD) x 100

Where AE is the absorbance of the reaction mixture containing the standard antioxidant or extract and AD is absorbance of the DPPH solution only.

4.2.5. Statistical analysis

Experiments were carried out using completely randomized design. All statistical analyses were performed using Statistix-10. Data were subjected to Analysis of Variance (ANOVA). Means were separated using Least Significant difference (LSD) at the 5% level of significance and presented as means.

4.3. RESULTS AND DISCUSSION

4.3.1. Changes in antioxidant activity by FRAP after FCM first and second instar infestation

In this study, ‘Midknight’ valencia fruit antioxidant activities measured by FRAP were significantly ($p < 0.05$) influenced by FCM larvae infestation (Figure 4.1). According to FRAP results, ‘Midknight’ valencia fruit infected with FCM larvae showed higher antioxidant activity in comparison to the control fruit. Furthermore, fruit infested with second instar larvae antioxidant activity were high when compared with first instar larvae and control fruit. The results of the current study were similar to those reported by Rubio-Melgarejo et al. (2020) and Rubio-Melgarejo et al. (2021), whereby, antioxidant activity quantified by FRAP significantly increased after anthracnose fungus (*Colletotrichum siamense*) and Fusarium solani (*Nectria haematococca*) infestations in ‘Soursop’ fruit. It was found that plants increased antioxidant activity assayed by FRAP, due to plant defence mechanism against pathogens by trapping iron to inhibit pathogen virulence and triggering oxidative stress (Rubio-Melgarejo et al., 2020). It was reported by Liu, et al. (2021) that plants distribute iron in an attempt

to limit pathogen growth. In addition, iron is stored in vacuoles, and pathogens and plants compete for iron during an attack in plants (Franza and Expert, 2013). Moreover, pathogens scavenge plant iron through different mechanisms during infection, while plants interfere with pathogen iron scavenging by disrupting iron signalling, sequestering iron in ferritin, and making defensins (Herlihy et al., 2020). A pathogen-induced iron deficiency activates the immune response. Thus, plants recognize pathogen-associated patterns to initiate a pattern-triggered immunity response (Bürger and Chory, 2019). Herlihy et al. (2020) suggested that pattern-triggered immunity and iron deficiency activate plant resistance to pathogens with the same hormone responses. In this current study, FCM larvae feeding caused an increase in antioxidant activity quantified by FRAP to limit pathogen growth in infested 'Midknight' valencia citrus fruit.

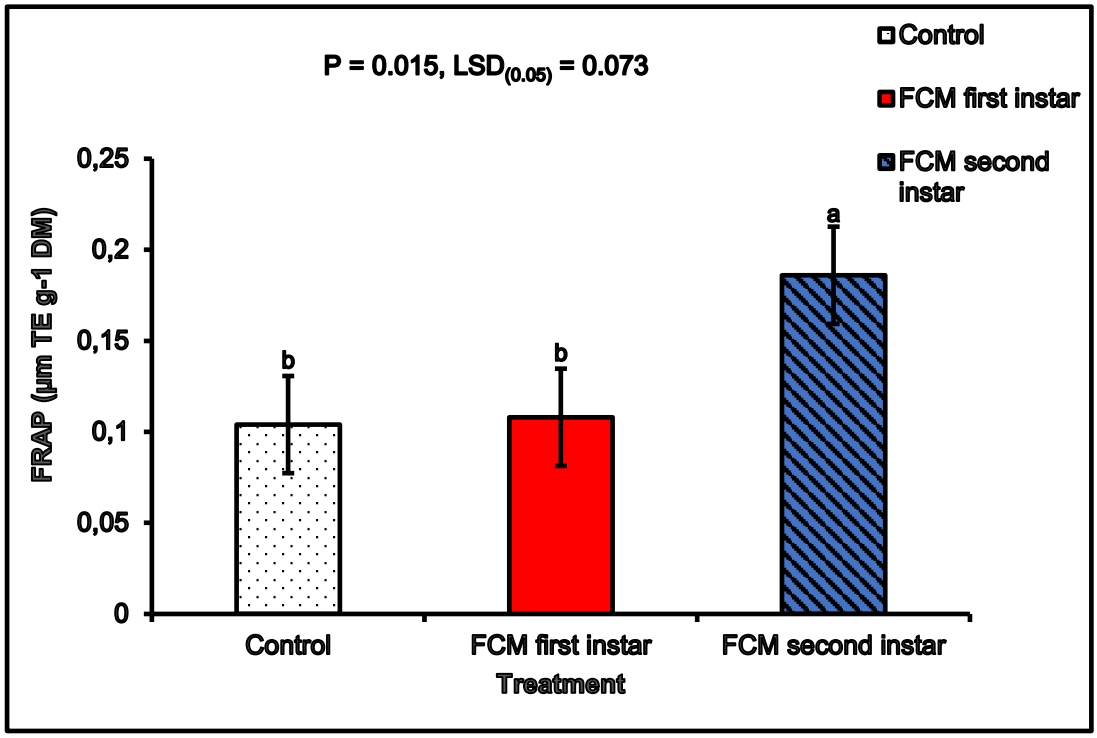


Figure 4. 1. Effects of false codling moth larval growth stages on 'Midknight' valencia citrus fruit antioxidant activity quantified by FRAP. Vertical bars with different letters and no overlapping error bars represent the significative differences between treatments according to the LSD test at p = 0.05

4.3.2. Changes in antioxidant activity by ABTS after FCM first and second instar infestation

In this study, there was no significant ($p > 0.05$) difference in antioxidant activity measured by ABTS between FCM instar larva-infected fruit and controls (Figure 4.2). However, ABTS analysis revealed that fruit inoculated with the first instar larvae of the FCM had higher antioxidant activity than that infested with the second instar larvae and control in 'Midknight' valencia citrus fruit. The results of this study were similar to those of Rubio-Melgarejo et al. (2020), who investigated how anthracnose fungus (*Colletotrichum siamense*) affects ‘Soursop’ fruit after harvest. The antioxidant activity of ‘Soursop’ fruit measured by ABTS was not significantly affected by anthracnose fungus (*Colletotrichum siamense*) inoculation in postharvest storage; however, it causes an increased (Rubio-Melgarejo et al., 2020). According to ABTS analysis, ‘Soursop’ fruit antioxidant activity increased because the fruit responded quickly to insects and pathogens (Rubio-Melgarejo et al., 2020). Mitra et al. (2019) found that pathogen attack and insect attack induce stress in ludwigia (‘Rubin’) resulting in ROS. Oxidation burst occurs as a result of reactive oxygen species, hence, the need for network signals to scavenge ROS (Bhattacharjee, 2012; Llorent-Martínez et al., 2017). The production of antioxidants, including phenolics, is also critical for scavenging ROS (Mitra et al., 2019). In this study, the increase in antioxidant activities assayed by ABTS could be due to fruit adaptation mechanisms that enable them to respond quickly to invaders or insect infection by producing secondary metabolites (Spoel and Dong, 2012).

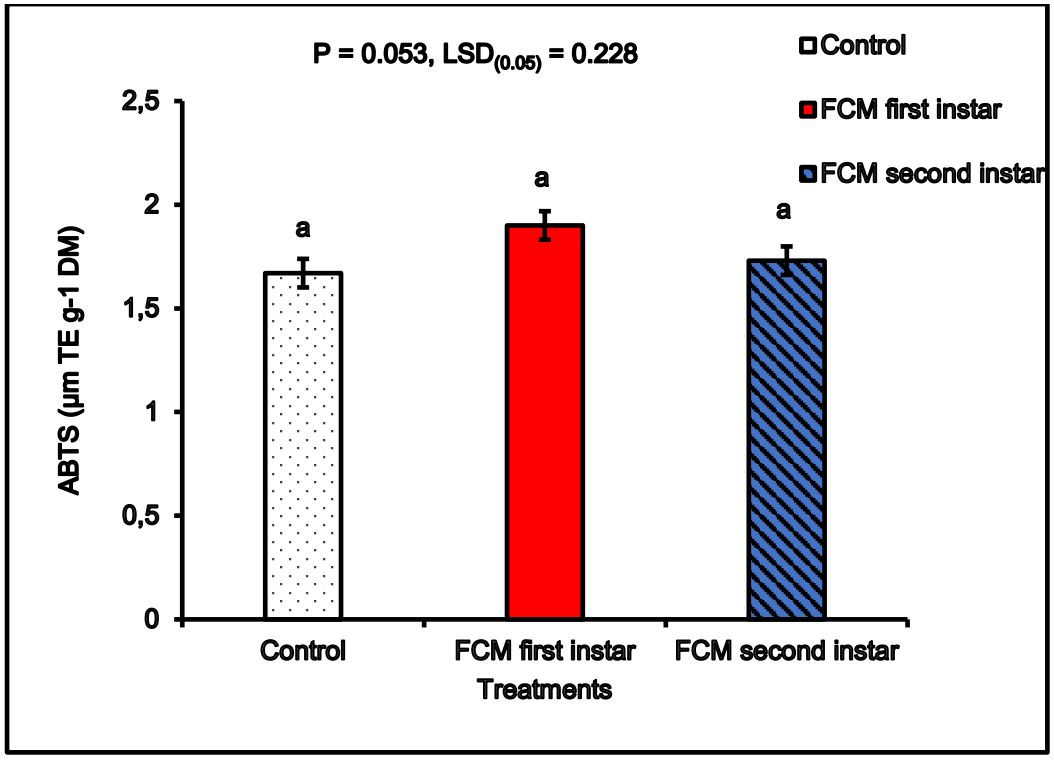


Figure 4. 2. Effects of false codling moth larval growth stages on 'Midknight' valencia citrus fruit antioxidant activity quantified by ABTS. Vertical bars with similar letters and overlapping error bars represent non-significant difference between treatments according to the LSD test at p = 0.05

4.3.3. Changes in antioxidant activity by DPPH after FCM first and second instar infestation

There was a significant ($p < 0.05$) difference in antioxidant activity measured by DPPH in 'Midknight' valencia citrus fruit after inoculation with FCM larvae (Figure 4.3). In this study, fruit inoculated with FCM first and second instar larvae resulted in higher antioxidant activity in comparison to the control fruit. Similarly, Ivancic et al. (2022) investigated the effects of brown marmorated stink bug (*Halyomorpha halys*) insect on various olive fruit cultivars (Istrska belica and Pendolino). Brown marmorated stink bug (*Halyomorpha halys*) (BMSB) inoculation significantly increased antioxidant activity of 'Istrska belica' olive fruit measured by DPPH when compared to control. The increase in antioxidant activity caused by BMSB feeding was associated with the fruit's defense strategy against insects (Ivancic et al. 2022). When insects attack plants, they cause cellular damage to tissues, thereby causing plants to produce reactive oxygen species

(Reyes et al., 2007). According to Arshiya (2013), reactive oxygen species in damaged tissues delay or prevent oxidative damage to lipids, nucleic acids, and proteins, thereby allowing antioxidants to detoxify ROS. In this present study, antioxidant activity assayed by DPPH in 'Midknight' valencia citrus fruit significantly increased with false codling moth larvae infestation; this may reflect the fruit's defensive response to insect pests (Reyes et al., 2007).

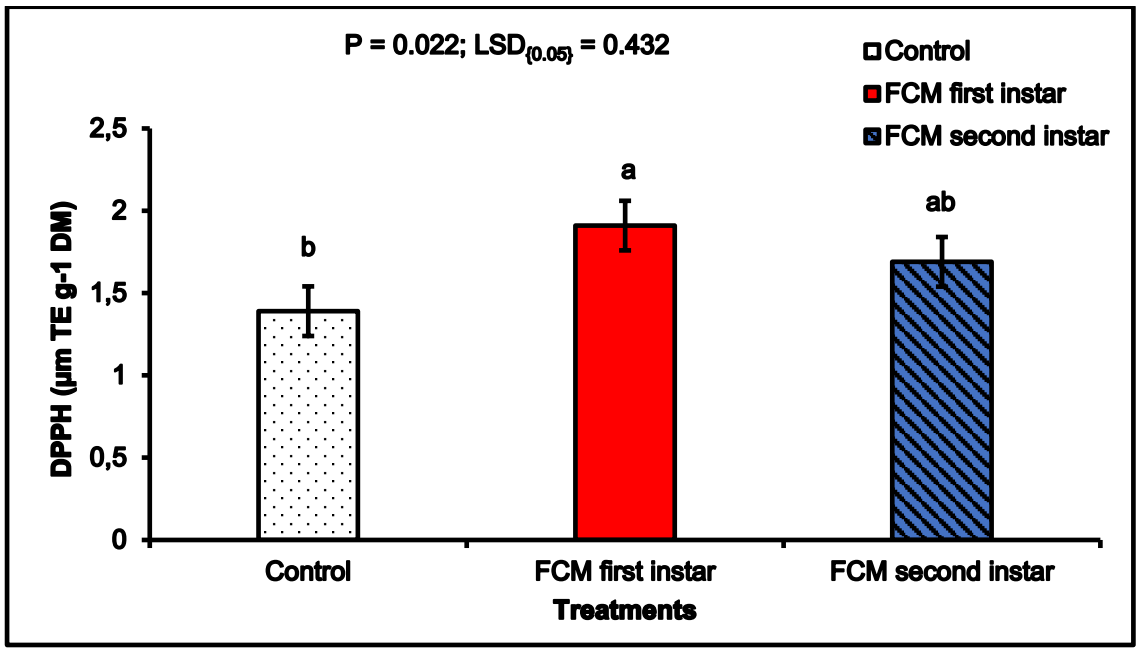


Figure 4. 3. Effects of false codling moth larval growth stages on 'Midknight' Valencia citrus fruit antioxidant activity quantified by DPPH. Vertical bars with different letters and no overlapping error bars represent the significative differences between treatments according to the LSD test at p = 0.05

4.4. CONCLUSION

This study found that the larval growth stages of the false codling moth affected antioxidant activities causing an increase in fruits. The changes affected 'Midknight' valencia citrus fruit quality, leading fruit to be unsuitable for consuming and processing. According to this study false codling moth infestation is a great challenge and there is need for eradication in infested orchards.

CHAPTER 5

SUMMARY, LIMITATIONS, CONCLUSION, RECOMMENDATIONS, AND FUTURE STUDIES

5.1. SUMMARY

This study evaluated the effect of false codling moth larval growth stages on ‘Midknight’ valencia citrus fruit biochemicals, secondary metabolites and antioxidant activity. False codling moth larvae inoculated ‘Midknight’ valencia citrus fruit showed a significant effect on TPC, TFC and antioxidant activity quantified by DPPH. For antioxidant activities quantified by FRAP, FCM only affected fruit infested with the second instar. However, there was no significant effect on TSS, TA, TSS:TA ratio and antioxidant activity assayed by ABTS.

False codling moth inoculation increased biochemicals in ‘Midknight’ valencia citrus fruit when compared to the control. Fruit infested with FCM larvae had an increased in TSS, TA, TPC and TFC while the control was lower. However, there was no significant difference in TSS and TA of ‘Midknight’ valencia citrus fruit exposed to FCM larval growth stages and the control.

‘Midknight’ valencia citrus fruit antioxidant activities were influenced differently by false codling moth larvae. Antioxidant activity of ‘Midknight’ valencia citrus fruit quantified by FRAP and DPPH were significantly affected by FCM larvae. Meanwhile, antioxidant activity quantified by ABTS was not significantly influenced by FCM larvae. However, FCM larvae caused an increase in antioxidant activity of ‘Midknight’ valencia citrus fruit when compared to the control.

5.2. LIMITATIONS

The limitations in this study include lack of information on how false codling moth first and second instar affects ‘Midknight’ valencia citrus fruit biochemicals, secondary metabolites and antioxidant activity. This study is also based on one fruit cultivar thus does not represent behaviour of other cultivars.

5.3. CONCLUSION

False codling moth first and second instar affected ‘Midknight’ valencia citrus fruit. False codling moth highly influenced TPC, TFC and antioxidant activity quantified by FRAP and DPPH meanwhile there was no effect on TSS, TA and antioxidant activity quantified by ABTS.

5.4. RECOMMENDATIONS

Based on the current findings, false codling moth larvae changes the chemical make-up of ‘Midknight’ valencia citrus fruit thus the need to entirely eradicate it in orchards. The eradication of false codling moth will keep the original chemical make-up of fruits and allow them to be marketable and boosting the country’s economy.

5.5. FUTURE STUDIES

False codling moth is a phytosanitary pest that is native to SA and known for feeding on citrus fruits. Furthermore, SA is also known to be one of the biggest citrus fruit exporters. However, the effect of this important phytosanitary pest on citrus fruit biochemical properties has not been determined. The effect of FCM on citrus fruit biochemicals should be made part of the future studies to understand further the influence of this phytosanitary pest in fruits. There is limited information on how this pest affects citrus fruit biochemicals thus this study should be repeated to substantiate the current findings.

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7. APPENDICES

Appendix 3.1 a: Analysis of variance for total soluble solids

Source	DF	SS	MS	F	P
Treatment	2	5,3047	2,65233	1,96	0,1610
Error	27	36,6140	1,35607		
Total	29	41,9187			

Appendix 3.1 b: Analysis of variance tor titratable acidity

Source	DF	SS	MS	F	P
Treatment	2	0,0002255	0,0001127	0,53	0,5931
Error	27	0,005716	0,000117		
Total	29	0,00591			

Appendix 3.1 c: Analysis of variance for TSS: TA

Source	DF	SS	MS	F	P
Treatment	2	2,278	1,13888	0,25	0,7775
Error	27	121,072	4,48414		
Total	29	123,350			

Appendix 3.2: Analysis of variance for total phenolic contents

Source	DF	SS	MS	F	P
Treatment	2	0,16970	0,08485	5,50	0,0099
Error	27	0,41668	0,01543		
Total	29	0,58638			

Appendix 3.3: Analysis of variance for total flavonoids contents

Source	DF	SS	MS	F	P
Treatment	2	0,19932	0,09966	20,30	0,0000
Error	27	0,13257	0,00491		
Total	29	0,33189			

Appendix 4.1: Analysis of variance for antioxidant activities quantified by FRAP

Source	DF	SS	MS	F	P
Treatment	2	0,09622	0,03207	5,91	0,0150
Error	27	0,19553	0,00543		
Total	29	0,29175			

Appendix 4.2: Analysis of variance for antioxidant activities quantified by ABTS

Source	DF	SS	MS	F	P
Treatment	2	0,27741	0,13871	3,28	0,0532
Error	27	1,14260	0,04232		
Total	29	1,42001			

Appendix 4.3: Analysis of variance for antioxidant activities quantified by DPPH

Source	DF	SS	MS	F	P
Treatment	2	0,04315	0,02158	4,92	0,0223
Error	27	0,11835	0,00438		
Total	29	0,16151			