Efficacy of enhanced freshness formulation as a novel postharvest treatment for gold kiwifruit (*Actinidia chinensis*.)

By

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Declaration

I, Sisanda, Sibusiso, Luyanda, Mthembu (student no.: 213558719), declare that this is my original work, except where acknowledged. I further declare that these results have not otherwise been submitted in any form for any degree or diploma to any university. The work does not contain any other people's information or data such as graphs, tables and pictures unless acknowledged to be found or sourced from other researchers.

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Summary

When exporting kiwifruit, the main limiting factors are excessive fruit softening and fungal decay. Furthermore, exposing kiwifruits to low temperatures induces the mechanisms involved in the softening process. In addition, kiwifruit become much more susceptible to fungal decay as they soften, which exacerbates deterioration of kiwifruit during transit. Fruit firmness is an important criterion for the market value of kiwifruit and storage life for the wholesale and retail trade. Therefore, loss in firmness is a serious problem resulting in postharvest and economic losses. Given that cold storage alone is not enough to optimise firmness retention of kiwifruit, the current study aimed to investigate the capacity of enhanced freshness formulation (EFF) to reduce kiwifruit softening. Kiwifruits were subjected to eight weeks of storage at 0 °C and 90 % relative humidity, followed by one week at ambient storage. The efficacy of EFF to regulate the mechanisms behind the softening process such as cell wall degradation, membrane deterioration and fungal infection was evaluated. Furthermore, the efficacy of EFF to preserve bioactive compounds was also evaluated to assess the effect of this treatment on these highly appreciated quality attributes.

The first study evaluated the efficacy of EFF to delay softening of kiwifruit harvested at both the mid and late maturity stage. The influence of EFF on cell wall polysaccharides (CWPs) and cell wall degrading enzymes (CWDEs) was assessed. The findings showed that EFF optimised firmness retention of kiwifruit by preserving CWPs through the suppression of CWDEs which are responsible for initiating fruit softening. Furthermore, EFF maintained fruit quality and marketability, characterised by significantly lower mass loss, total soluble solids and higher titratable acidity.

The second study examined the capacity of EFF to inhibit the excessive production of reactive oxygen species (ROS) which induce membrane damage, resulting in softening and senescence. EFF treatment effectively delayed the progression of kiwifruit senescence by reducing membrane deterioration and the accumulation of ROS. The mechanism by which EFF reduced membrane damage caused by ROS was attributed to its capacity to enhance the activities of antioxidant enzymes involved in neutralising ROS and suppressing Phospholipase D which destroys membrane integrity, resulting in improved storability of treated fruit.

The third study assessed the efficacy of EFF to inhibit the fungal growth of *Botrytis cinerea*, which one of the major postharvest pathogens of kiwifruit that contribute to excessive softening. The findings show that EFF effectively suppressed the fungal growth of *Botrytis*

cinerea in a dose-dependent manner. The results demonstrate that EFF can serve as a potential disease control strategy for kiwifruit at the postharvest stage. The mechanism by which EFF suppressed fungal decay can be attributed to the treatment's capacity to induce disease resistance against *Botrytis cinerea*, by enhancing the synthesis of secondary metabolites and the action of defence-related enzymes, thus conferring greater protection against pathogen attack.

The fourth study investigated the ability of EFF to retain the antioxidant quality of kiwifruit during prolonged storage. The bioactive compounds of EFF-treated kiwifruits were effectively maintained throughout the storage period. The findings of the present study further revealed that the mechanism by which EFF optimises antioxidant retention is due to its ability to regulate enzymes involved in the synthesis and oxidation of bioactive compounds. Despite the positive results, further research on transcriptome analysis is needed to elucidate how EFF regulates softening and antioxidant enzymes.

Thesis format and publications

The chapters of this thesis were written using journal manuscript format because they are intended for publication. One experimental chapter has already been published.

Article published in press:

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Preface

This dissertation is an anthology of various manuscripts presented disjointedly. Each chapter represents an independent article, and reiteration between chapters was unavoidable.

Table of Contents

Chapter One

General introduction

1. Introduction

Gold kiwifruits (*Actinidia chinensis*) have a high concentration of bioactive compounds such as ascorbic acid, phenolic and flavonoid compounds, which make them a good source of antioxidants (Choi et al., 2022). The high antioxidant contents of gold kiwifruits have resulted in an increasing global demand for this fruit (Ma et al., 2017). However, kiwifruits exhibit a climacteric ripening pattern and are extremely perishable (Benítez et al. 2013; Cha et al. 2019). During export, kiwifruit experience postharvest-related challenges such as rapid softening, fungal decay and loss in antioxidants (Cha et al., 2019; Li et al., 2020; Kumarihami et al., 2020). Kiwifruit destined for export must be able to withstand transportation, handling and arrive in satisfactory condition at the place of destination. The most serious defect is excessively soft fruit, based on the observation of kiwifruit arriving at European markets.

In the event of rapid softening during transit, the kiwifruit industry incurs economic losses due to the reluctance of wholesalers and retailers to purchase kiwifruits in non-optimally firm conditions (Asiche et al., 2017). In addition to rapid softening, fungal decay is another factor that results in significant economic losses of kiwifruit during storage and marketing (Fatemi et al. 2013). *Botrytis cinerea* (*B. cinerea*), is the most significant postharvest pathogen of kiwifruit (Williamson et al., 2007). Postharvest losses caused by *B. cinerea* can amount to 20% or greater than 30% in extreme cases (Michailides and Elmer 2000; Li et al., 2020). Kiwifruit become much more susceptible to fungal decay as they soften. Therefore, delaying fruit softening can potentially reduce fungal decay.

Cold storage is the conventional method used to preserve and prolong the storage life of kiwifruit (Xia et al., 2020). Regrettably, cold storage alone is not sufficient enough to delay rapid softening and loss of antioxidants (Mitalo et al., 2019; Choi et al., 2022). Furthermore, there is a high demand of optimally firm and decay-free kiwifruit from the northern hemisphere markets. This makes it important to incorporate postharvest treatments with cold storage to effectively optimise the quality of kiwifruits. The application of fungicides is an effective approach for inhibiting pathogen infections and they serve as the primary strategy for controlling fungal decay in kiwifruits (Thomidis and Prodromou, 2018). The adverse effects of fungicides on the environment and human health have resulted in stricter government

regulatory policies, which prompts producers to develop alternative management strategies (Mari et al. 2015). The application of eco-friendly alternatives such as heat treatments (Mahajan et al., 2014; Fallik and Ilic, 2017), ozone treatment (Horvitz and Cantalejo, 2014; Tabakoglu and Karaca, 2018), edible coatings (Mahajan et al., 2014; Dhaka and Upadhyay, 2018), controlled atmosphere storage (Irshad, 2018) and modified atmosphere packaging (Bueno and Martínez, 2010; Mattos et al., 2012) have been investigated to delay softening and control fungal growth in fruits. However, the limitations of these treatments such as high technology costs, inefficiency to control fungal growth and lack of uniformity of results, have made it difficult for their implementation within the kiwifruit industry (Salzano et al., 2019). These constraints make it necessary to introduce innovative postharvest treatments that are eco-friendly and economically feasible.

Enhanced freshness formulation (EFF) is a hexanal-based formulation that comprises of antioxidants such as geraniol, α-tocopherol and ascorbic acid (Paliyath and Murr, 2007). Hexanal; the active ingredient of EFF, has GRAS (generally recognized as safe) status and has been tested and approved by the United States Food and Drug Administration (Paliyath and Subramanian, 2008). Hexanal possesses antifungal properties against *Alternaria alternate*, *Penicillium expansum* and *Botrytis cinerea* (Hamilton-Kemp et al. 1992; Song et al. 1998). EFF is easily applicable to operations of any size, comparatively inexpensive, effective at improving firmness and antioxidant retention as well as controlling fungal decay in horticultural produce (Paliyath and Subramanian, 2008; Qi et al., 2011; Jincy et al., 2017; Cheema et al., 2014; Cheema et al., 2018). Therefore, the application of this treatment in the kiwifruit industry has the potential to serve as a substitute for the currently used preservation strategies.

In addition, the softening rate and antioxidant contents of kiwifruits is significantly influenced by harvest maturity (Tavarini et al., 2009; Lee et al., 2015; Choi et al., 2019; Tilahun et al., 2020; Nkonyane et al., 2022). These studies demonstrated that the softening rate and loss in antioxidants increases with an advancement in fruit maturity. In our extensive review of the literature, we have not found studies investigating the potential of a postharvest treatment at delaying softening and loss of antioxidants in kiwifruits harvested at different maturity stages. South African grown gold kiwifruits exported to international markets are harvested at both the mid and late maturity stages (Mahlaba et al., 2021). Evaluating the efficacy of a postharvest treatment to delay softening and optimize the antioxidant content of kiwifruits harvested at different maturity stages is warranted.

2. Problem Statement

Fruit firmness is an important criterion for the market value and storage life of kiwifruits. Rapid softening limits the storage potential and marketability of kiwifruits during export. Excessive softening leads to premature deterioration which results in significant postharvest and economic losses (Asiche et al., 2017; Ma et al., 2023). The kiwifruit industry relies on cold storage to optimise the storage life of kiwifruits (Shin et al., 2018; Cha et al., 2019). However, cold storage alone is insufficient at delaying the softening of kiwifruits, resulting in a continuation in the loss of produce and profitability. This is a critical problem that requires the kiwifruit industry to devise a strategy that can alleviate rapid softening to reduce postharvest and economic losses.

Furthermore, pathogen invasion exacerbates the deterioration and postharvest losses of kiwifruit. The use of fungicides to inhibit fungal decay has significant disadvantages such as handling hazards, fungicide residues and threat to human health and the environment. This puts South African kiwifruit growers under pressure to develop eco-friendly sanitisers, as they face many restrictions and strict standards for food safety from maximum residue limit (MRL) policies. If fungicide residues that exceed the importing country MRLs are detected offshore, a product may be recalled and/or destroyed at the expense of the grower, resulting in significant losses. These issues necessitate the need to explore an eco-friendly postharvest treatment that can delay both rapid softening and fungal decay of kiwifruit.

Additionally, harvest maturity is another factor that influence fruit softening with late maturing fruit exhibiting higher softening rates. In our extensive review of the literature, we have not found studies evaluating the efficacy of EFF in delaying fruit softening at various maturity stages. Considering that the export-orientated South African kiwifruit industry harvests at different maturity stages, devising a postharvest strategy that can effectively retard the rapid softening of kiwifruit harvested at different maturity stages is warranted.

3. Significance of the study

During the year 2023, South Africa exported just under 1200 tonne of kiwifruit, with exports expected to increase to 1500 tonne in the year 2024 (McGregor, 2024). In the past five years, 97% of South African kiwifruit were exported to Africa alone. Now more than half of South African kiwifruit are exported to overseas markets, with major destinations being Asia, Middle East and Europe (DALRRD, 2023). The rapid softening and fungal prevalence of kiwifruits can result in significant postharvest and economic losses during export, valued at 10 – 100

million US dollars annually (Brito et al., 2021). Therefore, employing techniques that can effectively preserve kiwifruits for prolonged storage periods is undeniably important. With the use of agrochemicals for fruit preservation becoming more difficult to justify, kiwifruit growers may be at risk of having their produce not being fit for export. It has been reported that stringent MRL policies have reduced the export of fruits by approximately 14% (Hejazi et al., 2022).

Therefore, adopting treatments that leave no residues on fresh produce, ensures the product will be suitable for all export markets. Enhanced freshness formulation has the capacity to serve as an effective tool for optimising storability and marketability of kiwifruits. This research has the potential to reduce the postharvest and economic losses of kiwifruits, attributed to rapid softening and fungal decay. Enhanced freshness formulation is an eco-friendly cost-effective investment that can be incorporated in operations of any size. This project has the potential to benefit kiwifruit producers by facilitating greater market participation, meeting the strict demands of northern hemisphere markets in a cost-effective and environmentally friendly manner. Thus, making the production of kiwifruit a lucrative business for the South African kiwifruit growers.

4. Research aim

To optimise the postharvest performance of gold kiwifruit at the pre-consumer level by optimising firmness and antioxidant retention along with reducing fungal decay using postharvest application of enhanced freshness formulation.

5. Research objectives

1. To evaluate the efficacy of enhanced freshness formulation to optimise firmness retention of kiwifruits by regulating the mechanisms involved in the softening process.
2. To assess the potential of enhanced freshness formulation to preserve cell membrane integrity and reduce deterioration by enhancing the antioxidant scavenging potential of kiwifruits.
3. Investigate the efficacy of enhanced freshness formulation to induce disease resistance against *Botrytis cinerea* in inoculated kiwifruit.
4. Assess the efficacy of enhanced freshness formulation to optimise the antioxidant retention of kiwifruits.

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Chapter Two

Efficacy of eco-friendly treatments in delaying fruit softening: A review

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Abstract

Fruit firmness is of commercial importance as it is a major determinant of shelf life and commercial value. Fresh produce markets incur severe financial losses due to excessive fruit softening. Therefore, developing technologies to control fruit softening by delaying the ripening process is crucial for maintaining fruit quality. Factors such as gaseous exchange, activity of cell wall degrading enzymes and pathogen invasion contribute to fruit softening. This review discusses the efficacy of eco-friendly treatments namely: hexanal, edible coatings, heat treatment, ozone and UV-C to suppress fruit softening. Eco-friendly treatments have been demonstrated to enhance firmness retention by suppressing ethylene biosynthesis, reducing the activity of cell wall degrading enzymes and inactivating fungal pathogens by enhancing the activity of enzymes involved in plant defence mechanisms against fungal infection. This review gives an insight on the mechanism of eco-friendly treatments in relation to delaying textural softening.

Keywords: Ethylene, enzymes, pathogens, hexanal, ozone, edible coatings

2.1. Introduction

Fruits play an essential role in providing human nutrition, by supplying nutrients, vital for the maintenance of normal health. Nutritionally, fruits are known for their high energy, roughage value, minerals, vitamins (B-complex, C and K in some instances), β-carotene (pro-vitamin A), and phenolics (antioxidants). However, fruits are perishable, and excessive ripening during storage affects their shelf life and consumer purchase (Pankaj et al., 2018).

The ripening of fruit is a process involving the changes in physiological, biochemical and organoleptic properties, resulting in the development of aroma, colour, flavour, nutritional quality and texture (Paliyath and Padmanabhan, 2018). During ripening, the membrane structure is altered due to an increase in sterol levels and catabolism of phospholipids and membrane proteins. This leads to a loss of cellular compartmentalization followed by tissue structure (Paliyath and Droillard, 1992). Textural changes are the major event in fruit softening and is an integral part of ripening.

The primary cell wall and middle lamella consist of pectins which contribute to the texture of fruit. Softening of fruit tissue is attributed to enzymatic degradation and solubilization of the protopectin into soluble pectins, which are found loosely bound to the cell walls (Sakai et al., 1993). Pectins are fundamental constituents involved in the mechanical strength of the primary cell wall (Sirisomboon et al., 2000) and pectin degradation during ripening is responsible for tissue softening of fruit (Prasanna et al., 2007). Fruit softening reduces cell wall adhesion by decreasing the hardness of the cell wall, which facilitates microbial invasion in the cell, resulting in decay and spoilage (Jeddi et al., 2014; Toivonen and Brummell 2008). Losses of fruit due to softening can cause severe economic damage as excessive softening leads to postharvest decay and consumer rejection (Saladié et al., 2007; Figueroa et al., 2010; Chea et al., 2019; Singh et al. 2019; Weber et al. 2020). Therefore, in order to reduce postharvest losses, technologies developed for controlling fruit softening by delaying the ripening process are

crucial for maintaining the quality and safety of fruits to avoid mechanical damage during transportation.

Factors such as postharvest pathogens, increased respiration, transpiration, ethylene production and loss of membrane integrity, contribute to accelerated ripening and senescence of fruit. Therefore, technologies developed to suppress premature softening of fruit are essential for maintaining quality and improving storage of fruits. Such technologies include the application of eco-friendly treatments as postharvest treatments to optimise prolonged storage of fruit. Proposed eco-friendly treatments that have been investigated by researchers include heat treatments (Zhang et al., 2019), ozone technology (Liu et al., 2021), ultraviolet C (UV-C) light radiation (Kan et al., 2021), edible coatings (Hira et al., 2022) and hexanal formulations such as enhanced freshness formulation (EFF) (Cheema et al., 2018). Studies conducted on the listed treatments have demonstrated their capacity to optimise shelf life and improve fruit quality by suppressing enzymes involved in the degradation of the cell membrane.

Generally recognised as safe (GRAS) is a United States Food and Drug Administration (FDA) designation, that determines if a substance added to food is considered safe by experts under the conditions of its intended use. Edible coatings, hexanal formulations, ozone and UV-C have been given GRAS approval by the USDA and the FDA. These eco-friendly treatments can serve as an alternative treatment, for growers that use agrochemicals to control postharvest pathogens which contribute to fruit softening. As updated regulations from countries restricting the use of agrochemicals puts them under pressure to explore alternative techniques to preserve quality of fresh produce. Previous studies which are further discussed in this review, have illustrated the potential of these eco-friendly treatments to serve as an alternative to agrochemicals.

At this present stage, there is no review that provides insight of fruit softening in relation to eco-friendly treatment application. Therefore, the current review summarises recent advances in the applications of eco-friendly treatments to suppress fruit softening. It further provides a summary of the biochemistry of fruit softening and the factors affecting fruit softening during postharvest storage. The application of eco-friendly treatments to suppress fruit softening by reducing enzymatic activity, inhibiting fungal pathogens and lowering gaseous exchange are discussed. Furthermore, the feasibility of commercialising these treatments is also discussed. The aim of this review is to gain an understanding of the mode of action of eco-friendly treatments on optimising firmness retention and encourage the adoption of these treatments in managing fruit softening and promote wide utilization in the food industry.

2.2. Factors contributing to fruit softening

2.2.1. Ethylene

Ethylene is a naturally produced, two carbon gaseous plant growth regulator that has numerous effects on the growth, development and storage life of horticultural produce (Paul et al., 2012). The main changes associated with ripening include colour development, tissue softening, taste and flavour. Apart from its beneficial effect on promoting fruit ripening, ethylene production can be detrimental by causing excessive softening of fruit. In climacteric fruit, ripening related processes such as softening, are regulated by ethylene (Yang and Hoffman, 1984).

Ethylene is synthesized from the amino acid methionine which gets converted to S-adenosyl methionine (SAM) via the enzyme methionine S-adenosyl transferase (Arc et al., (2013). SAM is converted to the four-carbon compound 1-aminocyclopropane-1-carboxylic acid (ACC) by the enzyme ACC synthase (ACS). However, ACC can undergo either two fates, which is being converted to malonyl ACC (an in-active end product) (Kader, 1992) or being converted to ethylene by the enzyme ACC oxidase (ACO), an ethylene forming enzyme (Adams and Yang, 1979). Suppressing ethylene biosynthesis and perception of ethylene by the receptor at suitable

ripeness, can reduce ethylene production and optimise fruit firmness. As the rate-limiting step in ethylene production is the formation of ACC which is catalysed by ACS (Fluhr and Mattoo, 1996).

Previous studies have illustrated that accelerated fruit softening is closely associated with ethylene production (Wang et al., 2009; Bapat et al., 2010). Cell wall degrading enzymes and proteins involved in the softening of fruit tissues such as polygalacturonase (PG; EC3.2.1.15), pectin methylesterase (PME; EC 3.1.1.11), cellulase (EC3.2.1.4) and expansin, are regulated by ethylene (Bennett and Labavitch, 2008; Brummell and Harpster, 2001; Lashbrook et al., 1994; Rose et al., 1997). Therefore, inhibition of ethylene production can result in a delay in fruit softening. As suppression of ethylene production inhibits the activity of enzymes that participate in cell wall degradation, which leads to fruit softening.

2.2.2. Respiration

The respiration rate (CO_2 production) is a major metabolic process taking place in fresh produce. Respiration is the process whereby the energy derived from oxidative catabolism of stored organic material such as carbohydrates, fats and proteins are used to keep the fruit alive and support developmental changes (Wilson et al., 1999). Respiration may lead to the loss of moisture of fruit through the transpiration process, resulting in a loss of fruit firmness (Ghebreslassie, 2003; Romero and Rose, 2019). The deterioration of fruit texture as a result of high respiration, is due to increased metabolic activities and respiration heat (Sharma et al., 2011; Bhatia and Asrey, 2019).

2.2.3. Pathogens

Ripening is related to the disassembly of the fruit cell walls, resulting in tissue softening. Softening of fruit tissue provides a favourable environment for fungal invasion (Cristescu et al., 2002). The degradation of the cell wall facilitates fungal invasion, as it reduces mechanical

barriers to pathogen attack and spread. Thus, increasing the likelihood of bruising which leads to further wounds for pathogen entry (Blanco-Ulate et al., 2016). Necrotrophic pathogens are the most devastating postharvest pathogens, as they kill host tissue, resulting in rotting. Examples of necrotrophic fungi are *Botrytis cinerea, Sclerotinia sclerotiorum*, *Monilinia* spp., *Alternaria* spp., *Rhizopus* spp., *Penicillium* spp., and *Fusarium* spp. (Nunes, 2012; Bautista-Baños, 2014; van Kan et al., 2014; Liang and Rollins, 2018; Petrasch et al., 2019a).

Botrytis cinerea is a common fungal pathogen with no evident host specificity and can infect more than 1000 plant species (Elad et al., 2016). Similar to other fungal pathogens, *B. cinerea* secretes extracellular enzymes such as polygalacturonase, pectin methylesterase, proteases and laccases to stimulate cell wall degradation and attack the host organs effortlessly as mechanical barriers reduce (Forlani et al., 2019; Petrasch et al. 2019b). In addition, a previous study has demonstrated the ability of *B. cinerea* to accelerate fruit softening by triggering ethylene, salicylic acid and abscisic acid (ABA) pathways (Forlani et al., 2019).

In the early stages, *B. cinerea* deploys sRNAs and effector proteins to suppress premature host cell death and immune responses. This enables the fungus to establish inside the host and accumulate biomass prior to the necrotrophic phase (Veloso and Van Kan, 2018). *B. cinerea* has the ability to secrete oxalic acid that lowers the pH of the host tissues and promote the activity of fungal enzymes such as laccases, pectinases and proteases (Manteau et al., 2003; Sharon et al., 2007; Prusky and Lichter, 2007; Fernández-Acero et al., 2010). Additionally, the accumulation of oxalic acid leads to Ca^{2+} chelation, which weakens pectin structures of the cell walls and inhibiting deposition of callose (Chakraborty et al., 2013).

In addition to *Botrytis cinerea*, *Colletotrichum* species are key fungal pathogens that infect and result in excessive softening of fruit. Amongst the species, *Colletotrichum acutatum* and *Colletotrichum gloeosporiodies* complexes are the main agents. Previous studies have

illustrated that *Colletotrichum gloeosporiodies* causes bitter rotting on apple (Grammen et al., 2019) and anthracnose in mangoes (Shao et al., 2019). Furthermore, pathogens like the genus *Penicillium* can cause blue rot of apples, pears and green rot of citrus. *Mucor piriformis* and *Rhizopus stolonifer* may result in decay of apples, berries, pears and tomatoes (Moss 2008). *Lasiodiplodia theobromae* causes pericarp browning and disease in longan fruit (Sun et al. 2018).

2.2.4. Enzymes

Textural changes resulting in fruit softening are attributable to enzyme-mediated alterations in the structure and composition of cell wall, partial or complete solubilization of cell wall polysaccharides such as pectins and cellulose (Tucker and Grierson, 1987). The primary cell wall contains 35 % pectin, 25 % cellulose, 20 % hemicellulose and 10 % structural, hydroxyproline-rich protein (Brownleader et al., 1999). Pectins are important constituents involved in the mechanical strength of the primary cell wall and middle lamella contributing to the fruit texture (Sirisomboon et al., 2000). Tissue softening is ascribed to enzymatic degradation and solubilization of the protopectin (Sakai et al., 1993). Protopectin is converted into soluble polyuronides, which results in fruit softening (John and Dey, 1986).

This tightly bound protopectin is degraded into soluble pectins, which are found loosely bound to the cell walls. Previous studies have illustrated that pectin degradation during ripening may be responsible for tissue softening (De Vries et al., 1984; Hobson et al., 1987; Redgwell et al., 1992). Pectin-degrading enzymes are classified, based on their mode of action on pectin and pectic substances into polygalacturonase (PG), pectin methylesterase (PME), pectate lyase, and pectin lyase (Wong, 1995; Sakai et al., 1993; Chauhan et al., 2001).

PG is a hydrolytic enzyme that acts on polygalacturonic acid, to hydrolyse the α-1,4-glycosidic bonds between the galacturonic acid residues in galacturonans. It is commonly accepted that

PG is predominantly responsible for dissolution of the middle lamella during fruit ripening (Jackman and Stanley, 1995; Voragen et al., 1995). Previous research has demonstrated that a decrease in firmness is associated with an increased activity of pectic enzymes, particularly PG (Crookes and Grierson, 1983; Watkins et al., 1988).

Pectin methylesterase (Pectin pectylhydrolase, EC 3.1.1.11) catalyses the hydrolysis of pectin methyl ester groups, resulting in de-esterification. PME is specific for galacturonide esters and its action is to remove methoxyl groups from methylated pectin by nucleophilic attack. This leads to the formation of an acyl enzyme intermediate with the release of methanol, followed by diacylation (hydrolysis) to generate the enzyme and a carboxylic acid. PME preferentially attacks methyl ester bonds of a galacturonate unit next to non-esterified galacturonate unit (Pilnik and Voragen, 1970). Thus, they de-esterify the esterified pectic substances, making them vulnerable for PG action. Its action may be a prerequisite for the action of PG during ripening.

The lyases or trans eliminases cleave the glycosidic bond by trans β-elimination mechanism, for example, elimination of hydrogen from the C-4 and C-5 position of the aglycone portion of the substrate (Whitaker, 1984). Pectate lyases (PL) catalyses the cleavage of de-esterified or esterified galacturonate units by a trans β-elimination of hydrogen from the C-4 and C-5 positions of galacturonic acid. Pectin lyase (PNL) (EC 4.2.2.10) catalyses the cleavage of esterified galacturonate units by trans β-elimination (Wong, 1995). β−Galactosidase (β-Gal) (EC 3.2.1.23), is a glycosidase that acts on short chain oligomers of galactose units present either as glycoprotein, glycolipid, or hetero/homopolysaccharides. This enzyme partially degrades the pectic and hemicellulosic components of the cell wall and is possibly related to the breakdown of polysaccharides during over-ripening.

In addition, the activities of β-Gal and b-xylosidase (bXyl) are linked with the removal of neutral sugar and hemicellulose degradation, respectively, which results in a decline of fruit firmness (Figueroa et al. 2010). The amount of hemicellulose declined progressively during ripening of mango fruit (Mitcham and McDonald, 1992). A loss in considerable levels of characteristic monomers of hemicelluloses viz. glucose, xylose, and mannose occur during ripening of fruit. Furthermore, a study conducted by Chin et al. (1999), illustrated that a decline in extractable hemicellulose was associated with a decrease in tissue firmness of carambola fruit as it ripened. Therefore, enzymes play an important role in cell wall depolymerization or solubilization.

A loss in fruit quality occurs during ripening, due to a loss in membrane integrity and senescence. Phospholipase-D (PLD) is a membrane lipid degrading enzyme (Paliyath and Subramanian, 2008), involved in the membrane deterioration pathway (Paliyath and Droillard, 1992). PLD initiates the phospholipid breakdown by removing the head group of phospholipids, and other enzymes that act downstream such as phosphatidate phosphatise, lipolytic acyl hydrolase and lipoxygenase (LOX), that act in tandem with PLD (Paliyath and Droillard, 1992). The downstream enzymes cannot directly cause the catabolism of phospholipids. Therefore, the entire catabolic pathway can be slowed down by inhibiting PLD activity. Hexanal inhibits PLD by blocking the hydrolysis at the active site of PLD, thus reducing membrane deterioration and delaying senescence (Tiwari and Paliyath, 2011). Lipoxygenases are a family of non-heme iron-containing enzymes which catalyse the deoxygenation of polyunsaturated fatty acids in lipids. LOX activity is involved in membrane deterioration, ripening and senescence. Suppressing the expression of LOX activity results in improved maintenance of membrane integrity and delays the fruit ripening process (Zheng et al., 2007).

2.3. Effect of eco-friendly treatments on factors contributing to fruit softening

As discussed previously, the softening of fruits is affected by a number of factors. Therefore, the suppression of fruit softening by eco-friendly treatments can be achieved by a number of mechanisms.

2.3.1. Hexanal formulations

2.3.1.1. Effect on ethylene production

Ethylene is synthesized from the amino acid methionine SAM via the enzyme methionine S-adenosyl transferase (Arc et al., (2013). SAM is converted to ACC by the enzyme ACS, which is converted to ACC oxidase, an ethylene forming enzyme (Adams and Yang, 1979). Suppressing ethylene biosynthesis and perception of ethylene by the receptor at apposite ripeness, can minimise ethylene action and optimise fruit firmness. The rate-limiting step in ethylene production is the formation of ACC which is catalysed by ACS (Fluhr and Mattoo, 1996). Hexanal has the ability to moderately reduce ethylene production by downregulating a single ortholog of ACS (Tiwari and Paliyath, 2011).

Based on past research (Table 2.1), hexanal and EFF has been proven to be effective at reducing ethylene production as well as delaying the ethylene peak of fruit. EFF applied on mango (Anusuya et al., 2016) and banana (Yumbya et al., 2018), exhibited a significant reduction in ethylene production in comparison to untreated fruit. Hexanal applied on oriental sweet melons (Qi et al., 2011), mango (Jincy et al., 2017) and banana (Yumbya et al., 2018) exhibited lower ethylene production in comparison to their untreated counterparts. Furthermore, Qi et al. (2011) and Yumbya et al. (2018) illustrated that EFF reduced ethylene production and delayed the ethylene peak. These studies demonstrate the efficacy of hexanal treatments to reduce ethylene production.

2.3.1.2. Effect on respiration rates

The efficacy of hexanal treatment to reduce the rate of respiration has been demonstrated by studies conducted on banana (Yumbya et al., 2018), mango (Anusuya et al., 2016) and strawberry fruit (Yuan et al., 2008). EFF increased the respiration rate of tomato (Tiwari and Paliyath, 2011; Pak Dek et al., 2018), however treated fruit remained firmer than the untreated fruit throughout the storage period. The study conducted by Cheema et al. (2018) yielded similar findings to studies mentioned above, where EFF was applied on sweet bell pepper. The firmness of EFF treated bell peppers was significantly higher than untreated peppers, even though the treated peppers exhibited a greater CO_2 production.

Suppression of phospholipase-D activity (a phospholipid degrading enzyme) by hexanal treatment has the potential to reduce the catabolism of free fatty acids liberated during the membrane phospholipid catabolism. This may be compensated by channelling more carbon intermediates into the respiratory cycle, which leads to an increase CO_2 production due to the lowered demand for substrates to replace membrane phospholipids that are broken down by PLD action. This may explain the reason why fruit treated with hexanal experienced an increase in respiration rate.

2.3.1.3. Effect on fruit softening enzymes

A loss in fruit quality occurs during ripening, due to a loss in membrane integrity and senescence. Phospholipase D (PLD) is a membrane lipid degrading enzyme (Paliyath and Subramanian, 2008), involved in the membrane deterioration pathway (Paliyath and Droillard, 1992). PLD initiates the phospholipid breakdown by removing the head group of phospholipids, and other enzymes that act downstream such as phosphatidate phosphatise, lipolytic acyl hydrolase and lipoxygenase (LOX), that act in tandem with PLD (Paliyath and Droillard, 1992).

The downstream enzymes cannot directly cause the catabolism of phospholipids. Therefore, the entire catabolic pathway can be slowed down by inhibiting PLD activity. Hexanal inhibits PLD by blocking the hydrolysis at the active site of PLD, thus reducing membrane deterioration and delaying senescence (Tiwari and Paliyath, 2011). Lipoxygenases are a family of non-heme iron-containing enzymes which catalyse the deoxygenation of polyunsaturated fatty acids in lipids. LOX activity is involved in membrane deterioration, ripening and senescence. Suppressing the expression of LOX activity results in improved maintenance of membrane integrity and delays the fruit ripening process (Zheng et al., 2007).

PME is an enzyme that is involved in the hydrolysis of methyl ester groups in pectin chains, leading to the formation of carboxylate groups (Jayani et al., 2005). PME removes the methyl group of galacturonic acid polymers of pectin. PME activity causes the de-esterification of the pectin chain, this increases the susceptibility of the pectin chain to polygalacturonase mediated degradation (Carpita and Gibeaut, 1993). This results in a rapid loss of cell wall structure. The efficacy of hexanal treatments to suppress the activity of PLD by blocking the hydrolysis at the active site of PLD, reducing the LOX activity by preserving the membrane cells, decreasing PME and polygalacturonase (PG) transcripts results in improved quality and firmness of fruit.

The inhibitory effect of hexanal on PLD activity has been demonstrated by studies conducted on tomato (Tiwari and Paliyath, 2011) and mango (Jincy et al., 2017), whereby treated fruit exhibited significantly lower PLD enzyme activity relative to the untreated fruit. Hexanal treatment's ability to inhibit PLD activity of tomato fruit, suggests that the ripening of tomato was delayed as a result of less generation of phosphatidic acid in hexanal treated fruit. Yuan et al. (2008) reported contradicting results, whereby hexanal treated fruit exhibited a higher activity of PLD. Regardless of the contradictory results, hexanal treatment was effective at reducing the LOX activity of the strawberry fruit. Tiwari and Paliyath, (2011) reported that EFF downregulated the transcript levels of PME in tomato fruit. PME activity of guava fruit

was significantly reduced by EFF treatment (Gill et al., 2015) and Anusuya et al. (2016), demonstrated that EFF applied on mango reduced PME and PG enzyme activity. The findings from these studies are indicative of less oxidative damage in hexanal treated mango fruit.

2.3.1.4. Effect on pathogens

The ability of hexanal to inhibit the growth of fungal pathogens has been demonstrated by studies listed in table 2.6. During hexanal treatment, fungal hyphae undergo dehydration and distortion and breakages in their mycelial strands (Fallik et al., 1998). This is characterised by swelling of the cell walls, greater vacuolization, severe twisting of mycelial strands and malformation of spores (Anusha et al., 2016). Furthermore, Knobloch et al. (1998) suggested that the fungicidal and fungistatic properties of hexanal may be attributed to its ability to act on the plasma membrane and cause hyphal deterioration of pathogens.

Hexanal significantly reduced mycelial growth and spore germination of *Colletotrichum gloeosporioides* and *Lasiodiplodia theobromae* of mango fruit (Anusha et al., 2016). Inhibitory effects were more pronounced at higher concentrations of hexanal with complete inhibition at 0.06 %. Though spore germination of both pathogens was not completely inhibited, it was apparent that hexanal effectively reduced the germination percentage relative to the untreated spores. A study conducted by Song et al. (2007), demonstrated that hexanal reduced spore germination of *Botrytis cinerea* and *M. fructicola* by 30 % and 29.8 %, respectively. Hexanal vapor on raspberry and peach fruit reduced mycelial growth of *Sclerotinia sclerotiorum*, *Alternaria alternata*, and *Colletotrichum gloeosporioides* (Song et al., 2007). Hexanal effects on mycelial growth and spore germination were more pronounced at greater concentrations.

All hexanal treatments reduced the mycelial growth and inhibited spore production of *Monilinia fructicola* and *Monilinia laxa* of peach fruit (Baggio et al., 2013). Hexanal did not completely suppress the development of peach brown rot, but it had an antifungal activity

against *Monilinia* spp. by reducing the increase of lesion size and completely inhibiting sporulation. The continuous application of hexanal vapour was demonstrated to be an effective antifungal treatment on growth of *Botrytis cinerea* on wound-inoculated tomatoes (Utto et al., 2008). Continuous application of hexanal in the above-mentioned study was far more effective than a single dose application of hexanal vapour. Continuous exposure provided effective inhibition whereas single dose treatment of tomato fruit exhibited partial antifungal activity. The growth of fungi on inoculated apple slices of 'Golden Delicious' and 'Jonagold' were completely inhibited by hexanal vapor (Song et al., 1996).

Hexanal vapour inhibited spore germination and mycelial growth of *Botrytis cinerea* on strawberry fruit under in vitro bioassay conditions (Fallik et al., 1998). The infection of *Botrytis cinerea* inoculated strawberry fruit, was greater for hexanal treated fruit compared to untreated fruit during storage during. At the end of storage untreated strawberries had higher infection than the treated fruit, however, both the untreated and treated fruit exhibited over 90 % infection after 11 days of storage. Regarding the quality of the fruit in question, hexanal treated fruit exhibited greater loss in firmness compared to the untreated fruit. These detrimental effects may have been caused by exposure to a high concentration of hexanal vapour.

Previous studies have made it evident that hexanal has fungicidal properties. However, the efficacy of hexanal to inactivate pathogens is dependent on concentration and exposure time. Studies discussed above make it evident that high concentrations of hexanal are required to provide complete inhibition of mycelial growth and spore germination. However, these high concentrations pose a threat to the quality of fruit. The capacity of hexanal to effectively reduce spore germination of various pathogens, illustrates its potential to be integrated in the management of pathogens of postharvest importance.

Table 2.1: Effect of hexanal treatments on factors affecting fruit softening and firmness retention

Factor	Fruit	Cultivar	Treatment	Storage Condition	Key findings	Author
Ethylene	Mango	Alphonso and Banganapalli	EFF	14 ± 1°C, 85 ± 5% RH and 28 ± 2°C	Reduced C_2H_4 production	Anusuya et al. (2016)
	Banana	Grand Nain	EFF	25 ± 1ºC, 60 ± 5% RH	Reduced C_2H_4 production	Yumbya et al. (2018)
	Mango	Neelum	Hexanal	25 ± 0.8°C, 60 ± 10% RH	Reduced C_2H_4 production	Jincy et al. (2017)
	Oriental sweet melons	Jinheng No. 2	EFF	10°C, 85% RH	Reduced C_2H_4 production	Qi et al. (2011)
Respiration	Mango	Alphonso and Banganapalli	EFF	14°C, 85% RH and 28°C	Reduced CO_2 production	Anusuya et al. (2016)
	Banana	Grand Nain	EFF	25 ± 1ºC, 60 ± 5% RH	Reduced CO_2 production	Yumbya et al. (2018)
	Strawberry	Darselect	Hexanal	4°C	Reduced CO_2 production	Yuan et al. (2009)
	Tomato	Rapsodie	EFF	15°C and RT	Increased CO_2 production	Tiwari and Paliyath (2011)
	Tomato	De Ruiter	Hexanal	RT	Increased CO_2 production	Pak Dek et al. (2018)
	Sweet bell pepper	Felicitas	EFF	12°C, 95% RH	Increased CO_2 production	Cheema et al. (2018)
Enzymes	Strawberry	Darselect	Hexanal	4°C	Reduced LOX activity	Yuan et al. (2009)
	Tomato	Rapsodie	EFF	15°C and RT	Inhibited PLD activity	Tiwari and Paliyath (2011)
	Guava	Allahabad Safeda	EFF	6 – 8°C, 95% RH	Reduced PME activity	Gill et al. (2015)
	Mango	Alphonso and Banganapalli	EFF	14°C, 85 RH and 28°C	Reduced PME and PG activity	Anusuya et al. (2016)
	Mango	Neelum	Hexanal	25°C, 60% RH	Reduced PLD activity	Jincy et al. (2017)
Firmness	Guava	Allahabad Safeda	EFF	6 – 8°C, 95% RH	Retained firmness	Gill et al. (2015)
	Strawberry	Jewel and Wendy	EFF	4°C	Retained firmness	Kayal et al. (2017)
	Sweet cherry	Bing	Hexanal	4°C, 95% RH	Retained firmness	Sharma et al. (2010)
	Oriental sweet melons	Jinheng No. 2	EFF	10°C, 85% RH	Retained firmness	Qi et al. (2011)
	Sweet bell pepper	Felicitas	EFF	12°C, 95% RH	Retained firmness	Cheema et al. (2018)

Relative humidity (RH), room temperature (RT), enhanced freshness formulation (EFF)

2.3.2. Edible coatings

2.3.2.1. Effect on ethylene production

The ability of edible coatings to reduce ethylene production of fruit has been demonstrated by studies listed in table 2.2. A study conducted by Guillén et al. (2013), showed that *Aloe vera* and *Aloe arborescens* gels significantly reduced ethylene production of peach and plum fruit. In this study, fruit were stored at 20℃ for six days and both coatings significantly reduced the ethylene production throughout the storage period. These findings are in agreement with those obtained by (Thakur et al., 2018), where plum fruit were coated with rice starch-ι-carrageenan coating under similar storage conditions but for a longer period of 21 days. Further studies illustrated that lemon coated with a combination of beeswax and coconut oil (Nasrin et al., 2020) and pear fruit coated with a combination of chitosan and alignate (Hira et al., 2022) significantly reduced ethylene production. Similar findings were reported by (Guerreiro et al., 2017) for fresh-cut apple coated with alginate and eugenol.

These findings illustrate that edible coatings can facilitate fruit in maintaining low levels of ethylene production at low (~4°C) and higher (~20°C) storage temperatures. However, the monolayer application of an individual coating is constrained by factors such as poor adhesion ability, uneven distribution on fruit surfaces and low water vapor permeability which reduce its performance (Poverenov et al., 2014). Improved performance of edible coatings has been achieved by combining more than one coating using a layer-by-layer approach (Nair et al., 2020). As depicted by studies conducted by Nasrin et al. (2020) and Hira et al. (2022), where the application of combined edible coatings performed better than the monolayer application of an individual coating.

Biosynthesis of ethylene is regulated by the enzyme ACC synthase and oxidase. ACC synthase converts the ACC into SAM to ACC, which is subsequently converted to ethylene via the action of a second enzyme ACC oxidase (Wills and Golding, 2016). Edible coatings repress

ethylene evolution by providing a gas barrier between the fruit and the surrounding atmosphere, which creates semi anaerobic conditions (increase in CO_2 and decrease in O_2) within the fruit. The semi anaerobic conditions formed inside the fruit decrease the catalytic activity of ACC oxidase, thus lowering ethylene production during storage (Valero and Serrano, 2010; Both et al., 2016; Deng et al., 2017).

2.3.2.2. Effect on respiration rates

The efficacy of edible coatings to suppress the respiration rate of fruit has been reported (table 2.2). *Aloe vera* and *Aloe arborescens* significantly reduced the respiration rates of peach and plum fruit stored for six days (Guillén et al., 2013). Sodium alignate lowered respiration and delayed the respiratory peak of coated peaches stored for eight days (Li et al., 2019). The respiration rate was reduced substantially in plum fruit coated with rice starch-ι-carrageenan over 21 days of storage (Thakur et al., 2018). Similar findings were reported by Nasrin et al. (2020) for coated lemon fruit stored for 18 days and pear fruit stored for 21 days (Hira et al., 2022).

Similar to findings reported above for ethylene production, the application of combination of coatings improved the efficacy of edible coatings to reduce respiration rates (Nasrin et al., (2020; Hira et al., 2022). In addition, these studies illustrated that edible coatings can reduce CO_2 production for both non-climacteric fruit (lemon) and climacteric fruit (peach). As well as maintain lower respiration rates short ($\leq$ 8 days) and longer ($\geq$ 18 days) storage periods. Edible coatings reduce CO_2 production of fruit by delaying the respiratory peak and facilitating the control of gaseous exchange (increase in CO_2 and decrease in O_2) between the fruit and the surrounding atmospheric environment (Mahajan et al., 2014).

The tricarboxylic acid (TCA) cycle in plants is the second stage of cellular respiration, whereby living cells are involved in the catabolism of organic fuel molecules in the presence of oxygen,

leading to CO_2 production (Liu et al., 2019). Genes involved in the TCA cycle such as 2-oxogluratalate dehydrogenase, malate dehydrogenase and pyruvate dehydrogenase, are associated with fruit ripening (Centeno et al., 2011; Araujo et al., 2012). Transcriptome analysis demonstrated that the application of edible coatings substantially downregulated the expression levels of genes involved with the TCA pathway, namely, citrate synthase (ACLA-3, PCP002422), isocitrate dehydrogenase (CICDH, PCP012811), succinate dehydrogenase 6 (SDH6, PCP002115), malate dehydrogenase (MDH, PCP005199), 2-oxogluratalate dehydrogenase (OGDH, PCP027756), and pyruvate dehydrogenase E1 (PDH-E1α, PCP021728) (Hira et al., 2022). This resulted in significantly lower respiration rates and significantly higher firmness levels of treated pear fruit.

2.3.2.3. Effect on fruit softening enzymes

As mentioned in section 2.4 of this review, pectin-degrading enzymes are associated with changes in pectins, which play a significant role in the softening of fruit tissues. Suppressing the activity of these enzymes has the potential to enhance firmness retention. Findings reported by Zhou et al. (2011) illustrated that the enzyme activity of PG, Pectinesterase (PE) and cellulase (CEL) in pears was significantly suppressed by edible coatings during 60 days of storage. In this study, prolonged storage (> 45 days) had an effect on the performance of the edible coatings. This is depicted by no significant differences observed between coated and uncoated fruit at the last day of storage for CEL and PE enzyme activities.

Chitosan effectively reduced the PG and PME activity in plums stored at 5°C for 20 days (Liu et al., 2014). A study conducted by Panahirad et al. (2020) demonstrated that the efficacy of carboxymethylcellulose to significantly suppress PG enzyme activity of plum fruit stored at 4°C. Similar findings were reported by Lo'ay and Taher, (2018), where chitosan/poly-vinyl-pyrrolidine coating reduced enzyme activities of CEL, LOX and pectinase (PT) in guava fruit stored at 27°C. Saleem et al. (2020) reported that gum arabic coating inhibited the CEL, PG

and PME enzyme activity in persimmon fruits. In addition, it is noteworthy to highlight that these findings demonstrate that edible coatings were able to suppress enzyme activities at low (≤ 5℃) and high (≤ 27℃) storage temperatures.

The activity of hydrolysing enzymes is dependent on the production of carbon dioxide and ethylene. An increase in carbon dioxide and ethylene production enhances the activity of cell wall degrading enzymes (Valero et al., 2013). The ability of edible coatings to control O_2 availability by modifying internal gas composition, lowers fruit respiration and ethylene production. This leads to a decrease in enzymatic activity, which delays the softening process of fruit (Chiabrando et al., 2015; Kowalczyk et al., 2017).

2.3.2.4. Effect on pathogens

Hydroxypropyl methylcellulose, beeswax (HPMC- BW) edible coatings significantly reduced incidence and severity of *Botrytis cinerea* on cherry tomatoes during cold storage at 5°C (Fagundes et al., 2014). However, the coatings did not prevent fungal decay and disease incidence, which reached 100% for all treatments when transferred to 20°C to simulate shelf-life conditions. The prolonged period of shelf-life simulation (7 days) may have had influence on lowering the performance of the coatings.

Candelilla wax effectively reduced the decay caused by *Rhizopus stolonifer* on strawberry fruit by more than 40 % (Oregel-Zamudio et al., 2017). Subsequently, disease severity was also significantly reduced during the storage period of six days at 25℃. The application of sodium alginate reduced the decay caused by *Penicillium expansum* in peach fruit stored for seven days at 28 ± 1°C (Li et al., 2019). Moulds are strictly aerobic and sodium alginate-based coatings have an effective barrier against gas exchange. This gives the coating the ability to slow down the growth of *Penicillium expansum* by creating an anaerobic environment (Li et al., 2019).

Chitosan coating was demonstrated to significantly reduce infection caused by *Botrytis cinerea*, *Rhizopus stolonifer* and *Aspergillus niger* on strawberry fruit (Melo et al., 2020). This study revealed that storage temperature had a profound effect on the performance of chitosan in inhibiting fungal growth. Strawberry fruit stored at 24°C exhibited a 90 % and 75.55 ± 13.46 % infection for *Botrytis cinerea* and *Rhizopus stolonifer*, respectively. Fruit stored at 12°C exhibited a 70 ± 17.32 % and 75 % infection for *Botrytis cinerea* and *Rhizopus stolonifer*, respectively. At low temperatures, the pathogenicity of the fungi is weakened due to a decrease in the physiological processes of the fruit (de Oliveira et al., 2014). As a result of this weakening, strawberries stored at cold temperature presented a lower percentage of *B. cinerea* infection.

In the case of *Rhizopus stolonifer*, the cold storage temperature contributed to a low dissemination capacity of this fungus. This is depicted by the lower percentage of infection for fruit stored at 12°C in comparison to fruit stored at 24°C. Contrarily, strawberry fruit infected with *Aspergillus niger*, exhibited a lower infection (18 ± 8 %) at 24°C and a higher infection (75 ± 15 %) at 12°C. According to Oliveira Junior et al. (2012), chitosan gels, tend to deposit on the surfaces of the hyphae. This results in shrunken hyphae and loss of cytoplasmic material (empty hyphae). From these results regarding disease incidence and severity, it is confirmed that the mode of action of edible coatings is fungistatic rather than fungicidal, because fungal growth was only retarded, but not completely inhibited.

Table 2.2: Effect of edible coatings on factors affecting fruit softening and firmness retention

Factor	Fruit	Cultivar	Treatment	Storage Condition	Key findings	Author
Ethylene	Apple	Bravo de Esmolfe	Alginate and eugenol	4°C	Reduced C_2H_4 production	Guerreiro et al. (2017)
	Peach and plum	Red Heaven and Santa Rosa	*Aloe vera* and *Aloe arborescens*	20°C, 85% RH	Reduced C_2H_4 production	Guillén et al. (2013)
	Plum	Doongara	Carrageenan	20°C, 85% RH	Reduced C_2H_4 production	Thakur et al. (2018)
	Lemon	Not reported	Coconut oil and beeswax	21 ± 2°C, 50 ± 5% RH	Reduced C_2H_4 production	Nasrin et al. (2020)
	Pear	Kosui	Chitosan and alignate	20°C	Reduced C_2H_4 production	Hira et al. (2022)
Respiration	Peach and Plum	Red Heaven and Santa Rosa	*Aloe vera* and *Aloe arborescens*	20°C, 85% RH	Reduced CO_2 production	Guillén et al. (2013)
	Plum	Doongara	Carrageenan	20°C, 55 ± 5% RH	Reduced CO_2 production	Thakur et al. (2018)
	Peach	Baihua	Sodium alginate	28 ± 1°C, 90% RH	Reduced CO_2 production	Li et al., 2019
	Pear	Kosui	Chitosan and alignate	20°C	Reduced CO_2 production	Hira et al. (2022)
	Lemon	Not reported	Coconut oil and beeswax	21 ± 2°C, 50 ± 5% RH	Reduced CO_2 production	Nasrin et al. (2020)
Enzymes	Pear	Huanghua	Shellac and Semperfresh	4°C, 95% RH	Reduced PG, PE and CEL activity	Zhou et al. (2011)
	Plum	Sanhuali	Chitosan	5 ± 1°C, 90 ± 5% RH	Reduced PG and PME activity	Liu et al. (2014)
	Plum	Golden Drop	Carboxymethylcellulose	4°C, 80 ± 5% RH	Reduced PG activity	Panahirad et al. (2020)
	Guava	Banati	Chitosan/poly-vinyl-pyrrolidine	27 ± 1°C, 48 ± 2% RH	Reduced CEL, LOX and PT activity	Lo'ay and Taher, (2018)
	Persimmon	Fuyu	Gum arabic	15 ± 2°C, 70% – 80% RH	Reduced CEL, PG and PME activity	Saleem et al. (2020)
Firmness	Papaya	Not reported	*Aloe vera*	25 ± 4ºC, 82 ± 2% RH	Retained firmness	Brishti et al. (2013)
	Plum	Not reported	Carrageenan	20°C, RH 55 ± 5% RH	Retained firmness	Thakur et al. (2018)
	Peach	Red Heaven	*Aloe vera* and *Aloe arborescens*	20°C, 85% RH	No effect on firmness	Guillén et al. (2013)
	Pear	Kosui	Chitosan and alignate	20°C	Retained firmness	Hira et al. (2022)

Relative humidity (RH)

2.3.3. Heat treatment

2.3.3.1. Effect on ethylene production

HT substantially enhanced ethylene production of apple (Klein and Lurie, 1990) stored at 0°C for a six-month period. In addition, the authors reported that increasing the exposure time (1, 2 and 4 days at 38°C) of heat treatment further exacerbated high ethylene production. Regardless of the elevated ethylene production, heat treated apples exhibited significantly higher firmness levels compared to untreated apples. In a study conducted by Nair et al. (2001), mango fruit were conditioned for 14 hours at 38 - 40°C followed by either hot water (HWT) or hot air treatment (HAT) at 46 - 48°C for ten minutes. The results illustrated that ethylene production was suppressed by the listed heat treatments. Which had a positive effective on firmness retention, as treated fruit registered a substantially higher firmness relative to untreated fruit after 35 days of storage at 5°C.

HWT at 48°C for ten minutes and HAT at 38°C for three hours, reduced ethylene production of treated peach fruit (Huan et al., 2017). As a result, the heat treatments enhanced the firmness retention of peach fruit after 35 days of storage at 4 ± 0.5°C. Silva et al. (2003) reported a higher initial ethylene emission rate for papayas subjected to HWT at 40°C for 20 minutes. In addition, the results show a displacement of the climacteric peek, meaning that the treatment causes a decrease of shelf life of fruit. Regardless of the higher initial ethylene production, treated fruit maintained lower ethylene production during storage.

Heat treatments have the capacity to inhibit the production of the ethylene precursor ACC (Lurie, 1998). Ethylene synthesis of fruit subjected to heat treatments, undergo a rapid loss of ACC oxidase activity (Paull and Chen, 1990). This is primarily due to the decrease in ACC oxidase mRNA and cessation of enzyme synthesis (Lurie et al., 1996). Which leads to the suppression of ethylene production. Furthermore, Lurie, (1998) stated that heat treatment can

inhibit endogenous ethylene production due to a loss or inactivation of ethylene receptors. However, no information is available on the response of ethylene receptors to heat treatments.

2.3.3.2. Effect on respiration rates

HT substantially decreased the respiration rates of apple fruit (Klein and Lurie, 1990) stored at 0°C for six months. Treatment effect was more pronounced with an increase in the exposure time (1, 2 and 4 days at 38°C). Heat treated apples exhibited significantly higher firmness levels compared to their untreated counterparts. Heat treatment at 40°C for 20 minutes, reduced the CO_2 production of papaya fruit (Silva et al., 2003). However, the results show a displacement of the climacteric peek, meaning that the treatment caused a decrease of shelf life of the papaya fruit. In a study conducted by Huan et al. (2017), peach fruit were subjected to HAT (48°C for 10 min) and HWT (38°C for 3h). The HAT outperformed the HWT treatment, characterised by lower CO_2 production and a delay in the respiratory peak. No significant differences were observed between HWT and untreated fruit.

Regardless, both heat treatments suppressed fruit softening and registered significantly higher firmness levels compared to the untreated fruit. Mango fruit subjected to conditioning at 14 hours at 38 - 40°C, followed by HAT and HWT at 46 - 48°C for ten minutes, exhibited higher respiration rates than the untreated fruit (Nair et al., 2001). Despite the elevated CO_2 production, treated fruit registered a substantially higher firmness in comparison to the untreated fruit. Similar findings were reported by (Hernández et al., 2017), where avocado fruit subjected to HWT at 38°C for one hour exhibited a significant higher CO_2 emission rate compared to the untreated fruit. The higher respiration rates caused by heat treatments may be attributed to the stress induced by exposure to a high temperature (Paull and Chen, 2000).

2.3.3.3. Effect on fruit softening enzymes

Cell wall hydrolases are the primary factors that cause the disassembly of the cell wall by decreasing cell-to-cell adhesion and cell dispersion at various stages of fruit development (Brummell, 2006). The combination of these processes in conjunction with the loss of pectin side chains, result in increased cell wall porosity that enable degradative enzymes increased access to substrates as fruit ripen.

HWT (50°C for 10 minutes) suppressed the enzyme activity of PG, PL and β-Gal in banana peels stored at 25°C for ten days (Amnuaysin et al., 2012). Suppression of the cell membrane enzymes resulted in a significant enhancement of firmness retention of treated fruit. HAT (38°C for 5 hours) inhibited the PLD and LOX in loquat fruit stored at 1°C for 35 days (Rui et al., 2010). Firmness was not measured in this study. However, treatment effect on membrane integrity parameters such as electrolyte leakage and lipid peroxidation measured as malondialdehyde content (MDA), were reported. MDA is a secondary end product of polyunsaturated fatty acid oxidation as an index of oxidative damage under environmental stress. MDA content in a tissue is a good indicator of the structural integrity of the membranes of plants (Posmyk et al., 2005). Heat treated loquat fruit registered lower electrolyte leakage and MDA content relative to the untreated fruit. Meaning that the efficacy of HAT in suppressing PLD and LOX enzyme activity preserved membrane integrity of the treated fruit.

HWT (52°C for 15 minutes) reduced the enzyme activity LOX in tomato fruit on day four of storage at 20°C (Imahori et al., 2016). Similar to the study mentioned above, firmness was not measured but MDA content was reported and no significant difference was observed between treated and untreated fruit. Hernández et al. (2017) reported that HWT (38°C for 1 hour) had no effect on PME and PG enzyme activity of avocado fruit. Similarly, Klein et al. (1995) reported that heat treatment (4 days at 38°C) had no effect on PME enzyme activity of apple

fruit during five months of storage at 0°C. However, treated fruit registered substantially higher firmness levels compared to the untreated fruit.

2.3.3.4. Effect on pathogens

The ability of HT to inhibit disease severity caused by fungal pathogens has been demonstrated by studies listed in table 2.6. In a study conducted by Wang et al. (2010), bayberry fruit were subjected to HAT at 48 °C for 3 hours and then stored at 20°C for 3 days or at 1°C for 12 days. HAT effectively suppressed the incidence of *Leptographium abietinum* in inoculated bayberry fruit at both storage temperatures. HWT (52°C, 2 min) inhibited the germination of *Penicillium italicum* on pericarp of mandarin fruit stored at 12 – 16°C for 60 days (Yun et al., 2013). In addition, treated fruit registered significantly higher firmness levels compared to the untreated fruit. Lignin is an essential component responsible for fruit firmness. Lignin is one of the major components of secondary cell walls, that provide cells with mechanical support and isolation from the environment. HWT delays the invasion of pathogens by inducing the accumulation of lignin. Which creates thickened cell walls which forms an effective physical barrier to pathogens. In addition, HWT enhanced fruit firmness as a result of a lignin thickened fruit cell wall.

HAT (38, 42 and 46°C for 96, 24 and 12 hours) reduced the growth of *Penicillium expansum* in inoculated apple fruits, stored at 20°C for 14 days (Fallik et al., 1995). The 96h at 38°C treatment effect exhibited the greatest fungal inhibition, followed by the 24h at 42°C and 12h at 46°C treatment effect. HAT (3 hours at 45 °C) reduced decay caused by *Botrytis cinerea* on strawberry fruit (Langer et al., 2018). The authors stated that the reduction in fungal decay in heat-treated fruits could be attributed to a well-preserved cell wall structure. This is characterised by significantly higher cell wall contents and firmness levels in heat treated fruit.

Table 2.3: Effect of heat treatment on factors affecting fruit softening and firmness retention

Factor	Fruit	Cultivar	Treatment	Storage Condition	Key findings	Author
Ethylene	Apple	Granny Smith	HAT	0°C, 90% RH	Increased C_2H_4 production	Klein and Lurie, (1990)
	Mango	Kensington Pride	HWT and HAT	5°C and 22°C	Reduced C_2H_4 production	Nair et al. (2001)
	Peach	Xiahui 5	HWT and HAT	4 ± 0.5°C, 85 ± 5% RH	Reduced C_2H_4 production	Huan et al. (2017)
	Papaya	Not reported	HWT	25°C, 62% RH	Increased C_2H_4 production	Silva et al. (2003)
Respiration	Apple	Granny Smith	HT	0°C, 90% RH	Reduced CO_2 production	Klein and Lurie, (1990)
	Peach	Xiahui 5	HAT	4 ± 0.5°C, 85 ± 5% RH	Reduced CO_2 production	Huan et al. (2017)
	Mango	Kensington Pride	HWT and HAT	5°C and 22°C	Increased CO_2 production	Nair et al. (2001)
	Avocado	Hass	HWT	5°C and 20°C, 60 ± 10% RH	Increased CO_2 production	Hernández et al., 2017
Enzymes	Banana	Hom Thong	HWT	25 ± 1°C	Reduced PLD and LOX activity	Amnuaysin et al. (2012)
	Loquat	Jiefangzhong	HAT	1 ± 0.5°C, 95% RH	Reduced LOX activity	Rui et al. (2010)
	Tomato	Sanibel	HWT	20°C	Reduced LOX activity	Imahori et al. (2016)
	Apple	Golden delicious	HT	0°C	No effect on PME activity	Klein et al. (1995)
	Avocado	Hass	HWT	5°C and 20°C, 60 – 70% RH	No effect on PME and PG activity	Hernández et al. (2017)
Firmness	Banana	Hom Thong	HWT	25 ± 1°C	Retained firmness	Amnuaysin et al. (2012)
	Peach	Xiahui 5	HWT and HAT	4 ± 0.5°C 85 ± 5% RH	Retained firmness	Huan et al. (2017)
	Apple	Granny Smith	HT	0C, 90% RH	Retained firmness	Klein and Lurie, (1990)
	Zucchini	Belle-308	HWT and HWFC	4 ± 0.5°C, 85 ± 5% RH	Retained firmness	Zhang et al. (2019)
	Mango	Kensington Pride	HWT and HAT	5°C and 20°C	Retained firmness	Nair et al. (2001)

Heat treatment (HT), hot water treatment (HWT), hot air treatment (HAT), hot water forced convection (HWFC), relative humidity (RH)

2.3.4. Ozone

2.3.4.1. Effect on ethylene production

Gaseous ozone treatment (0.15 ppm during the day and 0.3 ppm overnight) at 6°C, significantly reduced ethylene production of cantaloupe melon (Toti et al., 2018). The mechanism at which ozone suppresses ethylene production is due to its ability to suppress the key enzymes involved in ethylene biosynthesis namely, ACC synthase and ACC oxidase (Minas et al., 2014). Ozone application significantly reduced ethylene production in cantaloupes stored at 4 ± 0.5°C for 42 days (Chen et al., 2020). One of the key findings in this study is that higher doses of ozone concentration (6.432 - 15.008 mgm^{-3} for 1 hour) exhibited greater ethylene suppression. In addition, increasing ozone concentrations resulted in greater firmness retention and pectin content of cantaloupe. Triardianto and Bintoro, (2021) reported a similar trend for banana fruit, whereby ethylene production decreased with an increase in the concentration of ozone (0.3 – 0.5 ppm). Furthermore, the authors demonstrated that storage temperature had an effect on the efficacy of ozone in reducing ethylene production. Ethylene production increased with an increase in storage temperature, with the lowest ethylene production registered at 5℃, followed by 15℃ and 27℃ (Triardianto and Bintoro, 2021).

Ozone application significantly reduced the ethylene production of kiwifruit (Minas et al., 2018) at stored at 20℃. Ozone treatment has been proven to directly oxidize ethylene (Minas et al., 2010), leading to lower ethylene production. Ozone reduces ethylene production by reducing the concentration of ethylene biosynthesis intermediates and related enzyme activities. Minas et al. (2018), demonstrated the capacity of ozone to repress ethylene production by lowering the concentration of ethylene biosynthesis intermediates such as ACC, 1-malonyl-aminocyclo-propane-1-carboxylic acid (MACC) and inhibiting ACS and ACO enzyme activities.

2.3.4.2. Effect on respiration rates

Ozone treatment (10µL/L for 10 min) had no effect on the respiration rate of tomatoes, stored at 20℃ for nine days (Rodoni et al., 2010). The firmness of ozone treated tomato fruit in the above study registered significantly higher firmness, despite the treatment having no effect on respiration rates. In a study conducted by Minas et al. (2018), kiwifruit was stored for 2, 4 and 6 months at 0°C, 95% RH. Fruit stored for four and six months were exposed to 100 µL/L exogenous ethylene (20°C, 90% RH, 24 h). Ozone treatment substantially reduced the respiration rate of kiwifruit stored at 20°C. In addition, ozone treatment effectively lowered the softening rates below those of untreated fruit. Chen et al. (2020) illustrated that increasing the ozone concentration from 6.432 to 15.008 mgm^{-3}, exhibited greater suppression of CO_2 production in cantaloupes. Similarly, the capacity of ozone to retain firmness was more pronounced at higher doses of ozone.

Phosphoenolpyruvate carboxykinase (PEPCK) is an enzyme involved in the gluconeogenesis pathway, that converts oxaloacetate into phosphoenolpyruvate and carbon dioxide (Nardozza et al., 2013). Enolase (ENO) is an enzyme in the glycolytic pathway (which is the first step of cellular respiration) that catalyses the conversion of 2-phosphoglycerate to phosphoenolpyruvate (Kerbel et al., 1988). Minas et al. (2018) demonstrated that ozone treatment suppressed respiration and the expression of PEPCK in the gluconeogenic (sucrose biosynthesis) pathway and ENO in the glycolytic (CO_2 production) pathway was also suppressed. Regardless of the findings from this particular study, the mechanism on how ozone reduces fruit respiration was still not clear. Therefore, further research on how ozone application affects the tricarboxylic acid cycle is needed, in order to elucidate the mode of action of how ozone reduces the respiration rates of fruit.

2.3.4.3. Effect on fruit softening enzymes

Aqueous ozone application (1.4 mg/L for 30 min) inhibited the increase in β-Gal and α-Af enzyme activities but no effect on PG or PME in fresh-cut apple (Liu et al., 2021). The inhibitory effect on these enzymes reduced the degradation of cell wall substances (water-soluble pectin, protopectin and cellulose content). This resulted in a significantly higher firmness retention of treated fruit. Ozone treatment (0.15 ppm during the day and 0.3 ppm overnight) did not influence the enzyme activities of PG and PME but was effective in reducing the enzyme activity of α-ARA and β-Gal in melons (Toti et al., 2018). The reduction of the solubilisation of polysaccharides of the cell resulted in significantly higher firmness compared to the control.

Rodoni et al. (2010) reported that ozone application (10 µL/L, 10 min) had no significant effect on β-Gal and PG enzyme activities but was effective in inhibiting the enzyme activity of PME in tomatoes. The decrease in the enzyme activity of PME, pectin solubilization and depolymerization, led to reduced softening of ozone treated fruit. Gaseous ozone (0.3 µL/L) treatment had no significant effect on the enzyme activities of β-Gal and α-ara but was effective in lowering the enzyme activities of PG and EGase/glu in kiwifruit (Minas et al., 2014). The inhibitory action on fruit softening enzymes led to a delay in the solubilization of pectic polysaccharides, which enhanced firmness retention of treated fruit.

2.3.4.4. Effect on pathogens

The ability of ozone to suppress disease incidence of fungal pathogens has been demonstrated by studies listed in table 2.6. Ozone treatment (1.5, 2.5, 3.5 and 5.0µL/L for 24 h) effectively suppressed the growth of *C. gloeosporioides* in papaya (Ong and Ali, 2015). The authors found that ozone inhibited fungal growth by degrading the mitochondria of fungal spores. Furthermore, fruit exposed to higher concentrations of ozone (≥ 3.5µL/L) had increased decay compared to the lower doses of ozone. This indicates higher doses of ozone reduces the disease

resistance to anthracnose. Ozone treatment (1.6 - 60 mg kg^{-1}) suppressed the growth of *Penicillium digitatum* and *Penicillium italicum* in citrus (García-Martín et al., 2018), and reduced fungal growth of *Penicillium expansum* on apple fruits (Yaseen et al., 2015).

In defence enzyme systems, enzymes such as phenylalanine ammonia-lyase (PAL), peroxidase (POD) and polyphenol oxidase (PPO) are important biochemical indices for identifying plant disease resistance (Wang et al., 2014). POD may play a role in controlling the level of oxygen-free radicals induced by stress conditions, mainly in the removal of H_2O_2 in the reaction of lignin synthesis, to avoid cell damage (Sachadyn-Król et al., 2016). PPO participates in the lignification of plant cells during microbial invasion (Mukherjee et al., 2013). PAL serves as an important marker of induced disease resistance in plants because this enzyme is crucial in phenylpropanoid metabolism, which is closely related to the resistance of plants.

Pathogenesis-related proteins such as chitinase (CHI) and β-1,3-glucanase (GLU) are involved in plant defence mechanisms against fungal infection by hydrolysing the polymers of fungal cell walls (Zheng et al., 2011). Luo et al. (2019), demonstrated that ozone treatment enhanced pathogenesis-related proteins and defence-related enzymes which prevent fungal infection. This was characterised by an inhibition of mycelial development and spore germination of *Botrytis cinerea* and *Penicillium expansum* on kiwifruit. The capacity of ozone to enhance disease resistance by increasing the activities of defence-related enzymes, resulted in significantly higher levels of fruit firmness.

Table 2.4: Effect of ozone treatment on factors affecting fruit softening and firmness retention

Factor	Fruit	Cultivar	Treatment	Storage Condition	Key findings	Author
Ethylene	Kiwifruit	Hayward	0.3 µL L^{-1}	0°C, 95% RH and 20°C, 95%	Reduced C_2H_4 production	Minas et al. (2018)
	Cantaloupe	Caldeo	0.15 ppm and 0.3 ppm	6 ± 2°C, 90 ± 5% RH	Reduced C_2H_4 production	Toti et al. (2018)
	Cantaloupe	Not reported	6.4, 10.7 and 15 mg^{-3}	4 ± 0.5°C, 90 ± 5% RH	Reduced C_2H_4 production	Chen et al. (2020)
	Banana	Kepok	0.3, 0.4, and 0.5 ppm	5, 15, and 27°C	Reduced C_2H_4 production	Triardianto and Bintoro, (2021)
Respiration	Tomato	Not reported	10 µL L^{-1}	20°C, 90% RH	No effect on CO_2 production	Rodoni et al. (2010)
	Kiwifruit	Hayward	0.3 µL L^{-1}	0°C, 95% RH and 20°C, 95%	Reduced CO_2 production	Minas et al. (2018)
	Cantaloupe	Not reported	6.4, 10.7 and 15 mgm^{-3}	4 ± 0.5°C, 90 ± 5% RH	Reduced CO_2 production	Chen et al. (2020)
Enzymes	Apple	Fuji	1.4 mg L^{-1}	4 ± 1°C, 90% RH	Reduced β-Gal activity	Liu et al. (2021)
	Cantaloupe	Caldeo	0.15 ppm and 0.3 ppm	6 ± 2°C, 90 ± 5% RH	Reduced β-Gal and α-Af activity	Toti et al. (2018)
	Tomato	Not reported	10 µL L^{-1}	20°C, 90% RH	Reduced PG and EGase/glu activity	Rodoni et al. (2010)
	Kiwifruit	Hayward	0.3 µL L^{-1}	0°C, 95% RH and 20°C, 90% RH	Reduced PME activity	Minas et al. (2014)
Firmness	Tomato	Not reported	10 µL L^{-1}	20°C, 90% RH	Retained firmness	Rodoni et al. (2010)
	Kiwifruit	Hayward	0.3 µL L^{-1}	0°C, 95% RH	Retained firmness	Minas et al. (2018)
	Cantaloupe	Not reported	6.4, 10.7 and 15 mg^{-3}	4 ± 0.5 °C, 90 ± 5% RH	Retained firmness	Chen et al. (2020)
	Cantaloupe	Caldeo	0.15 ppm and 0.3 ppm	6 ± 2°C, 90 ± 5% RH	Retained firmness	Toti et al. (2018)
	Apple	Fuji	1.4 mg L^{-1}	4 ± 1°C, 90% RH	Retained firmness	Liu et al. (2021)

Relative humidity (RH)

2.3.5. Ultraviolet C (UV-C)

2.3.5.1. Effect on ethylene production

UV-C (4.1 kJ/m^2) treatment was effective in suppressing ethylene production of tomatoes stored at 20°C (Lu et al., 2016). UV-C has been reported to reduce ethylene production by inhibiting enzyme activity of ACC oxidase (Burana and Srilaong, 2010). In addition, the authors reported higher firmness retention for UV-C treated fruit. Similar results were reported by Mansourbahmani et al. (2017) for UV-C treated (1.5, 3 and 4.5 kJ m^{-2}) tomato fruit stored at 7°C. The results from these two studies show the capacity of UV-C to reduce ethylene production and softening at a low and higher temperature. UV-C (0.01 to 0.30 kJ m^{-2}) treated banana fruit exhibited significantly lower ethylene production in comparison to the untreated fruit (Mohamed et al., 2017). The peel and pulp firmness of treated fruit was not fully retained, as softening was temporarily delayed as ripening progressed.

UV-C (4 kJ m^{-2}) significantly reduced ethylene production and lowered the climacteric peak of peach fruit (Zhou et al., 2020). Kan et al. (2021) obtained similar results for UV-C treated peach fruit. Furthermore, Kan et al. (2021) demonstrated that UV-C was able to suppress ethylene production by downregulating the expression of genes related to ethylene biosynthesis. The results illustrated that UV-C treatment inhibited the expression of ACS gene and ethylene receptor, which contributed to the reduced ethylene production and thus delayed softening of peach fruit.

2.3.5.2. Effect on respiration rates

UV-C (4.1 kJ/m^2) treatment was effective in suppressing the respiration rates of tomato, with treated fruit registering higher firmness retention (Lu et al., 2016). UV-C irradiation significantly reduced the respiration rate of peaches. However, UV-C had no effect on the fruit firmness as no significant differences were observed between treatments (Zhou et al., 2020). Contrarily, UV-C was not effective in suppressing the respiration rate of banana fruit (Mohamed et al., 2017). UVC doses exhibited a delay in pulp softening with a relatively higher value of firmness than control fruit. However, the inhibitory effect was lost as ripening progressed, characterised by no significant differences among treatments towards the end of the storage period.

2.3.5.3. Effect on fruit softening enzymes

UV-C (0.04 kJ s^{-1} m^{-2} for 30, 90 and 120 s) treatment suppressed the enzyme activities of PME and PG in fresh-cut melon (Chisari et al., 2011). In this study, an exposure time of 30 s to UV-C radiation was the most effective at reducing the enzyme activity of PG and PME. In addition, the firmness of UV-C treated melons was higher than untreated at the end of storage. UV-C irradiated (4.2 kJ/m^{-2} for 8 min) tomato fruit exhibited significantly lower PG, PME and CEL enzyme activities compared to untreated fruit (Bu et al., 2013). Furthermore, the inhibition of these cell wall degrading enzymes by UV-C irradiation, resulted in the preservation of fruit firmness. Lu et al. (2016) reported that UV-C irradiation (4.1 kJ/m^2) inhibited PME and PG activities of tomato fruit. In addition, UV-C delayed the metabolism of pectin substances (protopectin and water-soluble pectin), which contributed to the retention of fruit firmness.

UV-C (1.5, 3, and 4.5 kJ m^{-2} for 5, 10 and 15 min) treatment significantly reduced the enzyme activity of PG and PME of tomato fruit (Mansourbahmani et al., 2017). The capacity of UV-C to decrease the activity of these cell wall degrading enzymes led to improved firmness retention of treated fruit. UV-C (2 kJ m^{-2} for 6 min, 4 kJ m^{-2} for 12 min, 6 kJ m^{-2} for 18 min, or 8 kJ m^{-2} for 24 min) irradiation significantly reduced PG, PME and CEL enzyme activities of pineapple (Ou et al., 2016). The initiation of fruit softening of pineapple was effectively delayed by suppressing the activity of the cell wall degrading enzymes. Repeated doses of UV-C irradiation reduced the activities of PG, PME and Glcase activities in strawberry fruit, which in turn delayed fruit softening (Araque et al., 2019).

2.3.5.4. Effect on pathogens

The ability of UV-C to repress fungal growth of pathogens has been demonstrated by studies listed in table 2.6. UV-C (0.001236 J/cm) treatment suppressed fungal growth of *Botrytis cinerea* on strawberries, with pronounced effect at 90 and 120 s, which resulted in complete inhibition (Janisiewicz et al., 2016). A UV-C dose of 6.16 kJ m^{-2} for 2 min significantly reduced the disease incidence and severity of anthracnose on mangoes (Sripong et al., 2015). UV-C irradiation at a dose of 13 kJ m^{-2}, significantly reduced the fungal growth of *Lasiodiplodia theobromae* on inoculated mangosteen fruit (Sripong et al., 2019). Pombo et al. (2011) reported similar results, with UV-C irradiation significantly reducing the fungal growth of *Botrytis cinerea* on strawberry fruit at a dose of 4.1 kJm^{-2}. The authors of these studies further demonstrated that UV-C suppressed fungal growth on fruit by inducing defence mechanisms within the plants. The enzyme activities of PAL, POD, CHI and GLU, which are

the defence mechanisms associated with plant defence, were enhanced by UV-C treatment. Furthermore, this led to an enhanced firmness retention of treated fruit.

The severity of crown rot disease on banana fruit was significantly reduced by UV-C irradiation with greater effect exhibited by the highest irradiation dose (0.30 kJ/m^2) (Mohamed et al., 2017). In this study, UVC treatment delayed fruit softening with treated fruit registering higher value of firmness compared to untreated fruit. However, UV-C irradiation did not retain the firmness of banana fruit at the end of ripening. The authors of this study examined the cell wall structure in the crown tissue of banana fruit using scanning electron microscopy (SEM). The SEM micrographs revealed that UV-C maintained the membrane integrity of treated fruit, which resulted in reduced tissue damage caused by the crown rot disease. Untreated fruit exhibited rapid cell wall degradation due to an intense colonization by fungal mycelia. Thus, the loss of barrier strength in the cell wall against pathogen penetration increases the decay process.

Table 2.5: Effect of UV-C treatment on factors affecting fruit softening and firmness retention

Factor	Fruit	Cultivar	Treatment	Storage Condition	Key findings	Author
Ethylene	Tomato	Zheza 205	4.1 kJ/m^2	20°C	Reduced C_2H_4 production	Lu et al. (2016)
	Tomato	Valouro	1.5, 3 and 4.5 kJ m^{-2}	7°C, 90% RH	Reduced C_2H_4 production	Mansourbahmani et al. (2017)
	Banana	Berangan	0.01 to 0.30 kJ m^{-2}	25 ± 2°C, 85% RH	Reduced C_2H_4 production	Mohamed et al. (2017)
	Peach	Beinong2 x 60–24–7	4 kJ m^{-2}	15 ± 2°C, 75 ± 5% RH	Reduced C_2H_4 production	Zhou et al. (2020)
	Peach	Xiahui 5	4 kJ m^2	10°C, 85% RH	Reduced C_2H_4 production	Kan et al. (2021)
Respiration	Tomato	Zheza 205	4.1 kJ/m^2	20°C	Reduced CO_2 production	Lu et al. (2016)
	Peach	Beinong2 x 60–24–7	4 kJ m^{-2}	15 ± 2°C, 75 ± 5% RH	Reduced CO_2 production	Zhou et al. (2020)
	Banana	Berangan	0.01 to 0.30 kJ m^{-2}	25 ± 2°C, 85% RH	No effect on CO_2 production	Mohamed et al. (2017)
Enzymes	Melon	Reticulatus	0.04 kJ s^{-1} m^{-2}	5°C, 90% RH	Reduced PME and PG activity	Chisari et al. (2011)
	Strawberry	Camarosa	4, 2 and 0.8 kJ/m^{-2}	0°C	Reduced PG, PME and Glcase activity	Araque et al. (2019)
	Pineapple	Not reported	2, 4, 6 and 8 kJ/m^{-2}	25°C, 85% RH	Reduced PG, PME and Glcase activity	Ou et al. (2016)
	Tomato	Zhenzhu1.	4.2 kJ/m^{-2}	18°C, 95% RH	Reduced PG, PME and CEL activity	Bu et al. (2013)
	Tomato	Zheza 205	4.1 kJ/m^2	20°C	Reduced PG and PME activity	Lu et al. (2016)
	Tomato	Valouro	1.5, 3 and 4.5 kJ m^{-2}	7°C, 90% RH	Reduced PG and PME activity	Mansourbahmani et al. (2017)
Firmness	Kiwifruit	Hayward	1, 0.5 and 0.25 kJ/m^2	0 - 1°C, 90 ± 5% RH	Retained firmness	Bal and Kok, (2009)
	Tomato	Zheza 205	4.1 kJ/m^2	20°C	Retained firmness	Lu et al. (2016)
	Tomato	Valouro	1.5, 3 and 4.5 kJ m^{-2}	7°C, 90% RH	Retained firmness	Mansourbahmani et al. (2017)
	Peach	Xiahui 5	4 kJ m^2	10°C, 85% RH	Retained firmness	Kan et al. (2021)
	Banana	Berangan	0.01 to 0.30 kJ m^{-2}	25 ± 2°C, 85% RH	No effect on firmness	Mohamed et al. (2017)
	Peach	Jinxiang	4 kJ m^{-2}	15 ± 2°C, 75 ± 5% RH	No effect on firmness	Zhou et al. (2020)

Relative humidity (RH), ultraviolet C (UV-C)

Table 2.6: Studies conducted on pathogen inactivation in response to eco-friendly treatments

Fruit	Cultivar	Treatment	Storage	Pathogen	Findings	Author
Apple	Golden Delicious and Jonagold	Hexanal	0°C	*Penicillium expansum, Botrytis cinerea*	Inhibited hyphal growth	Song et al. (1998)
Peach and Raspberry	Red Haven and Encore, Red Wings, K81-6	Hexanal	0°C and 20°C	*Sclerotinia sclerotiorum*, *Alternaria alternata*, *Colletotrichum gloeosporioides*	Reduced mycelial growth	Song et al. (2007)
Peach	Chiripá	Hexanal	20°C	*Monilinia fructicola, Monilinia laxa*	Suppressed pore germination	Baggio et al. (2013)
Tomato	Royale	Hexanal	20 ± 1°C, 99% RH	*Botrytis cinerea*	Reduced growth of *B. cinerea*	Utto et al. (2008)
Longan	Daw	Hexanal	5°C	*Lasiodiplodia theobromae*	Reduced incidence severity	Thavong et al. (2010)
Tomato	Josefina	EC	5°C and 20°C, 85 ± 5% RH	*Botrytis cinerea*	Reduced incidence and severity	Fagundes et al. (2014)
Strawberry	Albion	EC	25°C	*Rhizopus stolonifer*	Reduced incidence and severity	Oregel-Zamudio et al. (2017)
Peach	Baihua	EC	28 ± 1°C, 90% RH	*Penicillium expansum*	Reduced decay	Li et al. (2019)
Bayberry	Not reported	HT	1°C and 20°C, 90% RH	*Leptographium abietinum*	Suppressed mycelial growth	Wang et al. (2010)
Mandarin	Kamei	HT	12 ± 4°C, 90 ± 5% RH	*Penicillium italicum*	Inhibited fungal growth	Yun et al. (2013)
Apple	Golden delicious	HT	20°C	*Penicillium expansum*	Inhibited spore germination	Fallik et al. (1995)
Strawberry	Aroma	HT	4°C and 20°C	*Botrytis cinerea*	Suppressed mycelial growth	Langer et al. (2018)
Papaya	Sekaki	Ozone	25°C, 70% RH	*Colletotrichum gloeosporioides*	Suppressed fungal growth	Ong and Ali, (2015)
Mandarin	Fortune and Ortanique	Ozone	5°C and 20°C	*Penicillium digitatum, Penicillium italicum*	Suppressed fungal growth	García-Martín et al. (2018)
Orange	Navelate, Lanelate, Salustiana and Valencia	Ozone	5°C and 20°C	*Penicillium digitatum, Penicillium italicum*	Suppressed fungal growth	García-Martín et al. (2018)
Kiwifruit	Hayward	Ozone	0°C, 95% RH	*Botrytis cinerea, Penicillium expansum*	Inhibited spore germination	Luo et al. (2019)
Apple	Golden delicious and Fuji	Ozone	1 ± 1°C, 95% RH	*Penicillium expansum*	Suppressed fungal growth	Yaseen et al. (2015)
Strawberry	Toyonoka	UV-C	20°C	*Botrytis cinerea*	Suppressed fungal decay	Pombo et al. (2011)
Strawberry	Albion and monterey	UV-C	22°C	*Botrytis cinerea*	Suppressed fungal growth	Janisiewicz et al. (2016)
Mango	Chok-Anan	UV-C	13°C, 85 ± 5% RH	*Colletotrichum gloeosporioides*	Reduced incidence and severity	Sripong et al. (2015)
Banana	Berangan	UV-C	25 ± 2°C, 85% RH	Crown rot disease	Suppressed disease severity	Mohamed et al. (2017)
Mangosteen	Not reported	UV-C	25°C, 80 ± 5% RH	*Lasiodiplodia theobromae*	Reduced disease severity	Sripong et al. (2019)

Relative humidity (RH), enhanced freshness formulation (EFF), edible coating (EC), heat treatment (HT), ultraviolet C (UV-C)

2.4. Conclusion

Eco-friendly treatments have been successful in controlling factors that influence the softening process of fresh produce. Furthermore, scientific studies have provided an insight on the mechanism of each treatment in optimising firmness retention of fruit. These studies involved experiments related to gaseous exchange, inhibition of pathogens and activity of cell membrane enzymes. Results from these studies demonstrate the efficacy of eco-friendly treatments in suppressing the gaseous exchange, fungal growth and cell wall degrading enzymes. Which in turn, yielded fruit of superior quality in comparison to untreated fruit and improved firmness retention during storage. Hexanal formulations offer an affordable and cost-effective investment for adoption within the industry. Although high concentrations of hexanal provide complete inhibition of fungal growth, high concentrations have a detrimental effect on fruit quality. Therefore, further research needs to be conducted to establish optimum concentrations and exposure times that will provide maximum fungal inhibition without compromising on the organoleptic quality of fruit.

2.5. References

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Chapter Three

Mechanism of enhanced freshness formulation in delaying softening whilst optimising quality of gold kiwifruit harvested at two maturity stages

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Abstract

Kiwifruit have a limited storage life due to rapid softening, which results in significant postharvest and economic losses. Harvest maturity significantly influences the softening rate of kiwifruit. Considering that fruit firmness is the principal quality attribute of kiwifruit, this study sought to evaluate the efficacy of enhanced freshness formulation (EFF), a hexanal-based formulation containing antioxidants such as geraniol, α-tocopherol and ascorbic acid, in delaying the softening of 'Y368' kiwifruit harvested at the mid and late maturity stages (7 °Brix and 9 °Brix). Kiwifruit were treated with two treatments, namely, control (untreated fruit), 0.01 and 0.02% (v/v) EFF. Kiwifruit were stored for 8 weeks at 0 ºC and 90% relative humidity, then transferred to 20 °C for one week. Fruit quality parameters (firmness, total soluble solids, titratable acidity and mass loss), cell wall polysaccharides (CWPs) (pectin, cellulose and hemicellulose), and cell wall degrading enzymes (CWDEs) (pectin methylesterase, polygalacturonase, β-galactosidase and cellulase) were assessed. The results demonstrated that EFF significantly ($p < 0.05$) reduced fruit softening and maintained quality at both maturity stages, with a pronounced effect at the 0.02% treatment level. The PCA biplot showed that

EFF-treated kiwifruits were characterised by higher contents of CWPs, whereas control fruit exhibited higher activities of CWDEs. Furthermore, earlier harvested kiwifruit had superior quality and firmness retention than later harvested kiwifruit, which exhibited higher ripening and softening rates. Thus, EFF can improve the storage potential of gold kiwifruit by reducing rapid softening. CWDEs are triggered by ethylene production, thus, further research should investigate the inhibitory effects of EFF on ethylene biosynthesis.

Key words: *Actinidia chinensis*, cell wall, polysaccharides, enzymes, firmness, postharvest quality

3.1. Introduction

Gold kiwifruit (*Actinidia chinensis*) is an economically valuable horticultural commodity that has dominated the global commercial market, owing to its unique flavour and high nutritional value (Tilahun et al., 2020). However, gold kiwifruit is extremely perishable and softens rapidly, which limits its storage potential (Mack et al., 2017; Cha et al., 2019). This is of concern because it has an adverse effect on the economic performance of kiwifruit, as a limited storage life reduces profitability and leads to major postharvest losses (Kim et al., 2000; Jhalegar et al., 2011; Asiche et al., 2017; Ma et al., 2023). The postharvest losses of fruit due to rapid softening are estimated to be between 28 and 36% (Shafiee-Jood and Cai, 2016). Fruit firmness is dependent on the integrity of the cell wall, which is comprised of polysaccharides such as pectin, cellulose and hemicellulose (Zhang, et al., 2018). These cell wall polysaccharides (CWPs) play a crucial role in the maintenance of fruit firmness (Fullerton et al., 2020; Li et al., 2021). Fruit softening occurs due to the hydrolysis of CWPs, which is catalysed by enzymes such as pectin methylesterase (PME), polygalacturonase (PG), β-galactosidase (β-Gal) and cellulase (Cx) (Ramana-Rao et al., 2011).

In the cell wall, PME hydrolyses methoxylated pectin by hydrolysing the methyl group on the galacturonic acid (Zhang et al., 2019). This leads to the generation of demethylated pectin that is further hydrolysed by PG (Yi et al., 2016; Zhang et al., 2019). PG catalyses the hydrolysis of α (1→4) galacturonan linkages of demethylated pectin, causing the depolymerization and dissolution of pectin (Zhou et al., 2011). β-Gal hydrolyses pectin and hemicellulose by breaking the galactosidic bonds in the CWPs and removing galactose residues (Lin et al., 2018). Cx is involved in the degradation of cellulose and the β-1,4-glucan backbone of xyloglucan, which is a hemicellulosic polysaccharide (Zhou et al., 2011). The depolymerization of pectin

and hydrolysis of cellulose and hemicellulose weakens the adhesion between fruit cells which leads to fruit softening (Cybulska et al., 2015; Li et al., 2024).

Given that firmness is a key commercial quality attribute that affects grading, export and consumer acceptability (Li et al., 2016; Burdon et al., 2017), it is crucial to devise postharvest strategies that could be used to delay softening of kiwifruit (Wang et al., 2021). Low temperature storage facilitates the suppression of metabolic changes of fruit and is the main technology used to delay softening of kiwifruit during transit (Pranamornkith et al., 2012; Choi et al., 2022). However, kiwifruit can still experience rapid softening when stored at low temperatures during long distance transportation (Mitalo et al., 2019a; Xu et al., 2023). Studies have shown that storing kiwifruit at low temperatures (0 – 5 °C) induces softening genes (*AcXET2, AcPG, AcEXP1, AcPMEi, PbPG, PbPME* and *Pbβ-GAL*), which ultimately leads to rapid softening (Mworia et al., 2012; Asiche et al., 2018; Mitalo et al., 2019b; Xu et al., 2023). Therefore, exploring postharvest treatments that can suppress rapid softening of kiwifruit stored at low temperatures is needed to optimise their storability.

South African gold kiwifruits destined for export are harvested at the mid and late maturity stage (Mahlaba et al., 2022). Past research has shown that harvest maturity influences the softening rate of kiwifruit (Tavarini et al., 2009; Choi et al., 2019). Tavarini et al. (2009) and Mahlaba et al. (2022) determined that later harvested kiwifruits have a compromised postharvest performance. Mahlaba et al. (2022) demonstrated that late harvested gold kiwifruit experienced a 12% greater loss in firmness during storage. Therefore, it is critical to explore a postharvest strategy that can optimise firmness retention of kiwifruit harvested at different maturity stages.

Enhanced freshness formulation (EFF) is a hexanal-based formulation that comprises of antioxidants such as geraniol, α-tocopherol and ascorbic acid (Paliyath and Murr, 2007). EFF has been proven to optimise firmness retention of various fruit (Qi et al., 2011; Gill et al., 2015; El Kayal et al., 2017; Cheema et al., 2018). Enzyme and transcriptome analyses have demonstrated that EFF has the capacity to optimise firmness retention by suppressing the activity of enzymes and down regulating the transcript levels of genes involved in the degradation of CWPs (Tiwari and Paliyath, 2011). Therefore, EFF treatment could serve as a potential tool in optimising the storability of kiwifruit by delaying softening. In our extensive review of the literature, we have not found studies investigating the efficacy of EFF in suppressing rapid softening of kiwifruit.

Considering that fruit firmness is an important criterion that has a direct influence on the shelf life and marketability of kiwifruit. The current study aimed to evaluate the efficacy and mechanism of EFF to delay the softening of kiwifruit harvested at two maturity stages, under simulated export conditions. This was done by assessing the capacity of EFF on suppressing the action of enzymes involved in the degradation of CWPs, which contribute to fruit firmness. In addition to firmness, the mass loss, total soluble solids and titratable acidity are also essential parameters related to the quality of kiwifruit (Yi et al., 2016). Therefore, the efficacy of EFF on optimising these quality parameters was also evaluated.

3.2. Materials and methods

3.2.1. Fruit source

Kiwifruit (*Actinidia chinensis*) cv. 'Y368' were harvested from Roselands farm, a commercial kiwifruit farm located in the Richmond area (Latitude: 29.9033°S, Longitude: 30.2397°E), KwaZulu-Natal Province, South Africa. Kiwifruits were harvested when the total soluble solid content was greater than 6.2 °Brix (Mahlaba et al., 2022), at two maturity stages namely, maturity stage one (M1) and maturity stage two (M2) with 7 and 9 °Brix, respectively. Harvested fruit were immediately transported in a ventilated vehicle to the Postharvest Laboratory of the University of KwaZulu-Natal, where kiwifruit without blemishes, decay or physical damage were graded and selected for uniformity in size, then assigned to the respective postharvest treatments. All chemicals used to conduct the experiments in this study were purchased from Sigma-Aldrich, South Africa.

3.2.2. Preparation of postharvest treatments and storage

The enhanced freshness formulation was prepared by making a stock formulation comprising of 1% (v/v) hexanal, 1% (v/v) geraniol, 1% (w/v) α-tocopherol, 1% (w/v) ascorbic acid, 0.1% (w/v) cinnamic acid and 10% (v/v) Tween 20 dissolved in ethanol (10% v/v) (Paliyath and Murr, 2007). A 0.01 and 0.02% (v/v) hexanal concentration was prepared by mixing the stock solution in 100 L and 50 L of distilled water, respectively. Kiwifruit were immersed in 0.01 and 0.02% (v/v) EFF solution for 2.5 min, then air dried at room temperature. Control fruit were left untreated. The kiwifruits were stored for 8 weeks in a cold room with temperature set at 0 °C and relative humidity at 90 %, then transferred to 20 °C for one week to stimulate shelf life. Each treatment consisted of three replicates (n = 3); each replicate had three fruit per sampling interval for each maturity stage, with fruit sampled on week 0, 2, 4, 6, 8 and 9.

3.2.3. Firmness and mass loss

Fruit firmness was determined using a penetrometer (Selectech, mod. FT 327, Italy) with an 8 mm diameter head. Firmness was recorded as kilogram-force (kgf) and converted to newtons (N), where 1 kilogram-force (kgf) is equal to 9.81 newtons (N).

Mass loss (ML) was measured using a calibrated Ohaus digital scale (± 0.01 g) (Model SKX2202, Switzerland). Mass loss was calculated using Eq. 1

$$Mass\ loss\ (\%) = (IM - FM) / IM \times 100 \quad 1$$

Where, IM, the initial mass of fruit (g) and FM, the final mass of fruit (g).

3.2.4. Total Soluble Solids (TSS) and Titratable acidity (TA)

The desktop refractometer was used to measure TSS of the kiwifruit juice (Bellingham + Stanley Ltd, Model: RFM340+, UK). Titratable acidity was determined by titrating 10 mL of kiwifruit juice with 0.1 M NaOH to a pH value of 8.1, using a Mettler Toledo Potentiometric compact titrator (Model G20S, Greifensee, Switzerland). Titratable acidity was expressed as the percentage of citric acid equivalent on a fresh weight basis using Eq. 2.

$$TA\ (\%\ citric\ acid) = (0.0064\ x\ titre\ (NaOH)\ mL)/(10\ mL\ juice)\ x\ 100$$

3.2.5. Enzyme extraction procedure

Enzyme extraction was done according to the procedure described by Chen et al. (2017). One gram of kiwifruit was homogenized with 3.5 mL of 0.04 M sodium acetate buffer pH 5.2, containing 5% polyvinyl pyrrolidone, 2% β-mercaptoethanol and 0.1 M NaCl. Then, homogenate was centrifuged at 15 000 × g for 30 min at 4 °C. The supernatant was used to measure PG, β-galactosidase and cellulase enzyme activity according to the method described by Cao et al. (2021). In all assays, denatured enzyme was used as control and prepared by boiling for 10 min. All enzyme assays were run using a Shimadzu UV spectrophotometer (Model UV-1800 240V, Kyoto, Japan).

3.2.6. Quantification of enzyme activity

3.2.6.1.Polygalacturonase (PG, EC 3.2.1.15)

PG enzyme activity was assayed using the method described by Cao et al. (2021). The reaction mixture comprised of 1 mL of 0.5 M sodium acetate buffer (pH 5.5), 500 µL of 1% polygalacturonic acid and 500 µL of enzyme extract was incubated at 37 °C for 1 h. Thereafter, 1.5 mL of 0.63% 3,5-dinitrosalicylic acid (DNS) was added to the reaction mixture and boiled for 5 min. The concentration of the reducing groups was determined using D-galacturonic acid as a standard (0 – 20 nmol/mL, $R^2 = 0.996$) after measuring the absorbance at 540 nm. One unit of PG activity was expressed as nmol of galacturonic acid min^{-1} mg^{-1} protein.

3.2.6.2. β-galactosidase (β-Gal, EC 3.2.1.23)

β-Gal activity was assayed using the method described by Ali et al. (1995). Enzyme extract (100 µL) was added to 400 µL of 13 mM 2-nitrophenyl-β-D-galactopyranoside, 400 µL of 0.1% (w/v) bovine serum albumin and 500 µL of 0.1 M of sodium citrate (pH 4.1) and mixed thoroughly. After incubation for 15 min at 37 °C, the reaction was stopped by adding 2 mL of 0.2 M sodium carbonate. The concentration of released 2-nitrophenol was measured at a wavelength of 415 nm using 2-nitrophenol (100 – 1000 nmol/mL, $R^2 = 0.9947$) as a standard. One unit of β-Gal activity was expressed as nmol of 2-nitrophenol released min^{-1} mg^{-1} protein.

3.2.6.3. Cellulase (Cx, EC 3.2.1.4)

Cx enzyme activity was assayed using the method described by Cao et al. (2021) with modifications. The kiwifruit extract (100 µL) was added to 400 µL of 1% (w/v) carboxymethyl cellulose, 500 µL of 0.1 M sodium acetate buffer (pH 5.0), and placed at 37 °C for 1 hr. At the end, 1 mL of 1% DNS was added and the mixture was boiled for 5 min. The concentration of reducing groups released from carboxymethyl cellulose was measured at a wavelength of 540 nm using glucose (0 – 1000 µg/mL, $R^2 = 0.9954$) as a standard. One unit of Cx activity was expressed as µmol of glucose min^{-1} mg^{-1} protein.

3.2.6.4. Pectin methylesterase, PME (EC 3.1.1.11)

The PME activity was analysed following the spectrophotometric method of Hagerman and Austin (1986) modified by Amnuaysin et al. (2012). The enzyme assay mixture consisted of 2 mL of 0.5% (w/v) citrus pectin, 200 µL of 0.01% (w/v) of bromothymol blue (3 mM potassium phosphate buffer pH 7.5), 300 µL of water and 500 µL of enzyme extract. The reaction was

initiated by adding the enzyme and the absorbance was measured at 620 nm and recorded for 3 min. The enzyme activity was determined using D-galacturonic acid as a standard (0 – 42 µg/mL, R^2 = 0.9918). One unit of PME activity was expressed as µmol of methyl ester hydrolysed min^{-1} mg^{-1} protein.

3.2.7. Protein concentration

The Bradford assay (1976) was used to determine the protein concentration using bovine serum albumin as a standard (0 – 1 mg/mL, R^2 = 0.9957). The specific activities of cell wall degrading enzymes were presented with the unit of U/mg protein.

3.2.8. Protopectin (PP) and water-soluble pectin (WSP)

Pectin extraction was done according to the modified method described by Wang et al. (2023). Kiwifruit pulp (0.1 g) was homogenised in 2.5 mL of 95 % ethanol, followed by incubation in a water bath at 100 ℃ for 30 min. The ethanol was supplemented during the incubation process. The homogenate was then centrifuged at 12 000 × g for 10 min at 4 °C. The supernatant was discarded and another 2.5 mL of 95 % ethanol was added to the precipitate. The above procedures were repeated three times. After centrifugation, the supernatant was discarded, and 1.5 mL of distilled water was added to the precipitate, which was kept in a 50 °C water bath for 30 min, followed by centrifugation at 12000 × *g* for 15 min at 4 °C. The supernatant was collected as water soluble pectin (WSP). Protopectin (PP) was extracted by adding 1.5 mL of 0.5 M sulfuric acid to the precipitate. Thereafter, the homogenate was boiled for 1 h, followed by centrifugation at 12000 × *g* for 15 min at 4 °C. One mL of each pectin solution was boiled with 5 mL of concentrated sulfuric acid (98%, w/w) for 20 min, followed by the addition of 0.2 mL of 0.15% (w/v) carbazole-ethanol solution. The reaction mixture was stored in the dark for 30 min, the absorbance was measured at 530 nm. D-galacturonic acid was used as the standard (0 – 1 mg/mL, R^2 = 0.9906) and the results were expressed as mg/g FW.

3.2.9. Cellulose and hemicellulose

Cellulose content was determined according to the method described by Sun et al. (2022) and modified by Wang et al. (2023). Briefly, 1 g of kiwifruit pulp was added to 10 mL of 60 % sulphuric acid solution and hydrolysed at 4 °C for 12 h and filtered using Whatman #4 filter paper. Subsequently, 200 µL of filtrate was diluted to 10 mL and 200 µL of anthrone-sulfuric acid reagent was added. The mixture was placed in boiling water for 10 min and cooled. The

absorbance was measured at 620 nm and cellulose content was calculated using glucose as a standard (0 – 200 µg/mL, R^2 = 0.9923) and expressed as µg/g FW.

Hemicellulose was determined using the method previously described by Cheng et al. (2009) and modified by Sun et al. (2022). One gram of kiwifruit tissue was homogenised in 6 mL of cold distilled water for 6 h, followed by centrifugation at 8000 rpm for 15 min. The supernatant was discarded and the precipitate was immersed in 6 mL of 0.5 M HCl for 6 h. The homogenate was centrifuged and the supernatant was discarded. The precipitate was extracted with 6 mL of 1 M KOH containing 0.02 M NaBH4 for 6 h, followed by centrifugation. After the homogenate was centrifuged, the supernatant was designated as hemicellulose. Hemicellulose was hydrolysed with 60% (v/v) sulfuric acid at 100 °C for 1 h and diluted to 20 mL. Thereafter, 1.5 mL of 3,5-dinitrosalicylic acid was added to 2 mL of the supernatant and boiled for 5 min and diluted to 25 mL after cooling. The absorbance was measured at 540 nm and hemicellulose was estimated using glucose as a standard and results were expressed as mg/g FW.

3.2.10. Statistical analysis

All experiments were performed using a completely randomized factorial design. The experiment was comprised of three factors: treatment, harvest maturity and storage period. Statistical analysis, Pearson correlation and principle component analysis were performed in R software version 4.3.1 (R Development Core Team, 2023). Data were expressed as mean values ± SE and were subjected to analysis of variance (ANOVA) with a 5% level of significance.

3.3. Results and discussion

3.3.1. Fruit quality parameters

Retaining firmness is critical for mitigating fruit deterioration, as it is an essential parameter that affects fruit texture (Xu et al., 2023). Fruit firmness declined as the storage period progressed, with control fruit exhibiting the greatest loss in firmness (Figure 3.1 A). At week 9 of storage, firmness was approximately 5, 8 and 10N for the control, 0.01 and 0.02 % EFF treatment level, respectively. This represented a reduction of 91.48, 86.36 and 82.96 % from the initial firmness values at harvest. The sensory rejection point for kiwifruit is considered to be when firmness falls below 4 N, any fruit with firmness levels below this threshold are regarded unsuited for further storage (Zhang et al., 2021). The firmness values of treated fruit were well above the sensory rejection point. This suggests that the percentage reduction of firmness of treated fruit was in an acceptable limit for continued storage, indicating the

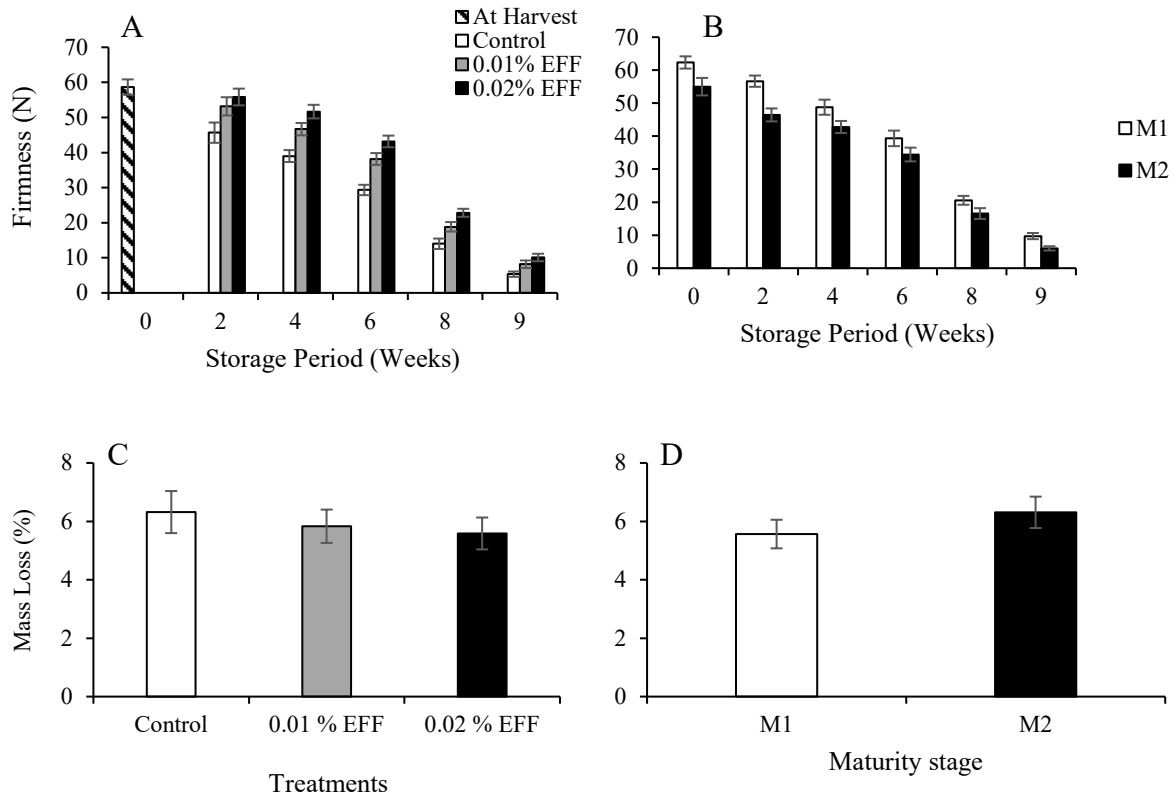
effectiveness of EFF treatment in delaying softening. The results are in accordance with those reported by Cheema et al. (2018), Sulaimankhil et al. (2021) and Silué et al. (2022), who showed that EFF optimised firmness retention of bell pepper, apple and mango fruit, respectively. Considering that the softening of kiwifruit is attributed to the degradation of CWPs (Wang et al., 2021). Our results indicate that the efficacy of EFF to reduce softening of kiwifruit is owed to its capacity to delay the degradation of CWPs (Figure 3.2). At the end of the storage period, firmness of kiwifruit harvested at M1 was approximately 38% greater than kiwifruit harvested at M2. These findings corroborate those reported by Tilahun et al. (2020), who showed that firmness of kiwifruit declines with an advancement in fruit maturity.

Mass loss contributes significantly to fruit deterioration which leads to a loss of marketability (Shiri et al., 2015). Treatments and maturity stage significantly ($p < 0.05$) influenced the mass loss of kiwifruit (Figure 3.1 C and D). Kiwifruit treated with 0.01 and 0.02% EFF had approximately 9 and 14% lower mass loss compared to the control, respectively. These results agree with those reported by Cheema et al. (2018), who showed that EFF reduced the mass loss of bell peppers. EFF can reduce the mass loss of fruit by preserving the membrane integrity, which lowers the moisture loss (Paliyath and Subramanian 2008; Tiwari and Paliyath 2011). The mass loss of kiwifruit harvested at M1 and M2 was registered at 5.71 and 6.20%, respectively. These results are comparable to those reported by Tavarini et al. (2009) and Tilahun et al. (2020), who showed that earlier harvested kiwifruits exhibited lower mass loss compared to later harvested fruit. These findings suggest that late harvested kiwifruits deteriorate quicker than earlier harvested fruit.

TSS is used as a maturity index and contributes to the flavour of kiwifruit (Meena et al., 2018). The 0.02% EFF-treated fruit exhibited significantly lower TSS than the control and the 0.01% treatment level. These results are in accordance with those reported by Jincy et al. (2017), who demonstrated that hexanal delayed the accumulation of TSS in mango fruit. As fruit ripen, TSS increases due to the conversion of polysaccharides into soluble sugars (Kaur et al., 2013). In addition, a rise in TSS can be attributed to moisture loss, which increases the concentration of soluble sugars (Nath et al., 2012). This corroborates the findings of the current study, which demonstrated that kiwifruit with the highest TSS (Figure 3.1 E and F) had the highest mass loss (Figure 3.1 C and D). Therefore, EFF can effectively delay TSS accumulation by lowering mass loss and the degradation of CWPs (Figure 3.2). Kiwifruits harvested at M2 exhibited approximately 21% higher TSS than fruit harvested at M1. Similar results were obtained by Tavarini et al. (2009) and Tilahun et al. (2020), who demonstrated that earlier harvested

kiwifruit had higher TSS than earlier harvested fruit. These findings suggest that the ripening rate of kiwifruit increases with an advancement in fruit maturity.

TA is linked to the concentration of organic acids in fruit and are important for the maintenance of fruit quality (Shiri et al., 2015). The treated kiwifruit exhibited significantly ($p < 0.05$) higher TA compared to the control fruit. TA for the control, 0.01 and 0.02% treatment level was registered at 0.146, 0.148 and 0.153%, respectively. Fruit harvested at M1 had approximately 7% higher TA than those harvested at M2. When fruit respire, organic acids are used as substrates in the respiratory cycle, which leads to a decrease in acidity (Kaur et al. 2013). Past research has illustrated that EFF treatment improves retention of TA by reducing the respiration rate (Yuan et al., 2009; Yumbya et al., 2018). Thus, we hypothesize that the retention of TA exhibited by treated kiwifruit may be attributed to the capacity of EFF to suppress respiration rates.



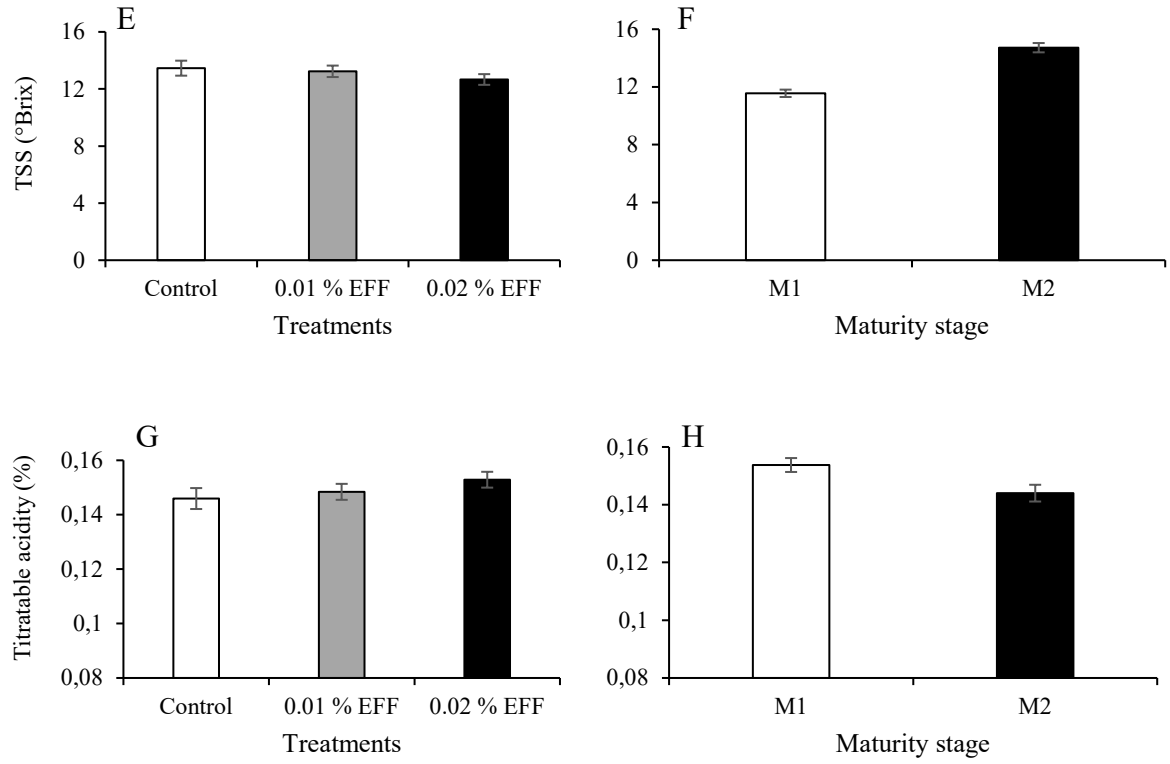


Figure 3.1. Effect of treatments (A) and the effect of interaction between maturity stage and storage time (B) on the firmness of kiwifruit. Effect of treatments (C) and maturity stage (D) on the mass loss of kiwifruit. Effect of treatments (E) and maturity stage (F) on the total soluble solids (TSS) of kiwifruit. Effect of treatments (G) and maturity stage (H) on the titratable acidity of kiwifruit. Values are the means ± SE (n = 3).

3.3.2. Cell wall polysaccharides (CWPs)

The interaction among treatment, maturity stage and storage time had no significant effect on PP (Figure 3.2). PP is a water insoluble form of pectic substances that contributes to the firmness and texture of fruits and degrades into WSP during fruit ripening (Zhang et al., 2019). PP was registered at 1.25, 1.36 and 1.44 mg/g FW for the control, 0.01 and 0.02% EFF treatment, respectively (Figure 3.2 A). These results are corroborated by those obtained by Gill et al. (2015), who demonstrated that EFF reduced the degradation of pectin in guava fruit. The PP of kiwifruit harvested at M1 and M2 was recorded at 1.45 and 1.24 mg/g FW, respectively (Figure 3.2 B). These findings are in alignment with those of reported by Tilahun et al. (2020), who demonstrated that earlier harvested kiwifruit registered a higher amount of pectin than later harvested ‘Hayward’, ‘Haegeum’ and ‘Hongyang’ kiwifruit cultivars, stored at 25 °C.

The interaction among treatment, maturity stage and storage time had no significant effect on WSP. The WSP content of the 0.01 and 0.02% treatment level was approximately 5 and 16% lower than the control, respectively (Figure 3.2 C). The WSP of kiwifruit harvested at M1 was approximately 25% lower in comparison to kiwifruit harvested at M2 (Figure 3.2 D). These results support the theory that the degradation of PP is characterized by an increase in WSP (Chen et al., 2017). Therefore, EFF can delay softening of kiwifruit by reducing the degradation of PP to WSP.

Cellulose is a structural polysaccharide of the cell wall that is involved in supporting and protecting the cell structure (Wang et al., 2021). Treatments had a significant ($p < 0.001$) influenced on the cellulose content. The 0.01 and 0.02% EFF-treated kiwifruit was 1.1 and 1.3-folds higher ($p < 0.001$) than the control, respectively (Figure 3.2 E). Additionally, the maturity stage significantly ($p < 0.001$) influenced the cellulose content. The cellulose content of kiwifruit harvested at M1 was 1.2-folds higher ($p < 0.001$) compared to M2 (Figure 3.2 F).

Hemicellulose is a heteropolysaccharide comprised of xyloglucan and xylose, which is connected with cellulose by hydrogen bond (Pos´e et al., 2019). The hemicellulose content of treated kiwifruit was approximately 15 and 20% greater ($p < 0.001$) for the 0.01 and 0.02% EFF treatment level, respectively (Figure 3.2 G). The hemicellulose content decreased as the storage period progressed. Kiwifruit harvested at M1 exhibited significantly ($p < 0.001$) greater hemicellulose compared to those harvested at M2 (Figure 3.2 H).

These findings indicate that EFF can effectively delay the softening of kiwifruit (Figure 3.1 A) by hindering the degradation of CWPs (Figure 3.2). The mechanism in which EFF delays the depolymerization of CWPs can be attributed to its ability to suppress the action of enzymes involved in the degradation of CWPs (Figure 3.3). This shows that EFF has the potential to impede the deterioration of kiwifruit attributed to rapid softening. Furthermore, the significantly lower CWPs exhibited by kiwifruit harvested at M2, suggests that the content of CWPs decreases with an advancement in maturity. This may explain why kiwifruit harvested at M2 were softer compared to M1 fruit (Figure 3.1 B). This observation demonstrates the impact of maturity stage on the firmness of kiwifruit, as softer fruit may be prone to deterioration during storage.

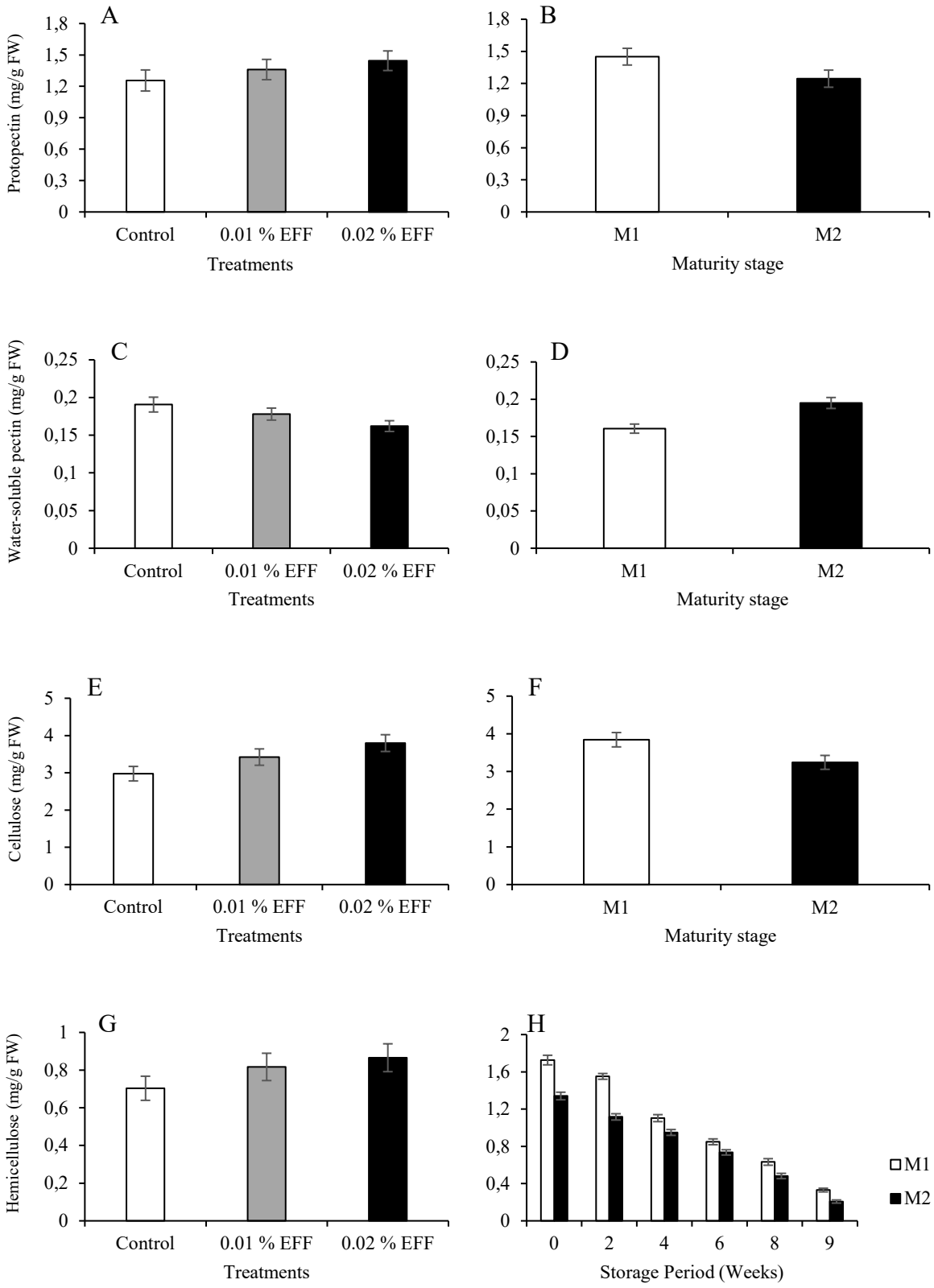


Figure 3.2. Effect of treatments (A) and maturity stage (B) on the protopectin content of kiwifruit. Effect of treatments (C) and maturity stage (D) on the water-soluble pectin content of kiwifruit. Effect of treatments (E) and maturity stage (F) on the cellulose content of kiwifruit.

Effect of treatments (G) and the effect of interaction between maturity stage and storage time (H) on the hemicellulose content of kiwifruit. Values are the means ± SE (n = 3).

3.3.3. Cell wall degrading enzymes (CWDEs)

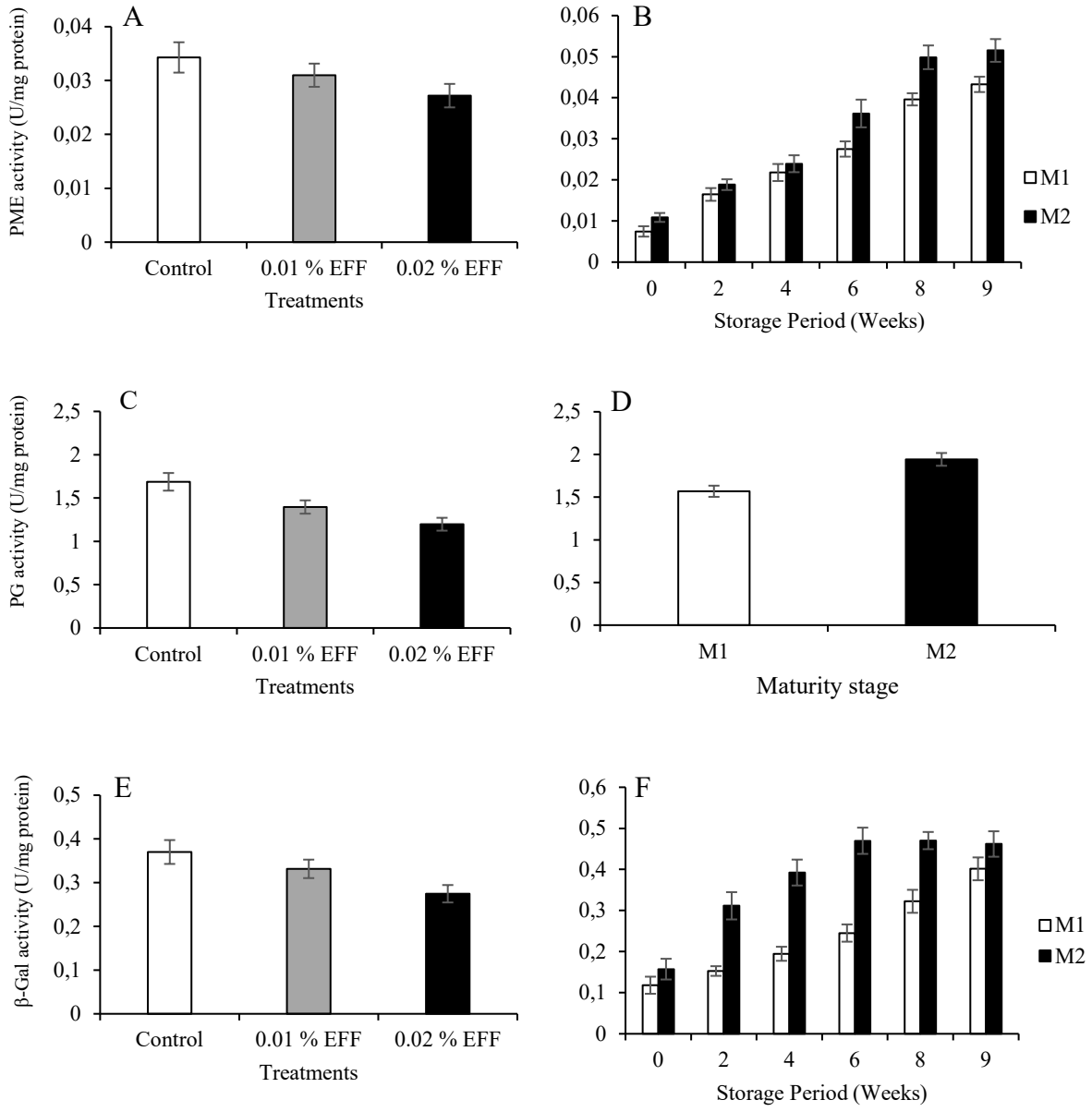
Treatments significantly ($p < 0.001$) influenced the PME activity, with the 0.01 and 0.02% EFF-treated kiwifruit registering approximately 12 and 21% lower activity than the control, respectively. These outcomes are in alignment with those reported by Gill et al. (2015), Kaur et al. (2019) and Sulaimankhil et al. (2021), who demonstrated that EFF suppressed the enzymatic activities of PME for guava, grapes and apples. Kiwifruit harvested at M2 exhibited significantly ($p < 0.05$) higher PME activity than M1 throughout the storage period.

The treatments had a significant ($p < 0.001$) influence on PG activity. The PG activity of the 0.01 and 0.02% EFF-treated kiwifruit was 10.82 and 19.59% lower compared to the control, respectively (Figure 3.3 C). These results are in accordance with those reported by Preethi et al. (2021), who showed that EFF suppressed PG enzyme activity in mango fruit. The maturity stage significantly ($p < 0.001$) influenced PG activity, showing a 19% increase in kiwifruit harvested at M2 compared to M1 (Figure 3.3 D). These findings are in alignment with those reported by Tavarini et al. (2009) and Tilahun et al. (2020), who demonstrated that later harvested kiwifruit exhibited higher PG activity than earlier harvested fruit.

β-Gal activity of the 0.01 and 0.02% EFF-treated kiwifruit was 1.1 and 1.4-folds lower ($p < 0.001$) than the control fruit, respectively (Figure 3.3 E). The enzyme activity of β-Gal increased as the storage duration progressed, with kiwifruit harvested at M2 exhibiting significantly ($p < 0.001$) higher β-Gal activity compared to M1 (Figure 3.3 F). EFF treatment significantly ($p < 0.001$) suppressed the Cx activity by approximately 7 and 13% for the 0.01 and 0.02% treatment level compared to the control (Figure 3.3 G). Furthermore, the Cx activity of kiwifruit harvested at M1 was approximately 6% lower than those of M2 (Figure 3.3 G).

CWDEs facilitate the degradation of CWPs, which results in fruit softening (Ramana-Rao et al., 2011). The mechanism in which EFF suppressed the action of CWDEs may be attributed to its capacity to down regulate the transcript levels of genes encoding PME, PG and β-Gal (Tiwari and Paliyath, 2011). Therefore, these results imply that EFF has the potential to optimise storability of gold kiwifruit by impeding rapid softening. Furthermore, the activity of CWDEs in kiwifruit harvested at M2 was significantly higher than M1. These findings show that the lower content of CWPs in kiwifruit harvested at M2 (Figure 3.2), may be attributed to

the higher activity of CWDEs. Which implies that later harvested kiwifruit had a higher softening rate than early harvested kiwifruit. This is consistent with results displayed in Figure 3.1 B, which show that kiwifruits harvested at M1 and M2 lost approximately 84 and 89% of their initial firmness, respectively.



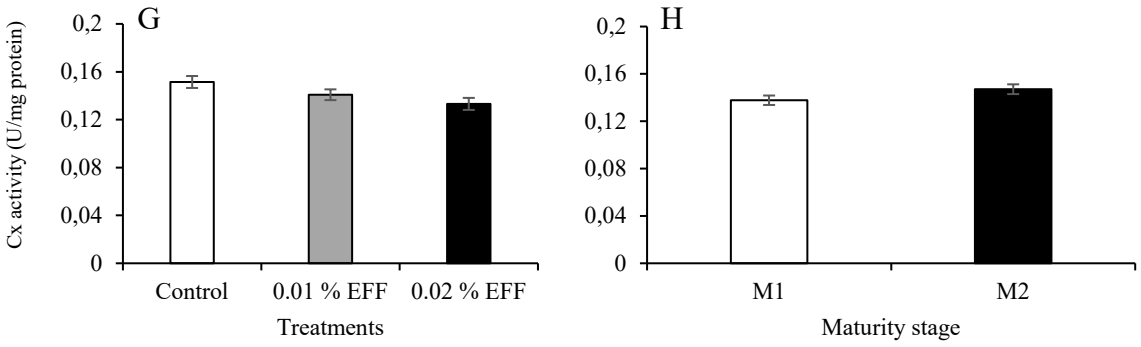


Figure 3.3. Effect of treatments (A) and the effect of interaction between maturity stage and storage time (B) on the PME activity of kiwifruit. Effect of treatments (C) and maturity stage (D) on the PG activity of kiwifruit. Effect of treatments (E) and the effect of interaction between maturity stage and storage time (F) on the β-Gal activity of kiwifruit. Effect of treatments (G) and maturity stage (H) on the Cx activity of kiwifruit. Values are the means ± SE (n = 3).

3.3.4. Pearson correlation coefficient and Principal component analysis

A correlation analysis was done to examine the relationships between the measured parameters (Figure 3.4). TSS was positively correlated with WSP ($r = 0.79$; $p < 0.001$), mass loss ($r = 0.67$; $p < 0.001$) and negatively correlated with firmness ($r = 0.68$; $p < 0.001$), PP ($r = 0.70$; $p < 0.001$), cellulose ($r = 0.76$; $p < 0.001$), hemicellulose ($r = 0.73$; $p < 0.001$) and TA ($r = 0.64$; $p < 0.001$). These findings support the theory that the increase in mass loss and the degradation of CWPs results in the accumulation of TSS (Zhang et al., 2019; Wang et al., 2021; Li et al., 2023; and Shinga et al., 2023). The activity of CWDEs was negatively ($p < 0.001$) correlated with firmness and CWPs. Therefore, it can be deduced that the softening of kiwifruit can be delayed by suppressing the activity of CWDEs, involved in the degradation of CWPs. Which in turn leads to optimised quality and storability of kiwifruit during storage.

Principal component analysis (PCA) was performed to distinguish which parameters were more dominant in categorizing the fruit from the treatments/maturities (Figure 3.5). PCA was produced for the first two dimensions where Dim1 and Dim2 accounted for 91.2% and 6.9% of the total variance, respectively. Factor loadings are categorized as strong, moderate, and weak based on their corresponding values, with >0.75 indicating strong, 0.75 – 0.50 indicating moderate, and 0.50 – 0.30 indicating weak loadings (Liu et al., 2003). In addition, factor loadings represent the correlation between the principle component and the original variable (Mooi et al., 2017). Dim1 exhibited positive correlations for PME (0.98), PG (1.00), β-Gal

(0.96), Cx (0.96), WSP (0.99), TSS (0.90) and mass loss (0.89) and negative correlations for PP (-1.00), Cellulose (-0.99), HC (-0.95), firmness (-1.00) and TA (-0.85) (Table 3.2).

The results demonstrate notable differences between control and treated kiwifruits across various parameters, suggesting the effectiveness of EFF treatment in mitigating softening and enzyme activity. Control fruit harvested at M1 and M2 exhibited higher mass loss and enzyme activity, indicating greater deterioration compared to treated fruits. Specifically, kiwifruits treated with EFF showed enhanced cellulose and hemicellulose content, particularly when treated with 0.02% EFF at M2, which also exhibited higher firmness, polyphenol content (PP), and titratable acidity (TA). Conversely, kiwifruits treated with 0.01 % EFF at M2 displayed elevated total soluble solids (TSS), water-soluble pectin (WSP), and β-Galactosidase enzyme activity. The findings suggest that while 0.02 % EFF concentration effectively preserved firmness at both maturity stages, 0.01 % concentration was insufficient for later-harvested fruits. Fruit harvested at M2 experienced rapid softening, which EFF treatment successfully alleviated by suppressing cell wall degrading enzyme (CWDE) activities involved in cell wall protein (CWP) degradation. This shows the potential of EFF to serve as a postharvest strategy to impede rapid softening of gold kiwifruit harvested at both the mid and late maturity stage. The current study primarily focused on the inhibitory effects of EFF on the action of CWDEs. Considering that CWDEs are activated by ethylene production (Bapat et al., 2010), further research can seek to clarify whether the suppression of CWDEs in kiwifruit is due to the inhibition of ethylene production in response to EFF treatment.

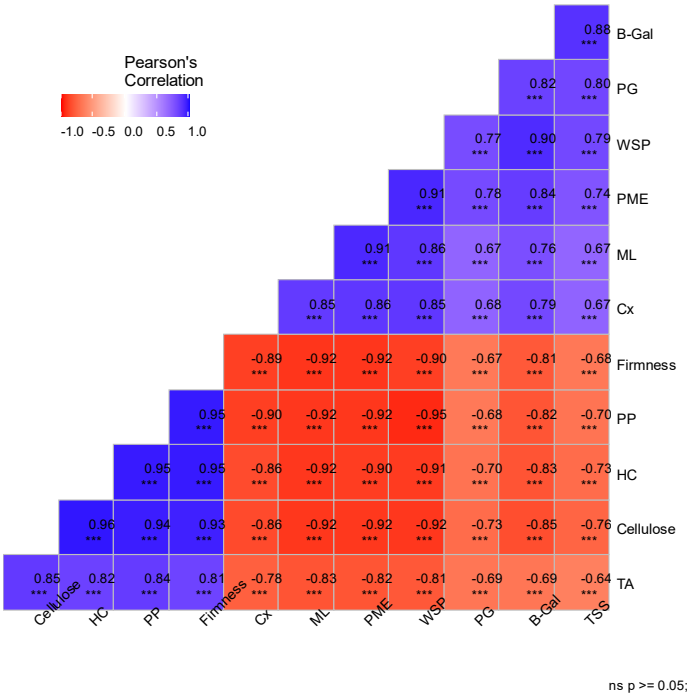


Figure 3.4. Pearson correlation matrix between pectin methylesterase (PME), polygalacturonase (PG), β-galactosidase (β-Gal) and cellulase (Cx), protopectin (PP), water soluble pectin (WSP), cellulose, hemicellulose (HC), firmness, total soluble solids (TSS), titratable acidity (TA) and mass loss (ML). The correlation coefficients are proportional to the colour intensity. Positive correlation is displayed in blue and negative correlation is displayed in red.

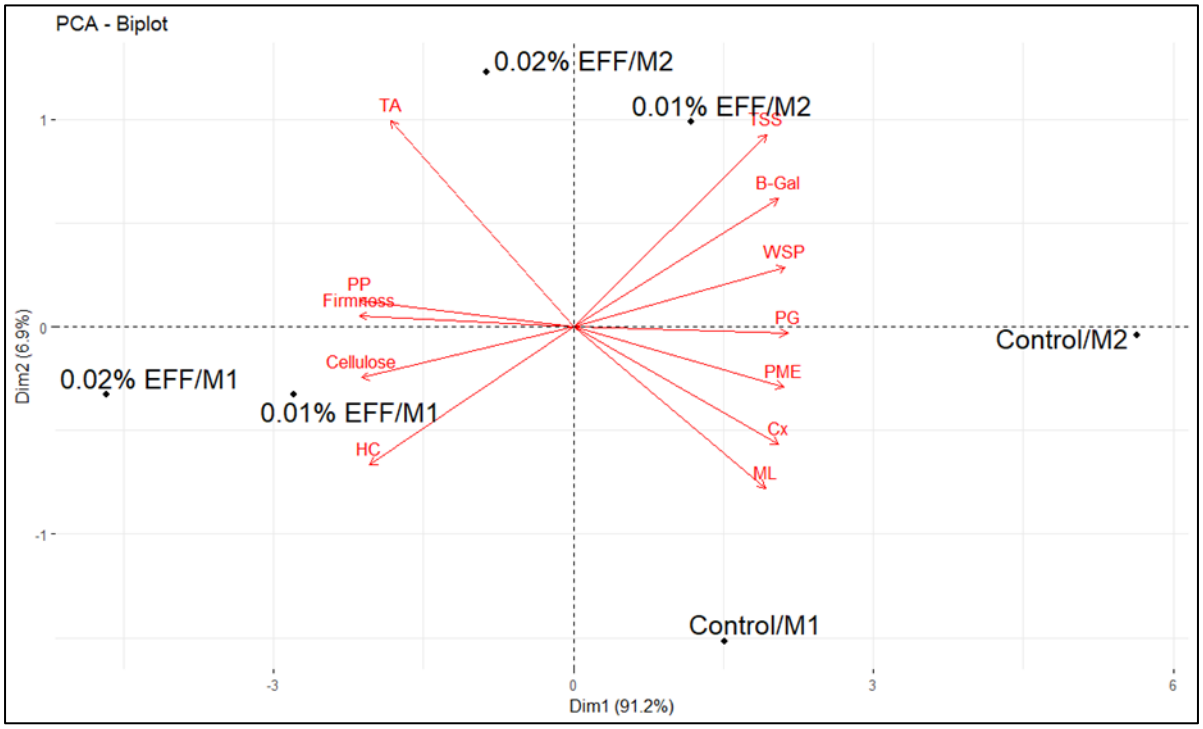


Figure 3.5. Principal component analysis biplot showing the correlations between the measured variables and factors (treatment and maturity stage). Dimension one (Dim1), Dimension two (Dim2), Pectin methylesterase (PME), polygalacturonase (PG), β-galactosidase (β-Gal), cellulase (Cx), protopectin (PP), water-soluble pectin (WSP), cellulose, hemicellulose (HC), firmness, total soluble solids (TSS), titratable acidity (TA) and mass loss (ML).

Table 3.1: Factor scores, loadings, eigenvalues and variance (%) for the first two dimensions (Dim1 and Dim2) based on cell wall polysaccharides, cell wall degrading enzymes and quality parameters of EFF-treated 'Y368' kiwifruit.

	Factor scores	
Observation	**Dim1**	**Dim2**
Control/M1	1.51	-1.52
0.01% EFF/M1	-2.79	-0.33
0.02% EFF/M1	-4.66	-0.33
Control/M2	5.63	-0.04
0.01% EFF/M2	1.18	0.99

0.02% EFF/M2	-0.87	1.23
Loadings		
PME	0.98	-0.13
PG	1.00	-0.02
β-Gal	0.96	0.29
Cx	0.96	-0.26
PP	-1.00	0.06
WSP	0.99	0.13
Cellulose	-0.99	-0.11
HC	-0.95	-0.31
Firmness	-1.00	0.03
TSS	0.90	0.43
TA	-0.85	0.46
ML	0.89	-0.36
Eigenvalue	10.95	0.83
Variance (%)	91.23%	6.95%
Cumulative (%)	91.23%	98.18%

Dimension one (Dim1), Dimension two (Dim2).

3.4. Conclusion

The outcomes of the study showed that EFF effectively delayed softening of kiwifruit by suppressing the activities of CWDEs and degradation of CWPs. Furthermore, the results revealed that a higher dose EFF treatment is required to suppress the higher softening rates exhibited by later harvested kiwifruits. The adoption of this treatment has the potential to alleviate postharvest and economic losses incurred in the kiwifruit industry, by reducing fruit deterioration caused by rapid softening and mass loss. Fruit softening is triggered by ethylene production, which regulates the activities of CWDEs. Therefore, future research is needed to investigate the effect of EFF on the ethylene biosynthesis, in order to gain more understanding on the mechanism of EFF in delaying softening of gold kiwifruit.

3.5. References

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Chapter Four

Enhanced freshness formulation preserves membrane integrity of kiwifruit by regulating reactive oxygen species

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Abstract

Membrane deterioration compromises quality and storability of kiwifruit. Oxidative damage caused by reactive oxygen species (ROS) such as superoxide radical and hydrogen peroxide and the enzymatic action of Phospholipase D (PLD), induces membrane deterioration and senescence of fruits. Membrane deterioration can be alleviated by enhancing the activities of antioxidant enzymes which scavenge ROS. Therefore, the aim of this study was to evaluate the efficacy of enhanced freshness formulation (EFF), a hexanal-based formulation containing antioxidants such as geraniol, α-tocopherol and ascorbic acid, to enhance the ROS scavenging potential of 'Y368' kiwifruit harvested at the mid and late maturity stages (7 °Brix and 9 °Brix). Kiwifruit were treated with three treatments, namely, control (untreated fruit), 0.01 and 0.02% (v/v) EFF. The fruit was thereafter stored for 8 weeks at 0 ºC and 90% relative humidity, then transferred to 20 °C for one week. The effect of EFF on the accumulation of ROS, membrane deterioration biomarkers (malondialdehyde and electrolyte leakage), PLD activity and activities of antioxidant enzymes were evaluated. The results demonstrated that EFF-treated kiwifruit exhibited significantly lower MDA, EL, ROS and PLD enzyme activity compared to the control. Furthermore, EFF-treated kiwifruit had substantially higher activities of antioxidant enzymes. The findings indicate that EFF delayed membrane deterioration of kiwifruit by decreasing ROS production, PLD activity and enhancing the activities of antioxidant enzymes. Further investigation on transcriptome analysis is needed to elucidate the mechanism in which EFF regulates antioxidant enzymes.

Key words: *Actinidia chinensis,* membrane integrity, oxidative stress, antioxidant enzymes, fruit maturity

4.1. Introduction

Kiwifruit have a climacteric ripening pattern and experience rapid deterioration and senescence during storage. Rapid deterioration and senescence of kiwifruit is characterised by mass loss and softening, which are limiting factors that compromise it's storage potential and marketability (Wang et al., 2020a; Cao et al., 2021). Accelerated fruit senescence and deterioration is attributed to the excess production of reactive oxygen species (ROS) such as

superoxide radical anion ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) (Goffi et al., 2019; Yan et al., 2023). High levels of ROS are detrimental to fruits as they cause lipid peroxidation, leading to the damage of cell membranes. Phospholipase D (PLD) is a phospholipid-degrading enzyme that initiates membrane degradation by hydrolysing membrane phospholipids to produce phosphatidic acid (Paliyath and Subramanian, 2008). This results in cell death through elevated levels of ROS (Park et al., 2004). Suppressing the production of ROS and PLD activity can reduce membrane degradation and delay the senescence of fruits (Tiwari and Paliyath, 2011).

The kiwifruit industry relies on cold storage to reduce deterioration and prolong the storage life (Goffi et al., 2019). However, abiotic stress conditions such as low temperatures can lead to the excessive production of ROS, resulting in oxidative damage (Hasanuzzaman et al., 2020). Research studies have shown that cold storage induces excessive production of ROS, which accelerates oxidative damage, senescence and softening in kiwifruit (Li et al., 2021; Liu et al., 2023; Wang et al., 2023). This challenge necessitates the need to develop strategies than can reduce oxidative stress and delay the senescence of kiwifruit during cold storage. The harvest maturity is another critical factor that has a significant effect on ROS activities of fresh horticultural produce. Studies by Wang et al. (2020b) demonstrated that harvest maturity significantly influences the production of ROS 'Hongyang' in kiwifruits. The authors established that kiwifruits harvested at the mid maturity stage exhibited less oxidative damage than late maturity kiwifruits, recommending mid maturity harvest for optimum quality and storability.

Plants can counteract oxidative stress via enzymatic antioxidant scavenging systems (Kaur et al., 2019). Enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), guaiacol peroxidase (POD) and glutathione reductase (GR) have the capacity to detoxify ROS and neutralise their deleterious effects (Wang et al., 2019). Therefore, maintaining an efficient ROS scavenging system is crucial in delaying membrane deterioration and senescence in plants (Hasanuzzaman et al., 2020). Enhanced freshness formulation (EFF) is a hexanal-based formulation that comprises of antioxidants such as geraniol, α-tocopherol and ascorbic acid (Paliyath and Murr, 2007 or Mthembu et al., 2023). EFF and hexanal can preserve membrane integrity by enhancing the ROS scavenging potential of fruits (Sharma et al., 2010; Qi et al., 2011; Jincy et al., 2017 and Cheema et al., 2018). Furthermore, hexanal can inhibit PLD activity by blocking the hydrolysis at the active site of PLD, resulting in enhanced cell membrane integrity (Paliyath et al., 1999; Jincy et al., 2017).

Therefore, EFF treatment has the potential to reduce membrane deterioration and optimise the storability of kiwifruit.

The export-orientated South African kiwifruit industry harvests at mid and late maturity stage (Mahlaba et al., 2021). As earlier indicated, cold storage and harvest maturity are two factors that strongly influence the induction of ROS and deterioration of kiwifruit. A postharvest treatment that can effectively reduce membrane deterioration of kiwifruits harvested at different maturity stages needs to be explored. In our extensive review of the literature, we have not found studies evaluating the efficacy of postharvest treatments in reducing oxidative damage in kiwifruit harvested at different maturity stages. Thus, the aim of this study was to investigate the efficacy of EFF in preserving the membrane integrity of South African grown gold kiwifruits harvested at two maturity stages, by reducing oxidative damage attributable to ROS.

4.2. Methods and materials

4.2.1. Fruit source

Kiwifruit (*Actinidia chinensis*) cv. 'Y368' were harvested from Roselands farm, a commercial kiwifruit farm located in the Richmond area (Latitude: 29.9033°S, Longitude: 30.2397°E), KwaZulu-Natal Province, South Africa. The fruit were harvested when the total soluble solid content was greater than 6.2 °Brix (Mahlaba et al., 2021), at two maturity stages namely, maturity stage one (M1) and maturity stage two (M2) with 7 and 9 °Brix, respectively. Harvested fruit were immediately transported in a ventilated vehicle to the Postharvest Laboratory of the University of KwaZulu-Natal, where kiwifruit without blemishes, decay or physical damage were graded and selected for uniformity in size, then assigned to the respective postharvest treatments. All chemicals used to conduct the experiments in this study were purchased from Sigma-Aldrich, South Africa.

4.2.2. Preparation of postharvest treatments and storage

The enhanced freshness formulation was prepared by making a stock formulation comprising of 1% (v/v) hexanal, 1% (v/v) geraniol, 1% (w/v) α-tocopherol, 1% (w/v) ascorbic acid, 0.1% (w/v) cinnamic acid and 10% (v/v) Tween 20 dissolved in ethanol (10% v/v) (Paliyath and Murr, 2007). A 0.01 and 0.02% (v/v) hexanal concentration was prepared by mixing the stock solution in 100 L and 50 L of distilled water, respectively. Kiwifruit were immersed in 0.01 and 0.02% (v/v) EFF solution for 2.5 min, then air dried at room temperature. Control fruit

were left untreated. Each treatment consisted of three replicates (n = 3); each replicate had three fruit per storage interval for each maturity stage. The kiwifruits were stored for 8 weeks in a cold room with temperature set at 0 °C and relative humidity at 90%, then transferred to 20 °C for one week to stimulate shelf life.

4.2.3. Membrane permeability

Membrane permeability was determined using a method described by Song et al. (2009). Cylinders of kiwifruit tissue were excised with a 10 mm diameter stainless steel cork borer from the equatorial region of the fruit and incubated in 30 mL of deionized water at 25 °C, followed by shaking for 30 min. Electrolyte leakage of the solution was measured using a Thermo Scientific Orion conductivity meter (Waltham, MA USA). The solution was then heated to 100 °C for 15 min and quickly cooled. The total electrolytes of the solution were then measured again. Relative leakage was expressed as a percentage of the total electrolyte leakage using Eq. 1

$$EC = \frac{\Sigma \mathrm{ECF} - \mathrm{ECI}}{n} \qquad 1$$

where EC = electrical conductivity; EC_I = initial reading; EC_F = final reading; n = number of samples.

4.2.4. Malondialdehyde (MDA)

MDA was determined using a method described by Song et al. (2009). Kiwifruit pulp (1 g) was homogenised with 5 mL of 10% trichloroacetic acid and then centrifuged for 10 min at 5000 x *g*. One millilitre of the supernatant was mixed with 3 mL of 0.5% thiobarbituric acid (TBA) dissolved previously in 10% trichloroacetic acid. The reaction mixture was heated for 20 min at 95 °C, cooled, and centrifuged for 10 min at 10 000 x *g*. Absorbance at 532 nm was measured and subtracted from the non-specific absorbance at 600 nm. MDA was calculated using an extinction coefficient of 155 m/M/cm, using Eq. 2 and expressed as nmol·g^{-1} of fresh weight (FW).

$$\mathrm{MDA} = (\mathrm{OD532} - \mathrm{OD600}) \times \mathrm{Vt} \times \mathrm{Vr} \times 1000/(\mathrm{Vs} \times \mathrm{m} \times 155) \qquad 2$$

where Vt, Vr and Vs were the total volume of the extract solution, the total volume of the reaction mixture solution and the volume of the extract solution contained in the reaction mixture solution, and m was the mass of samples.

4.2.5. Superoxide Anion (O2.-) content

The production rate of superoxide radical ($O_2^{\cdot-}$) was analysed using a method described by Singh et al. (2015). One gram of kiwifruit tissue was homogenized with 8 mL of 65 mM phosphate buffer (pH 7.8) containing 1mM ethylenediaminetetraacetic acid (EDTA), 1% polyvinylpolypyrrolidone (PVPP), and 0.3% Triton X-100. The homogenate was centrifuged at 5000 × *g* for 15 min at 4 °C. After centrifugation, 0.5 mL of the supernatant was added to 1 mL of 50 mM phosphate buffer (pH 7.8) and 0.5 mL of 10 mM hydroxylamine hydrochloride and incubated at 25 °C for 20 min in the dark. A 1 mL sample of the above reaction mixture was added to 1 mL of 19 mM p-aminobenzene sulfonic acid and 1 mL of 7 mM α-naphthylamine to formulate a new mixture, which was then incubated at 25 °C for 20 min in the dark. The $O_2^{\cdot-}$ content was calculated using sodium nitrite as the standard at 530 nm. The production rate was expressed as μmol NO_2 kg^{-1} s^{-1} FW.

4.2.6. Hydrogen peroxide (H_2O_2) content

Hydrogen peroxide content was determined using a method described by Velikova et al. (2000). One gram of kiwifruit tissue was homogenised in 5 mL of chilled 0.1% (v/v) trichloroacetic acid solution. The homogenate was centrifuged at 12 000 x *g* at 4 °C for 15 min and the supernatant was used to quantify H_2O_2. Thereafter, 0.5 mL extract was added to 0.5 mL of 10 mM potassium phosphate buffer (pH 7.0) and 1 mL of 1 M KI. The reaction was developed for 1h in darkness and absorbance was measured at 390 nm. Hydrogen peroxide was quantified using H_2O_2 as the standard and the results were expressed as μmol/g FW.

4.2.7. Phospholipase D (PLD EC 3.1.4.4.) assay

PLD assay kit (MAK 137, Sigma Aldrich, St. Louis, MO, USA) was used for the enzyme activity quantification. The enzyme was extracted using 0.2 M phosphate buffer (pH 7.0). The homogenate was centrifuged at 20 000 x *g* for 10 min. The assay was done in microtiter plate. To each well, 10 µL of sample and 90 µL of master reaction mix (assay buffer, enzyme mix, dye reagent and substrate) was added. The reaction mixture was incubated at 37 °C in the dark for 10 min. Thereafter, the initial absorbance was measured at 570 nm using a GloMax Discover microplate reader (Promega, Madison, Wisconsin, USA). The reaction mixture was incubated for an additional 20 min and the final absorbance was measured. PLD enzyme activity was determined using choline as the standard and expressed as mol g^{-1} FW.

4.2.8. Extraction procedure of antioxidant enzymes

One gram of kiwifruit tissue was homogenized with 2.3 mL of 100 mM potassium phosphate buffer (pH 7.0) containing 1.0 mM EDTA, 5% polyvinyl polypyrrolidone, and 1% Triton X-100. The homogenate was centrifuged at 13 000 × *g* for 20 min at 4 °C. The supernatant was used for the following antioxidant enzyme assays. The absorbance of the enzyme activities was recorded using a Jasco V-730 UV spectrophotometer and quantified using Beer Lambert's law:

$$A = \varepsilon \mathfrak{b} C \qquad 3$$

Where, A = absorbance, ε = molar absorptivity, b = path length, C = concentration.

4.2.9. Enzyme assays

4.2.9.1. Superoxide Dismutase (SOD, EC. 1.15.1.1)

The SOD assay kit (19160 SOD, Sigma Aldrich, St. Louis) was used for the enzyme assay. Twenty microliters of enzyme extract were plated in a 96-well microtiter plate. Then, 200 µL of WST solution ((2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium), monosodium salt and 20 µL of xanthine oxidase enzyme were added and incubated for 20 min at 37 °C. The absorbance was measured at 450 nm using a GloMax Discover microplate reader (Promega, Madison, Wisconsin, USA). The SOD inhibition rate was determined and expressed as enzyme U g^{-1} on fresh weight basis. Sample well with buffer was used as sample blank control to correct colour absorbance of samples.

4.2.9.2. Catalase (CAT) (EC. 1.11.1.6)

CAT enzyme activity was determined according to the method of Tian and Lei. (2006). The reaction mixture contained 1.5 mL 100 mM phosphate buffer (pH 7.0), 1.2 mL of 150 mM H_2O_2, and 0.3 mL of the enzyme extract. The decrease in absorbance at 240 nm due to the decomposition of H_2O_2 was recorded for 3 min at 240 nm. One unit of enzyme activity was defined as the amount that caused a change of 0.01 in absorbance per minute. Enzyme activity was expressed as U/g^{-1} FW using 6.93 x 10^{-3} mM^{-1} cm^{-1} as the extinction coefficient.

4.2.9.3. Ascorbate Peroxidase (APX) (EC. 1.11.1.11)

APX was assayed according to the method of Imahori et al. (2008). A total of 3 mL of the reaction mixture contained 50 mM Hepes-KOH (pH 7.6), 0.1 mM EDTA, 0.3 mL of 0.5 mM ascorbate, 0.6 mL of 1 mM H_2O_2 and 0.3 mL of the enzyme extract. The decrease in absorbance at 290 nm due to ascorbate oxidation was automatically recorded for 3 min. One unit of enzyme activity was defined as the amount that caused a change of 0.01 in absorbance per minute. The enzyme activity was expressed as U g^{-1} FW, using 2.8 mM^{-1} cm^{-1} as an extinction coefficient.

4.2.9.4. Guaiacol Peroxidase (POD) (EC. 1.11.1.7)

POD activity was assayed using the method described by Sripong et al. (2019). The reaction mixture comprised of 100 µL enzyme extract, 2.82 mL of 0.1 M sodium phosphate buffer (pH 7.0), 30 µL of 12.3 mM of H_2O_2 and 50 µL of 20 mM guaiacol. The increase in absorbance at 436 nm due to the oxidation of guaiacol was recorded for 2 min. One unit of enzymatic activity was defined as the amount of the enzyme that caused a change of 0.001 in absorbance per minute. The enzyme activity was expressed as U g^{-1} FW, using 25 mM^{-1} cm^{-1} as the extinction coefficient.

4.2.9.5. Glutathione Reductase (GR) (EC. 1.6.4.2)

The GR enzyme activity was assayed using a method described by Bhardwaj et al. (2022). The reaction contained 1.6 mL of 100 mM phosphate buffer (pH 7.8) containing 2 mM EDTA, 0.2 mM NADPH, 0.5 mM GSSG, and 0.4 mL of the enzyme extract. GR activity was recorded for 1 min at 340 nm and determined based on the oxidation of NADPH (extinction coefficient 6.22 mM^{-1} cm^{-1}), the results were expressed as U g^{-1} FW.

4.2.10. Statistical analysis

All experiments were performed using a completely randomized factorial design. The experiment comprised of three factors: treatments, fruit maturity and storage period. Statistical analysis was performed in R software version 4.3.1 (R Development Core Team, 2023). Data were expressed as mean values ± SE and were subjected to analysis of variance (ANOVA) with a 5% level of significance.

4.3. Results and discussion

4.3.1. Superoxide radical and hydrogen peroxide

During senescence and stress conditions in plants, a rise in the production of ROS such as $O_2^{-\bullet}$ and H_2O_2 occurs in cell compartments (Foyer and Noctor, 2005). The excessive production of ROS in fruits causes membrane lipid peroxidation which accelerates cell senescence and disintegration (Li et al., 2021). The EFF treatment significantly ($p < 0.01$) reduced the production of $O_2^{-\bullet}$ (Figure 4.1 A) throughout the storage period, with a pronounced effect at the 0.02% treatment level. These results are in accordance with those reported by Yuan et al. (2009) and Jincy et al. (2017), who showed that hexanal reduced the production of $O_2^{-\bullet}$ in strawberry and mango fruit, respectively. Kiwifruits harvested at M2 had approximately 11% higher ($p < 0.001$) $O_2^{-\bullet}$ content compared to those of M1 (Figure 4.1 B). These findings

corroborate those reported by Wang et al. (2020b) who demonstrated that late harvested kiwifruits exhibited significantly higher $O_2^{-\bullet}$ production.

EFF significantly ($p < 0.001$) reduced the production of H_2O_2, with a pronounced effect at the 0.02% treatment level (Figure 4.1 C). The H_2O_2 content of EFF-treated fruit was approximately 1.1 and 1.2-folds lower than the control. These results are similar to those reported by Jincy et al. (2017), who showed that hexanal lowered the production of H_2O_2 in mango fruit. Additionally, the H_2O_2 content of kiwifruit harvested at M2 was 1.2-folds ($p < 0.001$) greater than those of M1 (Figure 4.1 D), which is in accordance with findings obtained by Wang et al. (2020b). These findings indicate that EFF treatment can delay senescence and deterioration of kiwifruits during cold storage by reducing the production of ROS.

4.3.2. Malondialdehyde (MDA) and Electrolyte leakage (EL)

MDA serves as a biomarker for lipid peroxidation and cell membrane integrity (Li et al., 2021). The excessive production of ROS causes lipid peroxidation, resulting in the accumulation of MDA (Tian et al.,2013). The EFF treatments significantly ($p < 0.01$) lowered the accumulation of MDA throughout the storage period (Figure 4.1 E). These findings are in alignment with those reported by Jincy et al. (2017). The capacity of EFF to reduce the accumulation of MDA can be attributed to lower ROS levels in treated kiwifruits (Figure 4.1 A). These results suggest that EFF has the capacity to preserve the integrity of the cell membrane by reducing oxidative damage. Additionally, the MDA content of kiwifruits harvested at M2 was approximately 10% higher ($p < 0.001$) compared to kiwifruits harvested at M1 (Figure 4.1 F). These findings are comparable to those reported by Wang et al. (2020), who showed that late harvested kiwifruits exhibited significantly higher lipid peroxidation.

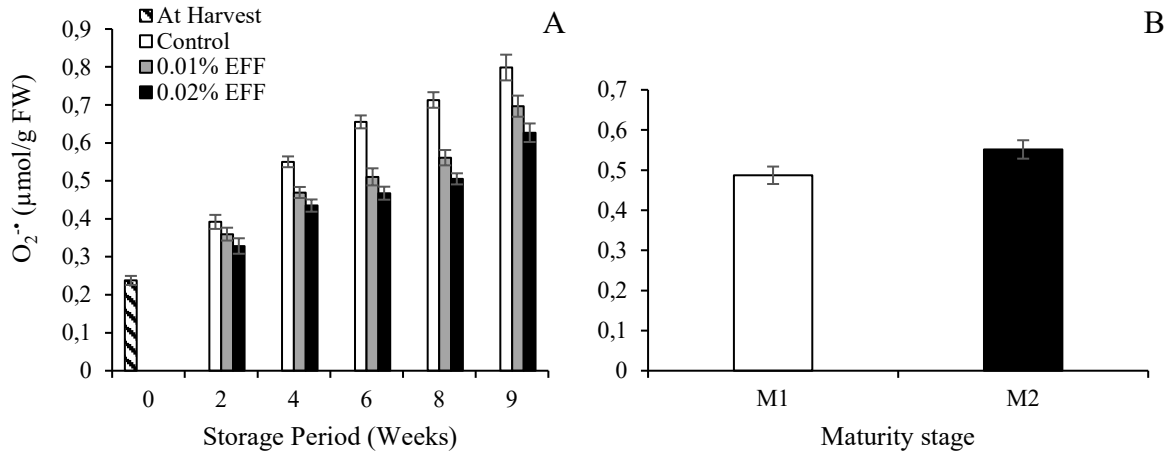
EL is an indicator of cell membrane permeability and deterioration (Hershkovitz et al., 2005; Feng et al., 2005). EFF-treated kiwifruits had significantly lower EL compared to the control fruit (Figure 4.2 A). The EL of treated kiwifruits was 1.1 and 1.2-folds lower ($p < 0.001$) than the control fruit. These results are similar to those obtained by Cheema et al. (2018), who demonstrated that hexanal treated bell peppers exhibited lower EL. Cheema et al. (2018) stated that a rise in EL is linked with the loss of cell membrane integrity. Therefore, the findings of the current study indicate that EFF can delay the senescence and optimise storability of kiwifruit by reducing membrane deterioration. The maturity stage had a significant ($p < 0.001$) influence on EL, with kiwifruit harvested at registering 1.3-folds greater EL compared to those

of M1 (Figure 4.2 B). These results are in accordance with those reported by Wang et al. (2020b).

4.3.3. Phospholipase D activity (PLD)

PLD is a membrane lipid degrading enzyme that initiates membrane deterioration, leading to the senescence of fruit (Paliyath and Subramanian, 2008). It hydrolyses phospholipids to produce phosphatidic acid (PA), resulting in the production of ROS (Sang et al., 2001). Thus, the inhibition of PLD enzyme activity can limit oxidative damage by reducing the generation of ROS. Treated fruit had substantially ($p < 0.001$) lower PLD activity compared to the control fruit (Figure 4.2 C). The PLD enzyme activity for the control, 0.01 and 0.02 % treatment level were registered at 0.49, 0.42 and 0.35 U/g FW, respectively. These results are in accordance with those reported by Tiwari and Paliyath (2011) and Jincy et al. (2017), who demonstrated the efficacy of EFF in suppressing PLD activity in tomato and mango fruit.

Studies by Pak Dek et al. (2018) and Padmanabhan et al. (2020) demonstrated that exogenous application of hexanal downregulated the expression of genes encoding PLD in tomato fruit and bell peppers. Therefore, we hypothesize that EFF suppressed the PLD activity in treated kiwifruit by downregulating the gene expression of PLD. The results obtained in this study suggest that EFF treatment reduced membrane deterioration by suppressing PLD enzyme activity. The PLD activity for fruit harvested at M2 was 11 % higher than the enzyme activity for fruit harvested at M1 (Figure 4.2 D). This indicates that the greater membrane deterioration exhibited by late harvested kiwifruit is attributed to a greater generation of PA, due to higher PLD activity, resulting in greater oxidative damage.



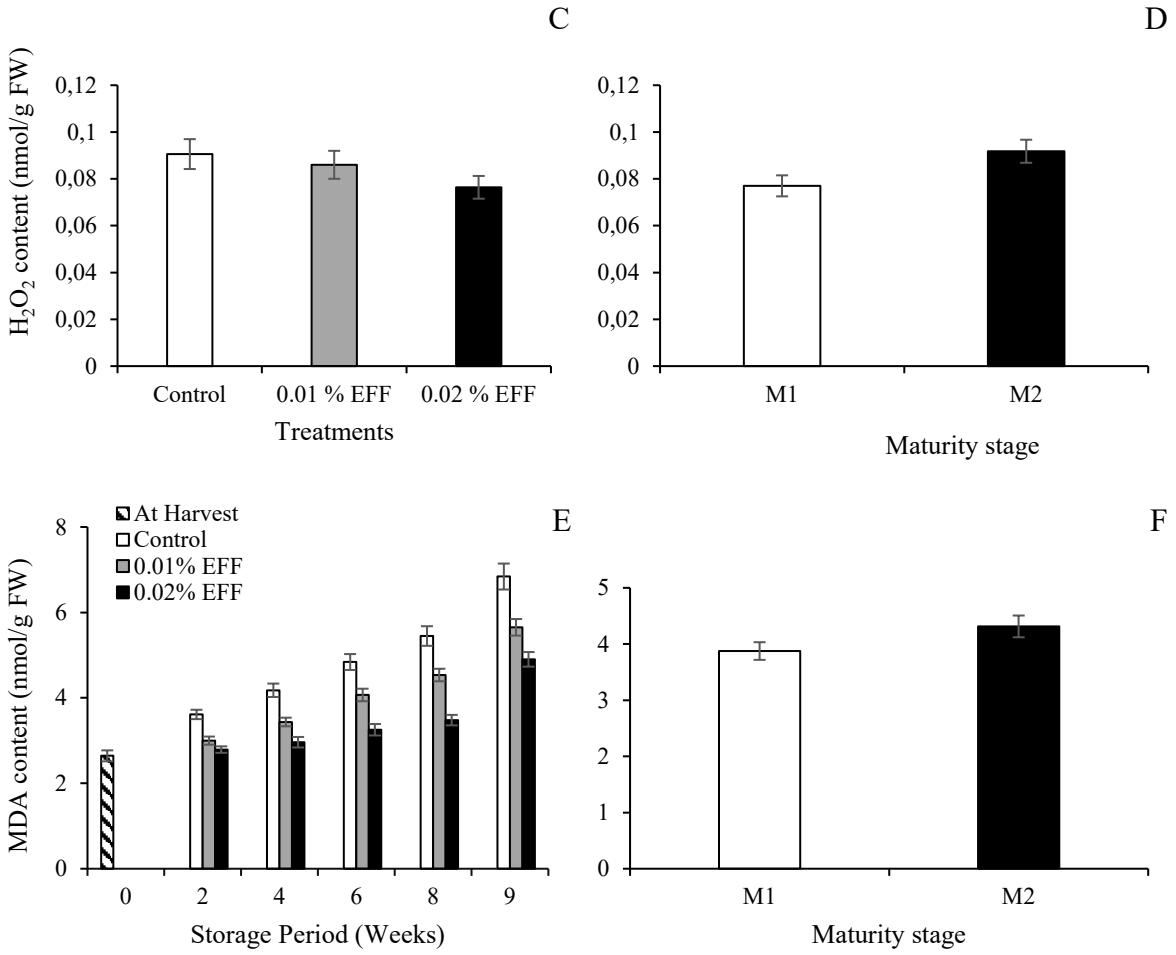
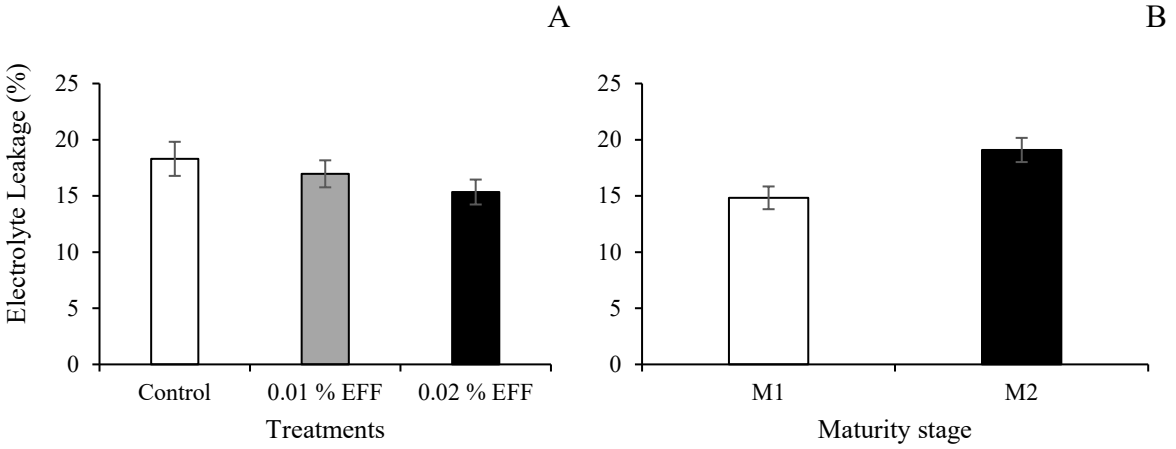


Figure 4.1: The effect of interaction between treatments and storage time (A) and maturity stage (B) on the $O_2^{-\bullet}$ content of kiwifruit. The effect of treatments (C) and maturity stage (D) on H_2O_2 content of kiwifruit. The effect of interaction between treatments and storage time (E) and maturity stage (F) on the malondialdehyde (MDA) content of kiwifruit. Values are the means ± SE (n = 3).



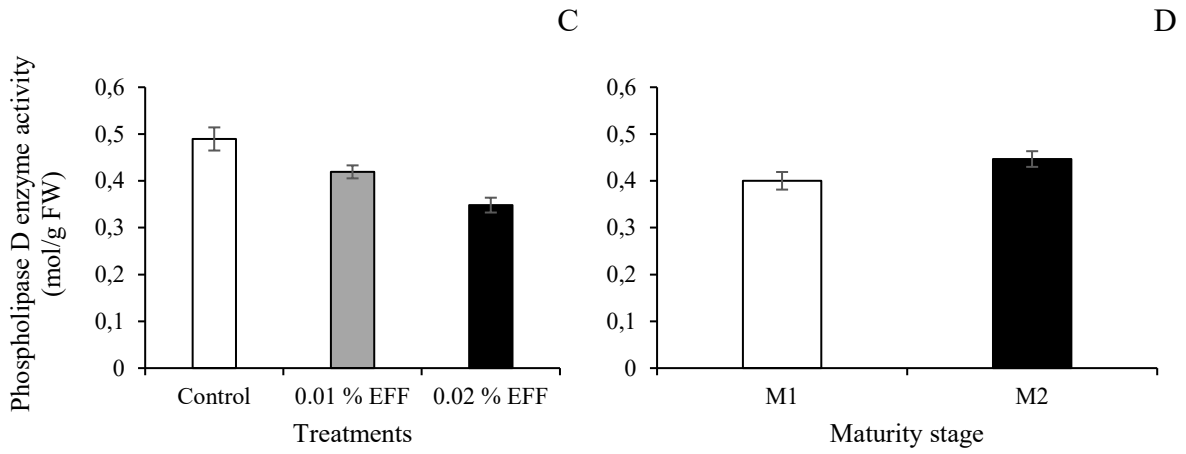


Figure 4.2: The effect of treatments (A) and maturity stage (B) on electrolyte leakage (EL) of kiwifruit. The effect of treatments (C) and maturity stage (D) on Phospholipase D (PLD) activity of kiwifruit. Values are the means ± SE (n = 3).

4.3.4. Antioxidant enzyme activities

An efficient antioxidant system has the ability to delay senescence and reduce oxidative damage of cell constituents by scavenging ROS. Within the cell, SOD establishes the first line of defence by catalysing the dismutation reaction of superoxide anion radical (O_2^-) to oxygen (O_2) and H_2O_2. CAT and POD catalyse the decomposition of H_2O_2 to generate H_2O and O_2. APX catalyses the decomposition of H_2O_2. GR is an NADPH-dependent enzyme that plays an important role in cell metabolism by facilitating the maintenance of a high ratio of GSH/GSSG by catalysing the reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH) (Beyer and Fridovich, 1987).

EFF significantly ($p < 0.001$) enhanced the SOD activity with a pronounced effect at the 0.02% EFF treatment level (Figure 4.3 A). The CAT activity of all treatments increased up to six weeks of storage, then gradually decreased (Figure 4.3 C). Throughout the storage period, the CAT activity of treated kiwifruits remained significantly ($p < 0.01$) higher compared to the control fruit. The APX activity of treated kiwifruits was 1.5 and 1.9-folds ($p < 0.001$) higher than the control fruits for the 0.01 and 0.02% treatment level (Figure 4.3 E), respectively. POD activity for the 0.01 and 0.02% treatment level was approximately 20 and 33% greater ($p < 0.001$) than the control (Figure 4.4 A), respectively. The treatments had a significant ($p < 0.001$) influence of the GR activity, with the control, 0.01 and 0.02% treatment level registering values of 0.01 and 0.012 and 0.014 U/g FW (Figure 4.4 C), respectively. These results agree with previous studies, which demonstrated that hexanal and EFF enhanced the activities of

antioxidant enzymes in cherries (Sharma et al., 2010), melons (Qi et al., 2011), mango (Jincy et al., 2017) and bell peppers (Cheema et al., 2018).

The findings of the current study suggest that EFF preserved cell membrane integrity by enhancing the ROS scavenging potential in treated kiwifruits. This was characterised by significantly lower accumulation of MDA, EL and ROS levels in treated kiwifruits (Figure 4.1 and 2). The mechanism in which EFF reduced oxidative damage in kiwifruits is in accordance with findings reported by Jincy et al. (2017) and Cheema et al. (2018). However, the mechanism in which EFF or hexanal enhances the activities of antioxidant enzymes is still unclear. Transcriptome analysis conducted by Padmanabhan et al. (2020) demonstrated that hexanal application upregulated the gene expression of APX.

Therefore, it can be hypothesized that hexanal enhances the activities of antioxidant enzymes by upregulating their gene expressions. However, further investigation on the effect of hexanal on gene expression of antioxidant enzymes is needed to validate this theory. The kiwifruit harvested at M1 exhibited significantly ($p < 0.05$) higher antioxidant enzyme activity compared to those of M2. These findings are similar to those reported by Wang et al. (2020b), who showed that mid harvested kiwifruit exhibited higher SOD and GR activity than their late harvested counterparts. These findings suggest that kiwifruits harvested at M1 were more effective at dealing with oxidative stress than those of M2. This observation is corroborated by the higher ROS levels and membrane degradation exhibited by M2 harvested kiwifruit (Figure 4.1 and 4.2).

The images displayed in figure 4.5, show that kiwifruits harvested at M2 had damaged flesh. This could be attributed to oxidative damage due to ROS production. This suggests that late harvested kiwifruits deteriorate quicker than their mid harvested counterparts. The images further show that EFF-treated kiwifruits harvested at both maturity stages had no damage on the fruit tissues. According to Yuan et al. (2009), fruits that lack any kind of decay, damage and shrivels are considered as marketable. Therefore, these findings indicate that EFF treatment has the capacity to optimise the marketability of kiwifruits harvested at both the mid (M1) and late (M2) maturity stages.

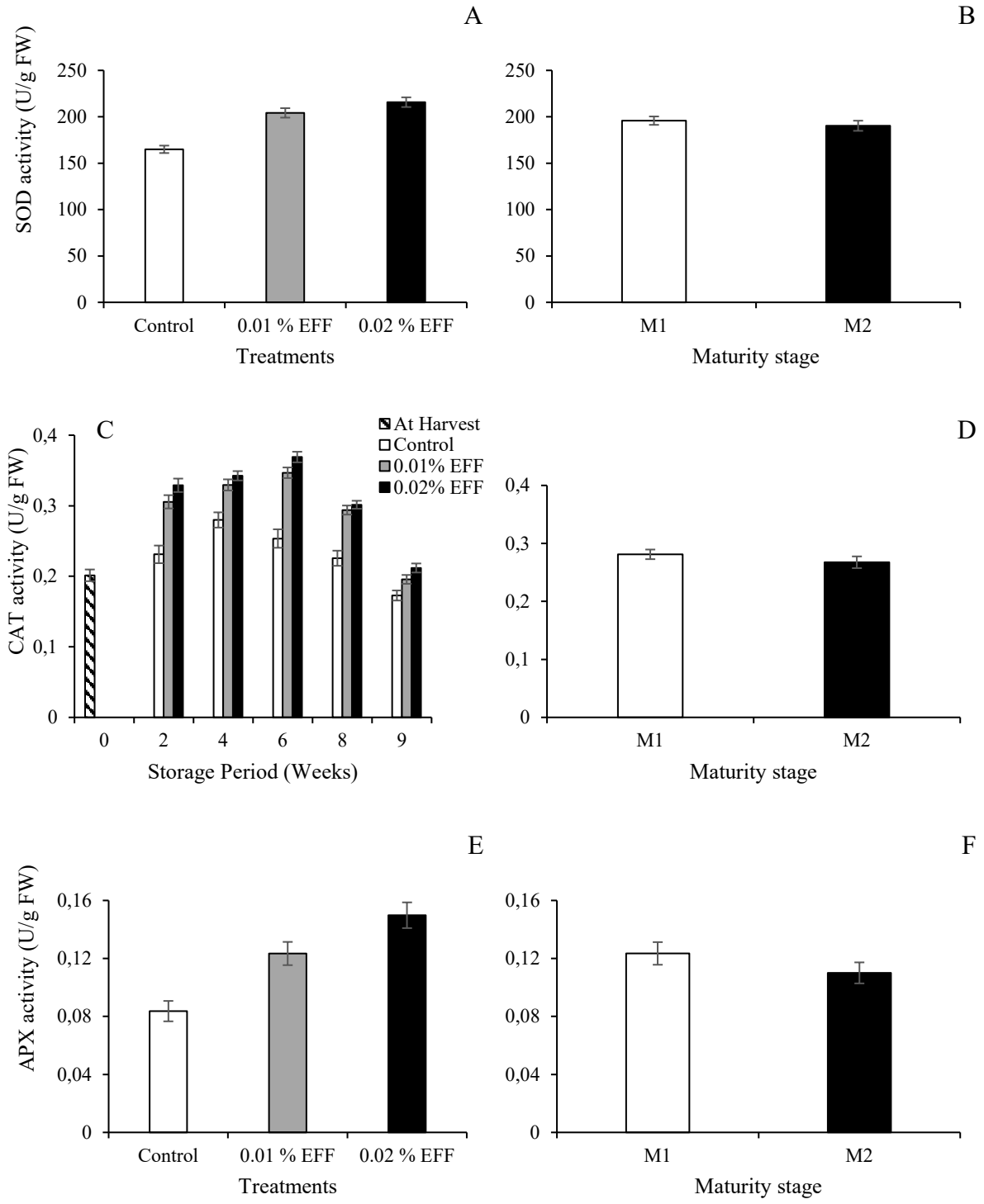


Figure 4.3: The effect of treatments (A) and maturity stage (B) on superoxide dismutase (SOD) activity of kiwifruit. The effect of interaction between treatments and storage time (C) and maturity stage (D) on catalase (CAT) activity of kiwifruit. The effect of treatments (E) and maturity stage (F) on ascorbate peroxidase (APX) activity of kiwifruit. Values are the means ± SE (n = 3).

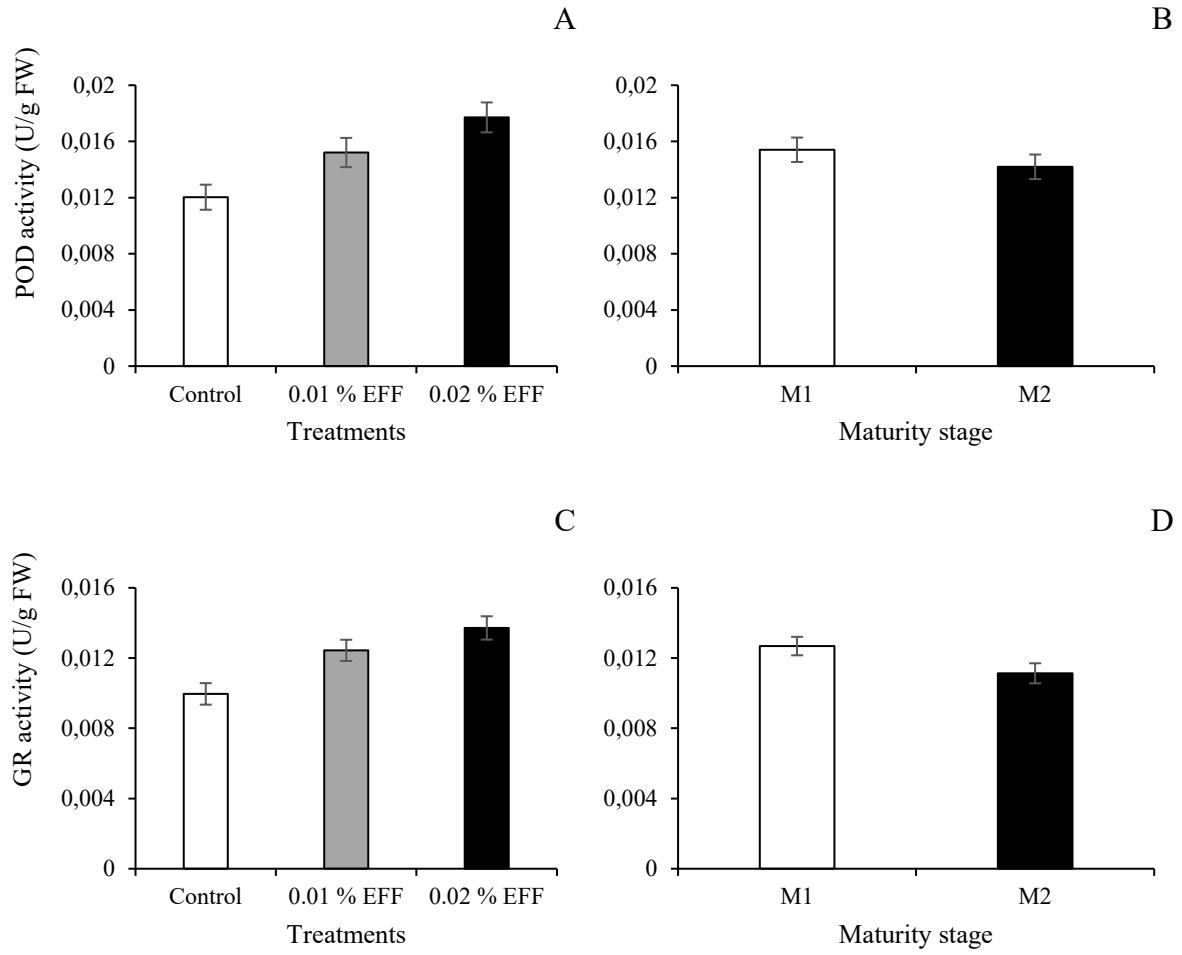


Figure 4.4: The effect of treatments (A) and maturity stage (B) on guaiacol peroxidase (POD) activity of kiwifruit. The effect of treatments (C) and maturity stage (D) on glutathione reductase (GR) activity of kiwifruit. Values are the means ± SE (n = 3).

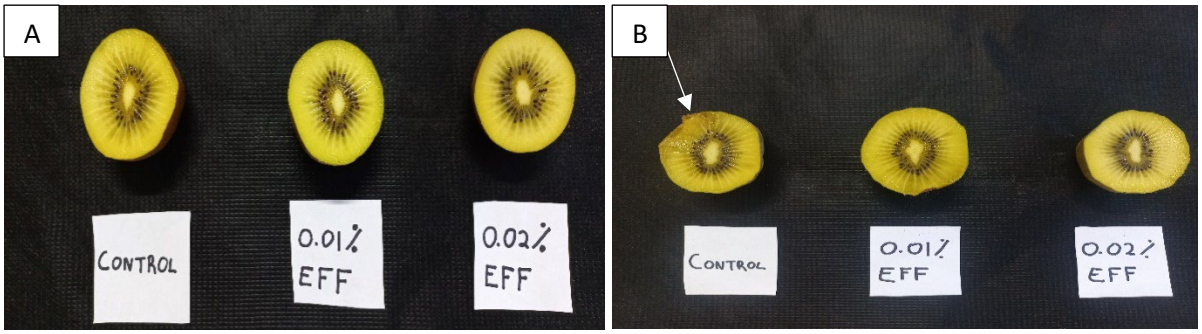


Figure 4.5. Images displaying the effect of enhanced freshness formulation on the fruit flesh kiwifruits harvested at M1 (A) and M2 (B).

4.4. Conclusion

The findings of this study demonstrated that EFF delayed membrane deterioration of kiwifruit by regulating ROS levels through the enhancement of antioxidant enzymes and suppression of PLD activity. Furthermore, the results showed that higher levels of membrane deterioration exhibited by late harvested kiwifruits, can be effectively delayed by increasing the dosage of EFF. These outcomes suggest that EFF can be used as a postharvest strategy to improve storability of kiwifruit by reducing oxidative stress. Therefore, EFF has the potential to reduce postharvest losses of kiwifruit attributed to membrane deterioration and senescence. Transcriptome analysis on the gene expression of antioxidant enzymes is warranted to elucidate the mechanism in which EFF enhances the activities of antioxidant enzymes.

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Chapter Five

Preserving Gold Kiwifruit Postharvest Quality: Efficacy and Mechanism of Enhanced Freshness Formulation to Suppress *Botrytis cinerea*

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Abstract

The study evaluated the effectiveness of enhanced freshness formulation (EFF) to inhibit the fungal decay caused by *Botrytis cinerea* in 'Y368' kiwifruit. *In vitro* and *in vivo* experiments with three treatments, namely, control, 0.03 and 0.06% EFF were set up. In the *in vitro* experiment (23 °C), mycelial growth and spore germination of *B. cinerea* exposed to EFF were examined. In the *in vivo* experiment (23 °C, 85 – 90% relative humidity), fruit quality parameters such as disease incidence, lesion diameter, total phenolics, total flavonoids and the activity of defence-related enzymes such as Phenylalanine Ammonia Lyase, Guaiacol Peroxidase, β-1,3-glucanase and Chitinase were evaluated. The results demonstrated that 0.03 and 0.06% EFF treatments effectively ($p < 0.001$) reduced mycelial growth of *B. cinerea* by 40.74 and 89.38%, respectively. Both EFF treatments reduced spore germination by 33 and 70%, respectively. The lesion diameter of the treated fruit was 28 and 76% smaller for the 0.03 and 0.06% EFF treatments, respectively. The concentrations of total phenolics in the control, 0.03 and 0.06% EFF treatments were 0.89, 1.06 and 1.12 mg GAE/g FW, respectively. Both EFF treatments exhibited significantly ($p < 0.001$) higher activity of defence-related enzymes compared to the control. Furthermore, EFF treatment effectively optimised the quality of

kiwifruit, with treated fruit registering 26 and 34% higher firmness than the control fruit for the 0.03 and 0.06% treatment level, respectively. The results provide compelling evidence of EFF efficacy in effectively suppressing *B. cinerea*. The higher activity of defense-related enzymes in EFF-treated fruit demonstrates their role in enhancing the fruit's resistance to decay. These findings underscore the potential of EFF as a valuable method in kiwifruit postharvest preservation, enhancing both the shelf life and quality of kiwifruit.

Keywords: Antifungal properties, antioxidants, chitinase, defence enzymes, guaiacol peroxidase, phenylalanine ammonia lyase, postharvest quality, hexanal

2.6. Introduction

Grey mould caused by *Botrytis cinerea* is a necrotrophic pathogen that results in severe deterioration of postharvest fruit quality in kiwifruit (Williamson et al., 2007). Postharvest losses attributed to *B. cinerea* are estimated between 20 and 30% of the total kiwifruit produce (Michailides and Elmer 2000; Li et al., 2020), resulting in economic losses valued at 10 – 100 million US dollars annually (Brito et al., 2021). The traditional approach to managing postharvest fungal diseases in kiwifruit involves the use of synthetic fungicides such as fludioxonil, fenhexamid and trifloxystrobin (Adaskaveg et al. 2005; Müller et al. 2011; Thomidis and Prodromou, 2018; Cheng et al., 2019). However, growing concerns surrounding issues such as fungicide residues, the emergence of fungicide-resistant strains, and environmental and food safety consideration have spurred researchers and growers to explore alternative strategies for pathogen management during postharvest storage (Moscetti et al., 2013; Li et al., 2017; Hua et al., 2019).

The European Union, the major market for South African kiwifruit industry, places a strong emphasis on food safety to safeguard consumer health. Maximum residue limit (MRL) regulations and thresholds exerts considerable pressure on growers and exporters to develop alternative disinfectants. When the chemical residues on a fresh produce surpass the MRLs of the importing country, it can lead to the costly recalls and/or destruction of the consignment, often borne by the grower or marketer, resulting in significant financial losses and reputational damage. Hence, there is pressing need to investigate alternative strategies to control fungal decay during storage, with the goal of reducing reliance on agrochemicals. Among the alternative control methods investigated are the biocontrol agents such as antagonistic yeasts, elicitors and products based on *Bacillus subtilis* (Oregel-Zamudio et al., 2017).

To date, the use of biological control agents is limited due to their inconsistent and unreliable performance, as well as their limited applicability in real-world field or supply chain settings (Pertot et al., 2017). As a result, these challenges have hindered the commercial adoption of this control strategy (Cheng et al., 2019). Other alternative treatments such as edible coatings (Romanazzi et al., 2017; Li et al., 2019; Melo et al., 2020), ultraviolet C treatment (Mohamed et al., 2017; Sripong et al., 2019), ozone treatment (Tanou et al., 2015; García-Martín et al., 2018; Luo et al., 2019) and heat treatment (Yun et al, 2013; Langer et al., 2018; Ge et al., 2020) have been evaluated as substitutes for fungicides. However, the majority of these control strategies remain in the experimental phase or not yet adaptable to commercial applications (Petrasch et al., 2019). Furthermore, the costs of technology associated with implementing these alternative control strategies have posed challenges for their adoption within certain segments of the horticultural sector including the kiwifruit industry (Salzano et al., 2019). The high costs associated with implementing these technologies leads to the continued reliance on more cost-effective and easy-to-apply synthetic chemical fungicides. However, this dependence can adversely affect kiwifruit producers who export their produce to international markets, with stringent standards for food safety

Hexanal is a promising alternative to fungicides which has been proven to be effective in suppressing fungal growth of various pathogens (Song et al., 2007; Utto et al., 2008; Thavong et al., 2010; Baggio et al., 2014). Hexanal is a naturally occurring volatile compound, specifically a C6 aldehyde formed from linoleic acid via the lipoxygenase pathway during lipid peroxidation in plants (Paliyath and Subramanian, 2008). Past research has yielded promising results in demonstrating the capacity of C6 aldehydes such as hexanal and (E)-2-hexenal in suppressing the fungal growth of *B. cinerea* (Song et al., 2007; Utto et al., 2008; Hyun et al., 2022; Zhang et al., 2023). However, these studies only focused on investigating the effect of hexanal as a vapour treatment. The main drawback of implementing vapour treatments is that it requires specialised equipment and facilities, which involves high capital investment. This constraint makes it less feasible for packhouses with limited resources to implement this treatment approach.

One advantage of hexanal over other vapour treatment methods is its versatility, as it can also be applied as a dip treatment. This is because hexanal as a dip treatment is straightforward, as it does not require specialised equipment or controlled environments. However, it is worth noting that there is a scarcity of studies assessing the efficacy of hexanal as a dip treatment for inhibiting fungal growth and *B. cinerea*, specifically. Furthermore, in our extensive review of

the literature, we have not found studies evaluating the efficacy of hexanal dip treatment in controlling fungal growth of *B. cinerea* in kiwifruit. Previous studies only focused on the effectiveness of hexanal as a dip treatment to inhibit fungal growth of *Lasiodiplodia theobromae*, *Collectotrichummusae* and *Penicillium expansum* in mango, banana and apples, respectively (Seethapathy et al., 2016; Pravin et al., 2019; Wang et al., 2022).

Therefore, the aim of this study was to investigate the efficacy of EFF as a dip treatment for controlling fungal growth of *B. cinerea* and investigate its capacity to induce disease resistance in kiwifruit, thus shedding light on the underlying mechanism of its antifungal action.

2.7. Materials and methods

2.7.1. Fruit source and pathogen

Kiwifruit (*Actinidia chinensis*) cv. 'Y368' were harvested from Roselands farm, a commercial farm located in the Richmond area (Latitude: 29.9033°S, Longitude: 30.2397°E), KwaZulu-Natal Province, South Africa. Kiwifruit were harvested when the total soluble solid content was greater than 6.2 °Brix (Mahlaba et al., 2022). Harvested fruit were immediately transported in a ventilated vehicle to the Postharvest Laboratory of the University of KwaZulu-Natal (UKZN), where postharvest treatments were applied, and *in vitro* and *in vivo* experiments were conducted. Isolates of *B. cinerea* from a pure culture prepared by single colony isolation, were obtained from the Plant Pathology Laboratory of UKZN.

2.7.2. Pathogen identification

The pure cultures were identified by molecular methods at Inqaba Biotechnical Industries (Pty) Ltd. DNA extraction was performed using the Quick-DNA™ Fungal/Bacterial Miniprep Kit (Zymo Research, Catalogue No. D6005). The Internal Transcribed Spacer (ITS) target regions were amplified using primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). The DNA extracted from the cultures was used for Polymerase chain reaction (PCR). Briefly, the PCR protocol was denaturation at 94 °C for 5 min, Annealing at 94 °C for 30 sec, Elongation at 50 °C for 30 sec, 68 °C for 1 min and 68 °C for 10 min. There were 35 cycles performed and a held at 4 °C. The PCR Amplicon fragments were enzymically purified using the ExoSAP procedure (NEB M0293L; NEB M0371). The amplicons were then purified for sequencing using the Zymo Research ZR-96 DNA Sequencing Clean-up Kit™, Catalogue No. D4050, and sequenced in the forward and reverse direction (Nimagen, BrilliantDye™ Terminator Cycle Sequencing Kit V3.1, BRD3-100/1000)

using the ABI 3730xl Genetic Analyzer (Applied Biosystems, Thermo Fisher Scientific). Molecular identification ascertained the pathogen as *B. cinerea*.

2.7.3. In vitro experiment

2.7.3.1. Mycelial growth

The effect of EFF on the mycelial growth of *B. cinerea* was evaluated at two concentrations, 0.03 and 0.06% using the agar dilution method (Anusha et al., 2016; Song et al., 2022). The appropriate volume of EFF was added to warm potato dextrose agar (PDA) medium to get the desired concentration. Each concentration of the amended medium was dispensed into 90 mm petri dishes, with each dish containing 20 mL, and then left to solidify. Mycelia of the pathogen were cut from actively growing culture and placed at the centre of the new petri plates and incubated at 23 °C. Each treatment consisted of three replications, with three petri dishes per replication. The mycelial growth was recorded after three, five and seven days. The efficacy of EFF was expressed as per cent inhibition in mycelial growth over control as demonstrated in Eq. 1.

$$\text{Per cent inhibition over control} = C - T \frac{x\,100}{C} \quad (1)$$

Where C = mycelial growth in control plate (mm) and T = mycelial growth in EFF amended plate (mm). The experiment was repeated three times.

2.7.3.2. Spore germination

Spores were collected from the cultures of *B. cinerea* grown in PDA medium. Spore suspensions were prepared by carefully adding the spores in each of the two EFF solutions. Similarly, spores were suspended in sterile water for use as control. A 100 µL aliquot of the spore suspension was placed on sterile slides and incubated in petri dishes containing water agar in order to maintain the required humidity for germination. The spores were incubated for 24 hours at 23 °C. After the incubation period, conidial germination was observed under a microscope (Zeiss AX10, ZEISS Group, Oberkochen, Germany) at 40-times magnification. Thereafter, the germination count was recorded as spore germination (%). Inhibition rate of spore germination was calculated using Eq. 2

$$\text{Inhibition rate of spore germination (\%)} = [(\mathrm{T} - \mathrm{G})/\mathrm{T}] \times 100 \quad (2)$$

where T and G are the total number of spores observed and the number of germinating spores, respectively.

2.7.3.3. Morphological changes in mycelium and spores induced by enhanced freshness formulation

The effect of EFF on the mycelial morphology and spores of *B. cinerea* (seven day old fungi) was observed under a scanning electron microscope (SEM) (Zeiss EVO LS 15, ZEISS Group, Oberkochen, Germany). The segments (2 x 2 mm) were cut from the PDA plates and immersed for three hours in 3% (v/v) glutaraldehyde for primary fixation. Thereafter, the segments were subjected to dehydration in a series of ethanol solutions, namely, 10, 30, 50, 70 and 90% for 10 min, respectively, followed by further dehydration in 100% ethanol for ten minutes (repeated three times). Thereafter, the samples underwent critical point drying, which involved heating and pressurising liquid carbon dioxide to its critical point using a critical point dryer (Quorom K850, Quorum Technologies, United Kingdom). Afterward, the dried samples were mounted on SEM stubs and transferred to a rotary pumped coater (Quorom Q150R ES, Quorum Technologies, United Kingdom). In the sputter coater, the samples were subjected to gold sputter coating to make the samples conductive to the electron beam. The specimens were viewed under a SEM.

2.7.4. In vivo experiment

2.7.4.1. Postharvest treatment and inoculation

Fruit disinfection and inoculation were done according to Ge et al. (2020) and Hyun et al. (2022). Kiwifruits were dipped in 2% (v/v) sodium hypochlorite solution for 2 min for disinfection. After air drying at room temperature, all inoculated fruit were divided at random into three groups for the following treatments: control (untreated), immersion in 0.03 and 0.06% (v/v) enhanced freshness formulation for 2.5 min. For inoculation, kiwifruits were wounded (depth 3 mm) at the equator with a sterile needle and inoculated with 15 µL of *B. cinerea* spore suspension (1 x 10^5 spores mL^{-1}). All the fruit were air-dried and then stored in polyethylene plastic bags at 23 °C, to maintain a high relative humidity of approximately 85 - 90% (Chen et al., 2015; Ge et al., 2020). Each treatment consisted of three replicates; each replicate had three fruit per storage interval and experiments were performed in triplicate.

2.7.4.2. Lesion diameter, disease incidence and index

Lesion diameter was measured by crossing method using only infected wounds. Disease incidence was calculated as the percentage of the fruit with apparent disease symptoms using Eq. 3. According to the area of rot on the fruit surface, the extent of disease severity was scored using the following scale: 0, no disease; 1, decay area < 20%; 2, decay area of 20% – 40%; 3, decay area of 40% – 60%; 4, decay area > 60%, and disease index was calculated using Eq. 4 as previously described by Ge et al. (2020).

$$\text{Disease incidence} = \text{number of symptomatic fruit} / \text{total number of fruit} \times 100 \quad (3)$$

$$\text{Disease index} = \text{Disease index} = \Sigma \frac{\text{disease scale x number of fruit at that scale}}{\text{total number of fruit x 4}} \quad (4)$$

2.7.4.3. Total phenolic and flavonoid compounds

The concentration of total phenolics was determined using a method described by Singleton et al. (1999), where 1 g of pulp sample was homogenized with 5 mL of 80% (v/v) methanol and centrifuged at 10 000 rpm for 15 min at 4 ºC. Thereafter, the mixture was supplemented with 2.6 mL of distilled water and 200 µL of Folin-Ciocalteu's phenol reagent was added to 200 µL of the methanolic extracts. After incubation of 6 min., 2 mL of 7 % (w/v) sodium carbonate was introduced to the reaction mixture. The absorbance was measured at 750 nm after 90 min using a Shimadzu UV spectrophotometer (Model UV-1800 240V, Kyoto, Japan). The concentration of total phenolics (TPC) was determined using gallic acid as a standard (0 – 300 mg/mL, $R^2 = 0.9953$) and expressed as mg gallic acid equivalents (GAE)/g fresh weight (FW) of kiwifruit.

The concentration of total flavonoids was determined by adding 3.2 mL of distilled water and 150 µL of 5% (w/v) sodium nitrite to 500 µL of methanolic extract. After 5 min, 150 µL of 10% (w/v) aluminium chloride was added. After 6 min, 1 mL of 1 M sodium hydroxide was added and the absorbance recorded at 510 nm using a Shimadzu UV spectrophotometer (Model UV-1800 240V, Kyoto, Japan). The concentration of total flavonoids (TFC) was determined using catechin as a standard (20 – 140 µg/mL, $R^2 = 0.9922$) and expressed as mg catechin equivalents (CE)/g fresh weight (FW) of kiwifruit.

2.7.4.4. Defence enzyme mechanisms

2.7.4.4.1. Phenylalanine ammonia lyase (PAL, EC 4.3.1.5)

PAL enzyme activity was assessed using a protocol previously described by Khan et al. (2003). One gram of kiwifruit pulp was homogenised in 10 mL of ice-cooled Tris-HCl buffer (50 mM, pH 8.5) containing 5% (w/v) polyvinylpolypyrrolidone (PVPP) and 14.4 mM β-mercaptoethanol. After centrifugation for 20 min at 10 000 x *g*, at 4 °C, the supernatant was recovered. PAL activity was quantified using a reaction mixture comprising of 800 µL of Tris-HCl buffer (0.5 mM, pH 8.0), 100 µL 6 µM of L-phenylalanine, and 100 µL of enzyme extract. After incubating the mixture at 40 °C for 1 h, the reaction was stopped by adding 100 µL of 5 N HCl. The absorbance was measured at 290 nm using a Shimadzu UV spectrophotometer (Model UV-1800 240V, Kyoto, Japan) and trans-cinnamic acid was used as a standard (5 – 35 µg/mL, $R^2 = 0.9903$) to calculate enzyme activity. Results were expressed as U/g of protein.

2.7.4.4.2. Guaiacol peroxidase (POD, EC. 1.11.1.7)

POD enzyme was extracted by homogenizing 3 g of kiwifruit pulp in 7 mL of 100 mM potassium phosphate buffer (pH 7.0) containing 1.0 mM EDTA, 5% (w/v) polyvinyl polypyrrolidone, and 1% (v/v) Triton X-100 at 4 °C. The homogenate was centrifuged at 13 000 x *g* for 20 min at 4 °C. POD enzyme activity was assessed using a protocol previously described by Sripong et al. (2019). The reaction mixture consisted of 100 µL enzyme solution, 2.82 mL of 0.1 M sodium phosphate buffer (pH 7.0), 30 µL of 12.3 mM H_2O_2 and 50 µL of 20 mM guaiacol. The increase in absorbance at 470 nm due to the guaiacol oxidation was recorded for 2 min, using a Shimadzu UV spectrophotometer (Model UV-1800 240V, Kyoto, Japan). One unit of enzymatic activity was defined as the amount of the enzyme that caused a change of 0.001 in absorbance per minute. The POD activity was determined using Beer Lambert's law: $A = \varepsilon \mathfrak{b} C$ and expressed as U/g of protein, using 25 mM^{-1} cm^{-1} as the extinction coefficient,

A = absorbance, ε = molar absorptivity, $\mathfrak{b}$ = path length, C = concentration.

2.7.4.4.3. Chitinase (CHI, EC 3.2.1.14)

Chitinase enzyme activity was assessed using a protocol previously described by Pombo et al. (2011). Briefly, 1 g of kiwifruit pulp sample was homogenised in 3 mL solution consisting of 10 mmol L^{-1} sodium acetate buffer and 2% (w/v) polyvinylpolypyrrolidone, adjusted to pH 5.

The homogenate was stirred for 3 h at 4 °C, thereafter, centrifuged at 12 000 × *g* for 30 min. The supernatant was recovered and used to analyse chitinase activity. The reaction mixture consisted of 0.739 mL of 0.2% chitin azure (Sigma) and 2.21 mL of extract. After incubating the reaction mixture at 37 °C for 1 h, 0.178 mL of 2 N HCl was added and immersed in ice to stop the reaction. Chitinase activity was measured at 575 nm using a Shimadzu UV spectrophotometer (Model UV-1800 240V, Kyoto, Japan) and *N*-acetylglucosamine was used as a standard (0 – 1 mg/mL, $R^2 = 0.9902$) and expressed as U/g of protein.

2.7.4.4.4. β-1,3-glucanase (GLU, EC 3.2.1.39)

GLU enzyme activity was assessed using a protocol previously described by Zheng et al. (2011). One gram of kiwifruit pulp was homogenised with 3 mL of 50 mM sodium acetate buffer (pH 5) and centrifuged at 17 000 x *g* for 30 minutes at 4 °C. The reaction mixture comprised of 100 µL of enzyme extract, and 50 µL of 0.4% laminarin and incubated at 37 °C for an hour. Thereafter, 200 µL of 0.63% 3,5-dinitrosalicylic acid was added and the reaction mixture was boiled for 5 minutes. The reaction was cooled and the absorbance was measured at 500 nm using a Shimadzu UV spectrophotometer (Model UV-1800 240V, Kyoto, Japan). Glucanase activity was determined as the amount of reducing glucose released from laminarin using glucose (0 – 20 mg/mL, $R^2 = 0.993$) as the standard. Enzyme activity was expressed as U/g of protein, where a unit is defined as 1 mg of glucose released per minute under these assay conditions.

2.7.4.4.5. Protein concentration

The Bradford assay (1976) was used to determine the protein concentration using bovine serum albumin as a standard (0 – 1 mg/mL, $R^2 = 0.9957$) and used to calculate the specific activity of defense mechanism enzymes.

2.7.5. Measurement of fruit quality parameters

To evaluate the effect of EFF on the postharvest quality of kiwifruits, fruits were divided into three treatment groups (control (untreated), 0.03 and 0.06% EFF) as stated above. After air-drying, kiwifruits were stored at 0 °C, 90% relative humidity and fruit quality parameters were assessed after eight weeks of storage.

2.7.5.1.1. Mass loss and fruit firmness

Mass loss was measured using a calibrated Ohaus digital scale (± 0.01 g) (Model SKX2202, Switzerland). Mass loss was calculated using Eq. 5

$$\text{Mass loss (ML)} = (\text{IM} - \text{FM}) / \text{IM} \times 100 \quad 5$$

Where, ML is mass loss (%), IM, the initial mass of fruit (g) and FM, the final mass of fruit (g).

Fruit firmness was determined using a penetrometer (Selectech, mod. FT 327, Italy) with an 8 mm diameter head. Firmness was recorded as kilogram-force (kgf) and converted to newtons (N), where 1 kilogram-force (kgf) is equal to 9.81 newtons (N).

2.7.5.1.2. Total soluble solids (TSS) and titratable acidity (TA)

TSS of kiwifruit juice was determined using a desktop refractometer (Bellingham + Stanley Ltd, Model: RFM340+, UK). Titratable acidity was determined by titrating 10 mL of kiwifruit juice with 0.1 M NaOH to a pH value of 8.1, using a Mettler Toledo Potentiometric compact titrator (Model G20S, Greifensee, Switzerland). Titratable acidity was expressed as the percentage of citric acid equivalent on a fresh weight basis using Eq. 6.

$$\text{TA (\% citric acid)} = (0.0064 \text{ x titre (NaOH) mL}) / (10 \text{ mL juice}) \text{ x } 100 \quad 6$$

2.7.5.1.3. Ascorbic acid

A method described by Malik and Singh (2005) and modified by Goffi et al. (2019), was used to determine the ascorbic acid content of kiwifruit. Ascorbic acid was extracted from 1 g of kiwifruit pulp using 4 mL of 16% (v/v) metaphosphoric acid, containing 0.18% (w/v) disodium ethylenediaminetetraacetic acid and centrifuged (Avanti J-265XP, Beckaman Coulter, Indianapolis, IN, USA) at 1100 x *g* for 10 min at 4 °C. The assay mixture contained 200 µL supernatant, Folin's reagent (1:5 v/v) and 0.3% (v/v) metaphosphoric acid in a final volume of 2 mL. The absorbance was measured at 760 nm, using ascorbic acid (1mg/mL, $R^2 = 0.9947$) as standard and expressed as mg/g FW.

2.7.6. Statistical analysis

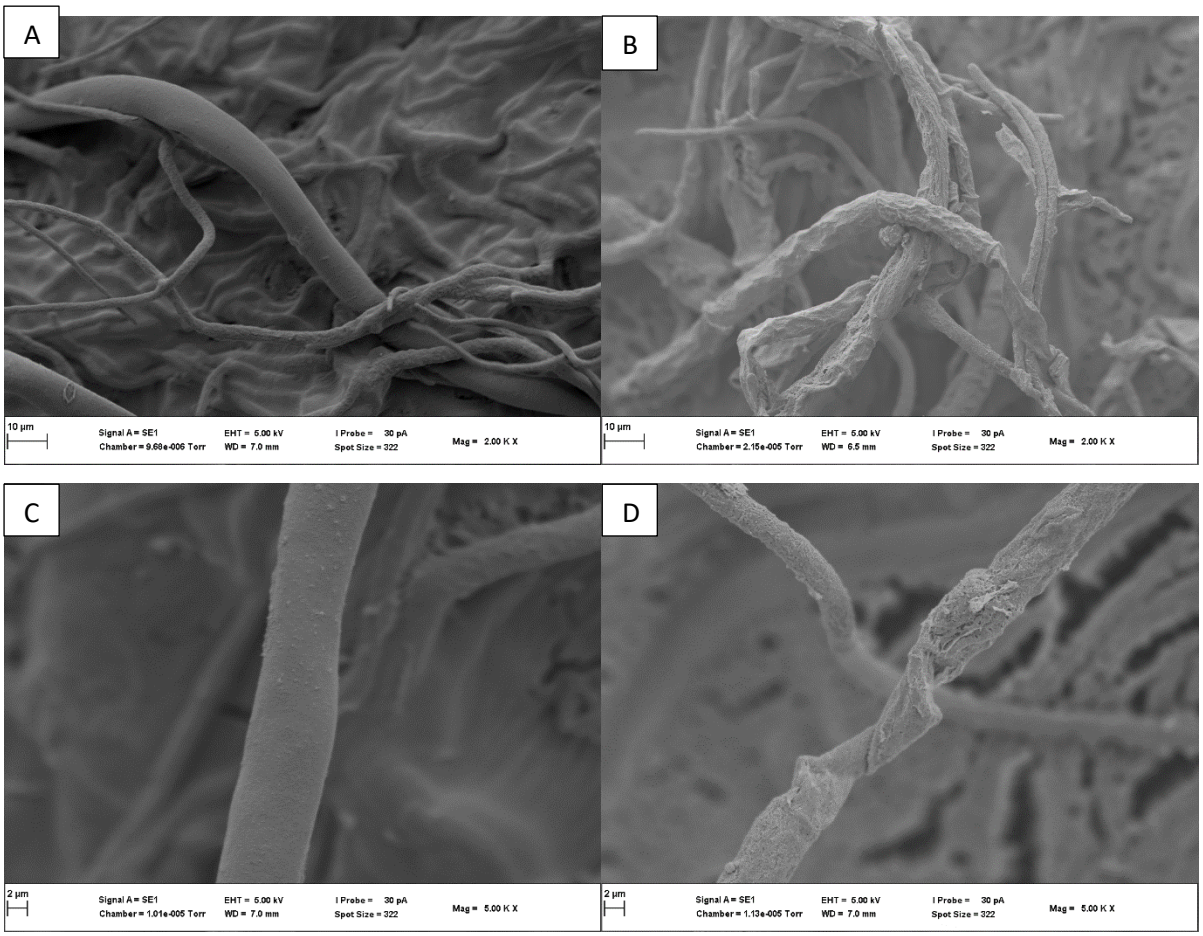
All experiments were performed using a completely randomized factorial design. The experiment comprised of two factors: treatments and storage period. Statistical analysis and

principle component analysis was performed in R software version 4.3.1 (R Development Core Team, 2023). Data were expressed as mean values ± SE and were subjected to analysis of variance (ANOVA) with a 5% level of significance.

2.8. Results and discussion

2.8.1. Morphological changes in mycelium and spores induced by enhanced freshness formulation

The SEM images showed that the EFF treatment had an adverse effect on the morphology of mycelia and spores of *B. cinerea* (Figure 5.1). The mycelia exposed to EFF treatment exhibited altered hyphae, which was shrivelled and twisted, with a damaged surface (Figure 5.1 A – D). Moreover, the spores of *B. cinerea* exposed to EFF treatment were collapsed and severely distorted (Figure 5.1 E, F).



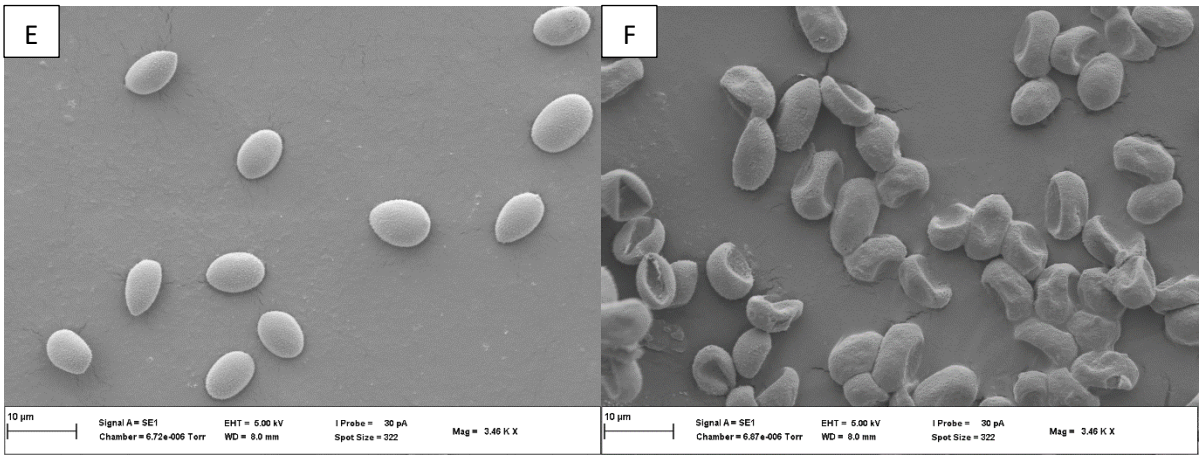


Figure 5.1. Scanning electron microscope images displaying the effect of enhanced freshness formulation on mycelia and spore morphology of *B. cinerea*. Control mycelia (A, C) and spores (E). EFF treated mycelia (B, D) and spores (F).

2.8.2. Mycelial growth and spore germination

The results showed that EFF effectively reduced the mycelial growth of B. *cinerea* (Table 5.1), with the 0.03 and 0.06% exhibiting an inhibition rate of 40.74 and 89.38%, respectively. Similarly, the EFF treatments proved effective in reducing spore germination, which was 1.6 and 3.7-folds lower for the 0.03 and 0.06% treatment level, respectively. The 0.03 and 0.06% EFF treatment level inhibited spore germination by 33.71 and 70.32%, respectively. The ability of EFF to suppress the growth of *B. cinerea* may be attributed to its capacity to distort fungal hyphae by twisting, breaking and damaging the membrane (Figure 5.1 A – D). Furthermore, suppression of spore germination may be owed to the capacity of EFF to induce the malformation of fungal spores, as illustrated by SEM images (Figure 5.1 E, F).

The results in this study are comparable to those reported by Anusha et al. (2016), who demonstrated that hexanal treatment distorted fungal hyphae and spores of *Colletotrichum gloeosporioides* and *Lasiodiplodia theobromae*. These findings depict that EFF can be used as an antifungal treatment to control *B. cinerea*. The *in vitro* experiment of this study solely focused on the effect of EFF on the morphology of *B. cinerea* and not its inhibitory mechanism. Therefore, further investigation on the effect of EFF on ergosterol biosynthesis and energy metabolism is needed to elucidate the antifungal mechanism in which EFF supresses the growth of *B. cinerea*.

Table 5.1: Effect of enhanced freshness formulation on the inhibition of mycelial growth and spore germination of *Botrytis cinerea*.

Treatment	Inhibition (%)	Spore germination (%)	Inhibition rate of spore germination (%)
Control	0 ± 0c	90.87 ± 1.59a	0 ± 0c
0.03% EFF	40.74 ± 0.56b	58.35 ± 1.35b	33.71 ± 1.21b
0.06% EFF	89.38 ± 0.71a	26.95 ± 0.40c	70.32 ± 0.75a
p value	<0.001	<0.001	<0.001

Values are the means ± SE (n = 3). Means with different letters indicate a statistically significant difference ($p < 0.05$).

2.8.3. Lesion diameter, disease incidence and index

The lesion diameter increased for all treatments; however, EFF treatment substantially reduced the size of the lesion. At seven days post inoculation (dpi), the lesion diameter of treated fruit was approximately 28 and 76% smaller for the 0.03 and 0.06% EFF treatment level, respectively, compared to the control. Similarly, the disease incidence also increased as the storage time progressed. At 7dpi, the disease incidence of 0.03% treated fruit was equal to that of control fruit. The 0.06% treatment level significantly lowered the disease incidence, recording a value of 41.67% at 7dpi. The EFF-treated fruit exhibited significantly lower disease index, which was registered at 90.71, 64.96 and 6.70% for the control, 0.03 and 0.06% EFF treatment level, respectively at 7dpi.

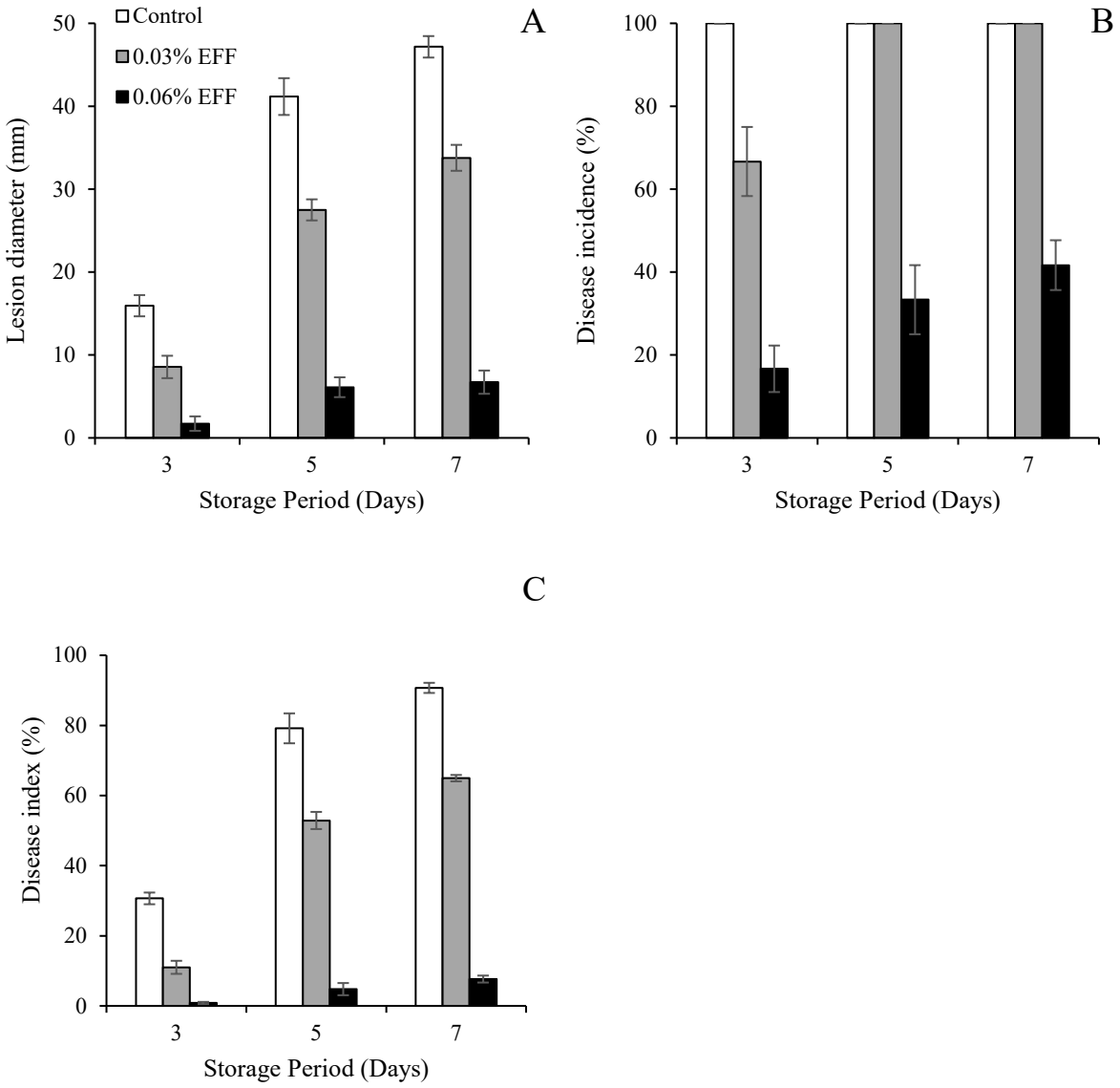


Figure 5.2. Effect of enhanced freshness formulation on lesion diameter (A), disease incidence (B) and disease index (C) of 'Y368' kiwifruit after inoculation with *B. cinerea*. Values are the means ± SE (n = 3). Means with different letters indicate a statistically significant difference ($p < 0.05$).

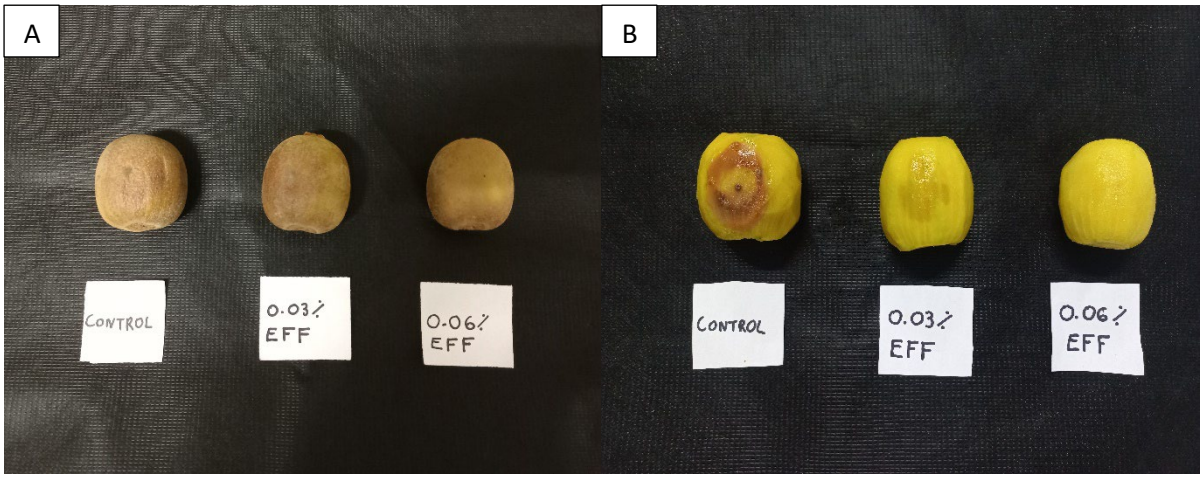


Figure 5.3. Images displaying the effect of enhanced freshness formulation on kiwifruit inoculated with *B. cinerea*. Unpeeled kiwifruit (A) and peeled kiwifruit (B).

2.8.4. Defence mechanism enzymes

The enzyme activity of PAL increased during storage, with treated fruit exhibiting significantly higher enzyme activity relative to the control. At 7 dpi, PAL enzyme activity of 0.03 and 0.06% EFF-treated kiwifruit was approximately 20 and 33% greater than the control, respectively. The enzymatic activity of POD increased significantly with more pronounced effect at the 0.06% treatment level, followed by the 0.03% and control treatment. The POD enzyme activity of control fruit declined after day 5, whereas treated fruit continued to exhibit an increase in enzyme activity. At 7dpi, the POD enzyme activity of the 0.03 and 0.06% treatment level was 5 and 6.5-folds higher than the control, respectively.

The GLU enzyme activity increased for all treatments, then declined on day 7. GLU enzymatic activity was registered at 10.97, 12.92 and 14.26 U/g for control, 0.03 and 0.06% EFF at 7 dpi, respectively. The chitinase enzyme activity of treated kiwifruit increased as the storage period progressed and remained substantially higher than the control. The enzyme activity of the control fruit decreased after 5dpi, however, the change in chitinase enzyme activity was not significant. At 7 dpi, chitinase activity of treated fruit was 32.13 and 42.35% higher than the control, respectively.

C6 volatile aldehydes such as (Z)-3-hexenal, (E)-2-hexenal, and hexanal function as fungicidal and signalling compounds that induce defence responses in plants (Kishimoto et al., 2008). Recent studies investigated the mechanism in which exogenous application of C6 aldehydes induce disease resistance, providing a plausible rationalisation for the underlying mechanism of this process (Padmanabhan et al., 2020; Hyun et al., 2022; Zhang et al., 2023). Padmanabhan

et al. (2020) showed that hexanal vapour induced the transcript levels of defence-related genes in pepper fruit. Hyun et al. (2022) showed that (E)-2-hexenal vapour activated defence pathways by modulating signalling molecules, resulting in the production of defence enzymes and secondary metabolites in treated kiwifruit.

Furthermore, Zhang et al. (2023) demonstrated that the upregulation of defence-related genes in response to (E)-2-hexenal vapour, enhanced the activities of defence-related enzymes and the accumulation of phenolic compounds. The enhancement of defence enzymes and phenolic compounds, induced disease resistance in treated tomato plants (Zhang et al., 2023). Based on these findings, it can be deduced that exogenous application of C6 aldeyhdes upregulates the gene expression of signalling molecules and defence genes. This results in the increase of signalling molecules, defence-related enzymes and secondary metabolites, which confers enhanced disease resistance in treated fruit.

The defence-related enzymes involved in plant defence mechanisms are PAL, POD, GLU and CHI. PAL is an enzyme that is part of the phenylpropanoid pathway and is involved in the synthesis of secondary metabolites such as phenolic compounds (Luo et al., 2015). Phenolic compounds play a vital role in disease resistance by acting as toxins to the invading pathogen (Shao et al., 2010). POD is an antioxidant enzyme that is involved in the synthesis of lignin and protects against pathogens by strengthening the cell wall (Mandal and Mitra 2007). CHI and GLU are responsible for degrading the cell walls of pathogens (Ge et al., 2020). CHI enzyme protects against pathogen attack by degrading chitin, which is a key constituent of the pathogen cell wall (Tian et al., 2007). Whereas GLU works in tandem with CHI, by releasing oligosaccharides which induce defence reactions, leading to the inhibition of fungal growth (Tian et al., 2007).

The results of the current study suggest that the suppression of fungal growth (Figure 5.2) in response to EFF treatment can be attributed to the enhancement of defence-related enzymes (Figure 5.4) which confer disease resistance. These findings are similar to those reported by Seethapathy et al. (2016), Pravin et al. (2019) and Dhakshinamoorthy et al. (2020), who demonstrated that hexanal treatment induced disease resistance by enhancing the activites of defence-related enzymes in mango and banana fruit, respectively. Exogenous application of hexanal and (E)-2-hexenal are known for inducing disease resistance by upregulating the expression of defence-related genes (Padmanabhan et al., 2020; Hyun et al., 2022; Zhang et al., 2023). Therefore, we hypothesize that EFF induced disease resistance in kiwifruit by

triggering the activity of defence-related enzymes, through the upregulation of defence-related genes. However, transcriptome analysis showing the effect of EFF on signalling molecules and defence-related genes will add value to this hypothesis.

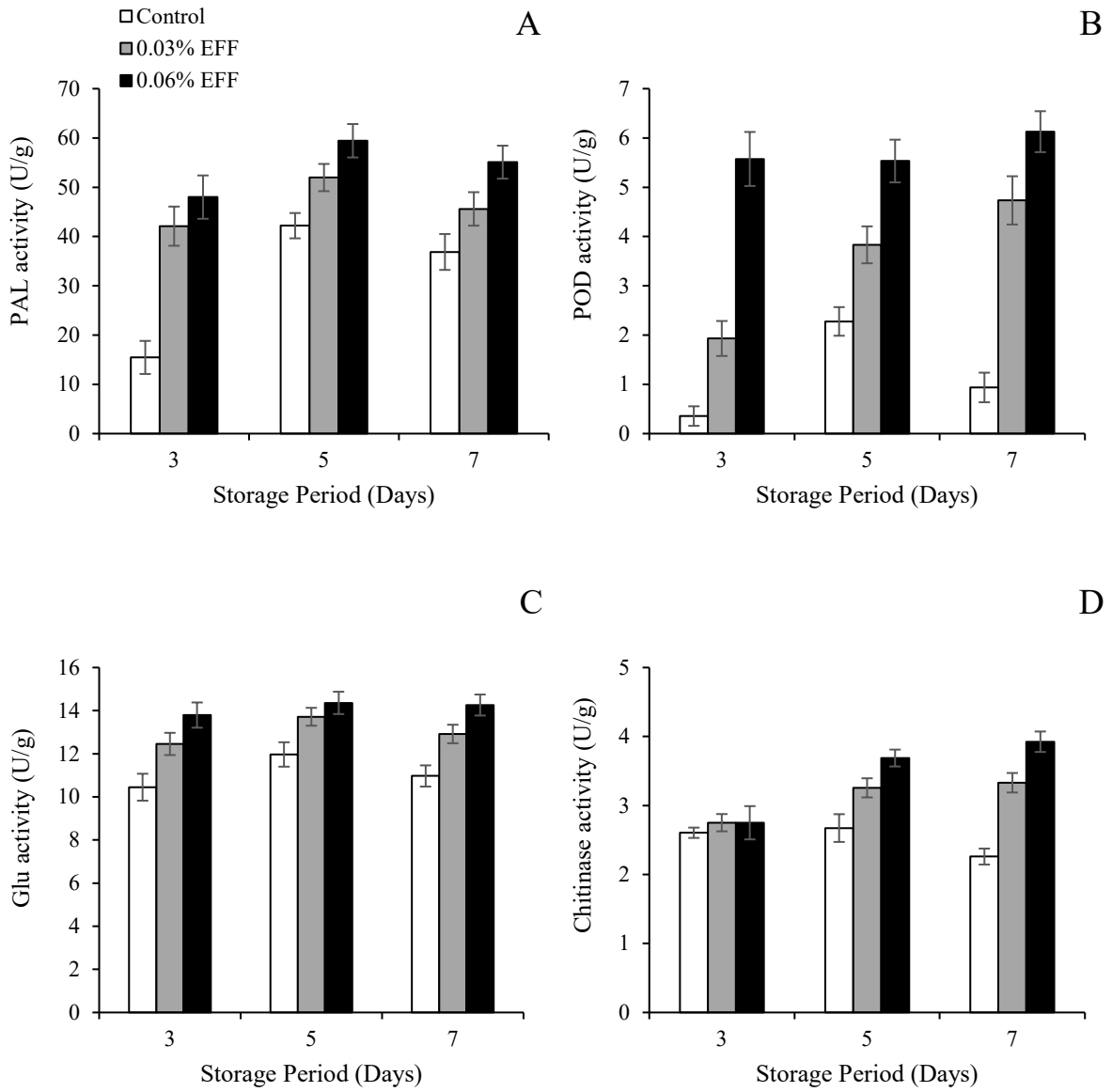


Figure 5.4. Effect of enhanced freshness formulation on phenylalanine ammonia lyase (PAL) (A), guaiacol peroxidase (POD) (B), β-1,3-glucanase (Glu) (C) and Chitinase (D) of 'Y368' kiwifruit after inoculation with *B. cinerea*. Values are the means ± SE (n = 3). Means with different letters indicate a statistically significant difference ($p < 0.05$).

2.8.5. Total Phenolic and flavonoid compounds

The treatments had a significant influence on TPC, with EFF-treated fruit exhibiting higher TPC. At 7 dpi, TPC was registered at 0.89, 1.06 and 1.12 mg GAE/g FW for the control, 0.03

and 0.06% EFF treatment level, respectively. Similar to TPC, the treatments had a substantial effect on the TFC, with treated fruit exhibiting greater TFC. At 7 dpi, no significant differences ($p > 0.05$) were observed between the control and 0.03% treatment level. However, the 0.06% treatment level remained substantially higher relative to the other two treatments. Phenolic and flavonoid compounds play a vital role in disease resistance by acting as toxins to the invading pathogen (Shao et al., 2010; Hyun et al., 2022). The EFF-treated fruit exhibited significantly greater concentrations of TPC and TFC (Figure 5.5), and lower fungal growth (Figure 5.2) compared to the control fruit.

This observation suggests that the enhancement of total phenolic and flavonoid compounds enhanced the kiwifruits resistance against *B. cinerea*. These findings are comparable to those reported by Dhakshinamoorthy et al. (2020), Hyun et al. (2022) and Zhang et al. (2023). These authors showed that the application of hexanal and (E)-2-hexenal enhanced disease resistance in banana fruit, kiwifruit and tomato plants, by inducing the synthesis of phenolic and flavonoid compounds. Hexanal and (E)-2-hexenal enhances the synthesis of phenolic and flavonoid compounds by upregulating the genes involved in the phenylpropanoid and flavonoid biosynthesis pathway (Padmanabhan et al., 2020; Hyun et al., 2022; Zhang et al., 2023). Therefore, we hypothesize that EFF induced disease resistance in kiwifruits by enhancing the concentrations of TPC and TFC, through the upregulation of genes involved in the phenylpropanoid and flavonoid biosynthesis pathways.

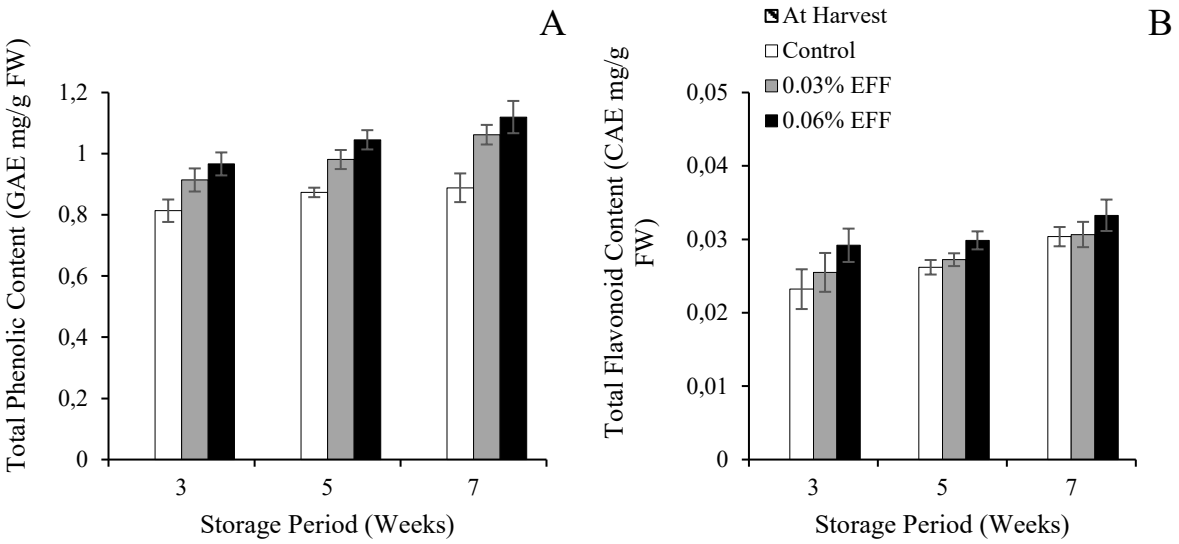


Figure 5.5. Effect of enhanced freshness formulation on total phenolics (A) and total flavonoids (B) of 'Y368' kiwifruit after inoculation with *B. cinerea*. Values are the means ± SE (n = 3). Means with different letters indicate a statistically significant difference ($p < 0.05$).

2.8.6. Principal component analysis

Principal component analysis (PCA) was performed to examine the relationship among the measured parameters and their response to the treatments over time. PCA was produced for the first two dimensions, where Dim1 and Dim2 accounted for 66.1 and 22.5% of the total variance, respectively (Figure 5.6). A scatter distribution of the data showed that the interaction between treatments and storage time distinguished the inoculated kiwifruit. Factor loadings are classified as strong, moderate and weak, when corresponding to loading values >0.75, 0.75 – 0.50 and 0.50 – 0.30, respectively (Liu et al., 2003). The PCA scores can be interpreted using factor loadings which represent the correlation between the principle component and the original variable (Mooi et al., 2017). Dim1 exhibited positive correlations for PAL (0.85), POD (0.96), Glu (0.96), CHI (0.85), TPC (0.88) and TFC (0.66) and negative correlations for lesion diameter (-0.64), disease incidence (-0.78) and disease index (-0.65) (Table 5.2).

The results show that EFF-treated kiwifruit was characterised by higher measurements of defence mechanisms, whereas control fruit exhibited greater fungal growth. These findings suggest that EFF had an influence on the defence mechanisms, with a greater effect at the 0.06% treatment level. Therefore, the results presented in the PCA biplot (Figure 5.6) further demonstrate that EFF treatment induced disease resistance in kiwifruit by enhancing the activities of defence-related enzymes (Figure 5.4) and the accumulation of TPC and TFC (Figure 5.5). These results are in accordance with findings from prior studies that demonstrated that hexanal and (E)-2-hexenal treatment induced disease resistance through these mechanisms (Seethapathy et al., 2016; Pravin et al., 2019; Dhakshinamoorthy et al., 2020 Dhakshinamoorthy et al., 2020; Hyun et al., 2022 and Zhang et al., 2023). Therefore, the outcomes of the current study show that EFF treatment has the potential to reduce the postharvest and economic losses of kiwifruit caused by *B. cinerea*. These findings suggest that EFF could serve as an eco-friendly alternative to chemical fungicides. The adoption of this treatment has the potential to reduce the reliance on agrochemicals, which may alleviate the pressure felt by kiwifruit producers from stringent MRL policies.

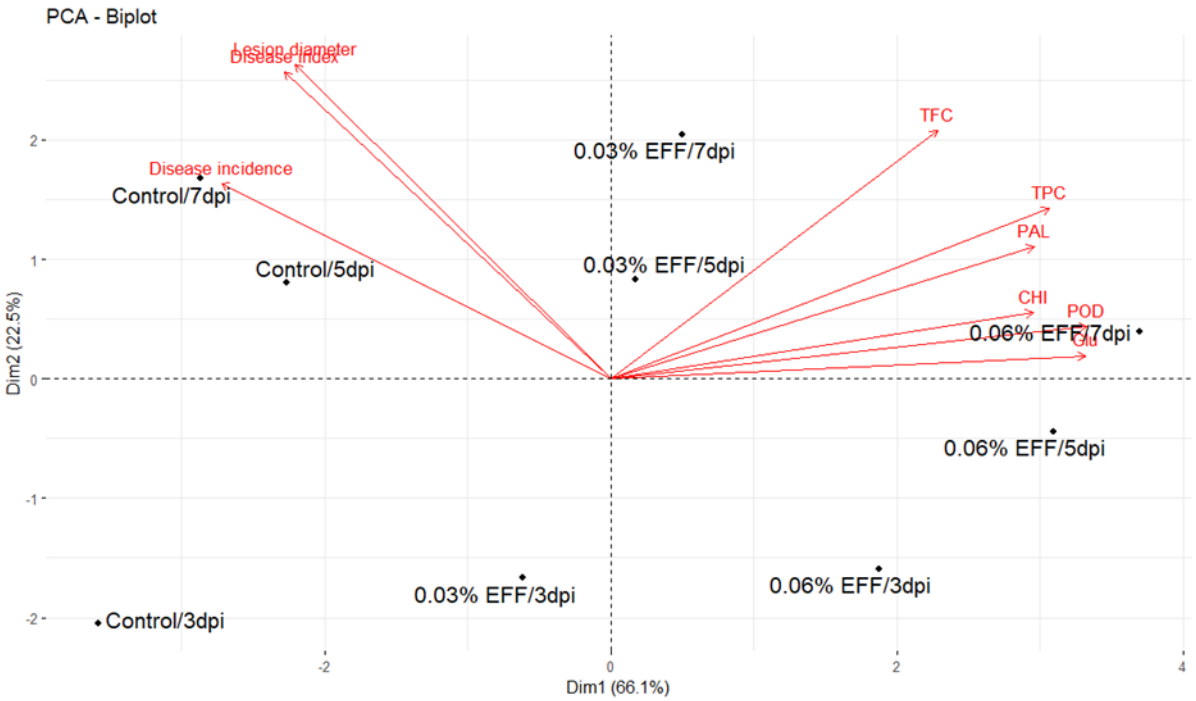


Figure 5.6. Principal component analysis biplot showing the correlations between the measured variables and factors (treatment and storage time). Dimension one (Dim1), Dimension two (Dim2), Enhanced freshness formulation (EFF), days post inoculation (dpi), total phenolic content (TPC), total flavonoid content (TFC), Phenylalanine ammonia-lyase (PAL), Guaiacol peroxidase (POD), β-1,3-glucanase (Glu) and Chitinase (CHI).

Table 5.2: Factor scores, loadings, eigenvalues and variance (%) for the first two dimensions (Dim1 and Dim2) based on fungal growth and defence mechanisms of EFF-treated 'Y368' kiwifruits.

	Factor scores	
Observation	**Dim1**	**Dim2**
Control/3dpi	-3.58	-2.05
0.03% EFF/3dpi	-0.61	-1.67
0.06% EFF/3dpi	1.87	-1.60
Control/5dpi	-2.27	0.81
0.03% EFF/5dpi	0.17	0.83
0.06% EFF/5dpi	3.09	-0.45
Control/7dpi	-2.87	1.68
0.03% EFF/7dpi	0.50	2.04
0.06% EFF/7dpi	3.69	0.40

Loadings		
Lesion diameter	-0.64	0.76
Disease incidence	-0.78	0.47
Disease index	-0.65	0.74
TPC	0.88	0.41
TFC	0.66	0.60
PAL	0.85	0.32
POD	0.96	0.13
Glu	0.96	0.06
CHI	0.85	0.16
Eigenvalue	5.95	2.03
Variance (%)	66.09%	22.52%
Cumalative (%)	66.09%	88.61%

Dimension one (Dim1), Dimension two (Dim2).

2.8.7. Quality parameters

2.8.7.1. Mass loss and firmness

Mass loss and softening are deteriorating factors that limit the quality and storage potential of fruit (Shiri et al., 2015; Xu et al., 2023). EFF effectively reduced the mass loss of treated kiwifruit, which is in accordance with findings obtained by Cheema et al. (2018). EFF treatment can reduce moisture loss by preserving the membrane integrity, which facilitates the retention of fruit mass (Paliyath and Subramanian 2008; Tiwari and Paliyath 2011). EFF-treated fruit had significantly higher firmness compared to the control fruit and similar findings were obtained by Sulaimankhil et al. (2021). The efficacy of EFF to optimise firmness of treated kiwifruit could be attributed to its capacity to suppress the action of cell wall softening enzymes (Gill et al., 2015).

2.8.7.2. TSS and TA

TSS is regarded as an index of fruit maturity and contributes to the flavour of kiwifruit (Meena et al., 2018). During fruit ripening, the conversion of starch into soluble sugars and an increase in moisture loss, results in the accumulation of TSS in kiwifruits (Xu et al., 2023). This is consistent with the findings of the current study, which demonstrated that kiwifruits with the highest TSS had the highest mass loss (Table 5.3). The results indicate that EFF can delay the accumulation of TSS by controlling moisture loss, which is in accordance with findings reported by Jincy et al. (2017). A loss in TA is attributed to the metabolic breakdown of organic

acids during respiration (Kaur et al. 2013). EFF delays the loss of TA by reducing fruit respiration (Yumbya et al., 2018). Therefore, we hypothesize that the higher TA values exhibited by treated kiwifruit may be attributed to the capacity of EFF to reduce respiration rates.

2.8.7.3. Ascorbic acid

Ascorbic acid is one of the widely recognised antioxidants that contributes to the nutritional quality of kiwifruit (Kumarihami et al., 2022). During storage, ascorbic acid is oxidised to monodehydroascorbate by the enzyme ascorbate oxidase (Chatzopoulou et al., 2020). Gill et al. (2015) reported that EFF retains ascorbic acid by decreasing the oxidation rate. Our findings corroborate those reported by Gill et al. (2015), who demonstrated that EFF-treated fruit maintained significantly higher ascorbic acid content.

Table 5.3: Effect of enhanced freshness formulation on the quality parameters of 'Y368' kiwifruit stored at 0 °C for eight weeks.

Treatment	Mass loss (%)	Firmness (N)	TSS (°Brix)	TA (%)	Ascorbic acid (mg/g FW)
Control	9.11 ± 0.538a	42.27 ± 3.786b	14.16 ± 0.201a	0.13 ± 0.005b	2.49 ± 0.227b
0.03 % EFF	7.14 ± 0.451ab	55.26 ± 3.055a	13.92 ± 0.321ab	0.14 ± 0.005a	3.37 ± 0.262ab
0.06 % EFF	6.12 ± 0.549b	59.29 ± 2.887a	13.25 ± 0.202b	0.15 ± 0.004a	3.88 ± 0.322a
p value	<0.05	<0.05	<0.05	<0.05	<0.05

Values are the means ± SE (n = 3). Means with different letters indicate a statistically significant difference ($p < 0.05$).

2.9. Conclusion

The exogenous application of EFF induced disease resistance against *B. cinerea* by enhancing defence mechanisms in kiwifruit. These findings suggest that EFF dip treatment has the potential to serve as an alternative treatment for controlling pathogen infection caused by *B. cinerea*. Furthermore, EFF optimised quality and preserved the nutritional value of kiwifruit under simulated export conditions. The present study focused on assessing the efficacy of inhibiting fungal growth using a protective method, which involves treating the fruit before

inoculation with the desired pathogen. This resulted in positive results but complete inhibition was not achieved. Further research should focus on attempting to achieve complete inhibition of *B. cinerea* by either using a curative method which involves applying a treatment after inoculation, testing with higher concentration levels or combining EFF with other cost-effective treatments.

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Chapter Six

Mechanism of Enhanced Freshness Formulation in Optimising Antioxidant retention of gold kiwifruit (*Actinidia chinensis*) harvested at two maturity stages

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Abstract

Kiwifruit exhibits a climacteric ripening pattern and has as an extremely perishable nature. Considering that high perishability leads to a loss in antioxidants and overall nutritional quality. This study aimed to examine the efficacy of enhanced freshness formulation (EFF), a hexanal-based formulation containing antioxidants such as geraniol, α-tocopherol and ascorbic acid, on maintaining the bioactive compounds of gold kiwifruit (cv. 'Y368') harvested at two maturity stages. Kiwifruits were treated with three treatments, namely, control (untreated fruit), 0.01 and 0.02 % (v/v) EFF. Fruits were treated with 8 weeks of cold storage at 0 ºC and 90 % relative humidity, then transferred to 20 °C for 8 days. Three bioactive compounds (ascorbic acid, total phenolics and flavonoids), antioxidant capacities using DPPH and FRAP assays, polyphenol oxidase, ascorbate oxidase, phenylalanine and tyrosine ammonia lyase enzyme activities were evaluated. The results showed that EFF significantly ($p < 0.05$) influenced bioactive compounds, antioxidant capacities and the activity of enzymes involved in the synthesis and oxidation of bioactive compounds. The maturity stage significantly influenced the content of bioactive compounds. Later harvested kiwifruit had greater content of bioactive compounds, compared to earlier harvested kiwifruit. The total phenolic content was 0.77, 1.09 and 1.22 mg GAE g^{-1} FW for control, 0.01 and 0.02 % EFF, respectively. The FRAP antioxidant concentration was 0.76, 0.91 and 0.96 µmol Fe (II) g^{-1} FW for control, 0.01 and

0.02 % EFF. The findings illustrate the capacity of EFF to optimise bioactive compounds and storability of kiwifruit during postharvest storage.

Keywords: Hexanal, postharvest quality, maturation, antioxidant capacity, gold kiwifruit

2.11. Introduction

The action of bioactivities including ascorbic acid, carotenoids and various flavanones and polyphenols contribute to the health beneficial properties of kiwifruit (Pérez-Burillo et al., 2018). Gold (yellow-fleshed) kiwifruit possesses significant nutritional value and an array of bioactive compounds which make it a good source of antioxidants. The high nutritional value of kiwifruit has resulted in an increasing global demand for kiwifruits (Ma et al., 2017). According to the Post-Harvest Innovation (PHI) Programme, it takes approximately 25 days to export South African fresh produce to Europe. However, deterioration of logistical infrastructure and shortage of refrigerated containers causes congestion and shipping delays at South Africa's ports (Brodie, 2022). In addition to this, the delivery of kiwifruit exported by sea is delayed by at least three weeks, due to delays that transpire at the European port (Eichstaedt, 2022).

Delays experienced along the supply chain result in extended storage duration of kiwifruit. These challenges present a problem since several kiwifruit cultivars have an extremely perishable nature due to their high metabolic activity (Asiche et al., 2017). This leads to a significant loss in bioactive compounds of kiwifruit (Huang et al., 2017; Cha et al., 2019; Kumarihami et al., 2020). The kiwifruit industry highly relies on cold storage, which facilitates in prolonging the storage of kiwifruit (Cha et al., 2019; Xia et al., 2020). However, cold storage alone is not enough to optimize quality if the storage duration is prolonged. Findings from previous research have illustrated that prolonged storage under cold conditions (0 -5 °C) results in a significant loss of bioactive compounds of kiwifruit (Goffi et al., 2019; Jeong et al., 2020; Xia et al., 2020; Choi et al., 2022). The importance of incorporating postharvest treatments with cold storage to reduce the loss of bioactive compounds is well established in kiwifruit.

High demand from European markets necessitates South African growers and exporters to develop strategies for preserving kiwifruit during extended storage periods, aiming to meet the increasing demand for high-quality kiwifruit with health-promoting characteristics. Fruit maturity is an essential factor that influences the concentration of bioactive compounds and the postharvest performance of kiwifruit (Lee et al., 2015; Nkonyane et al., 2022). Literature has demonstrated that kiwifruit should be harvested when the total soluble solid content is at a

minimum of 6.2 °Brix, in order to optimize fruit quality during storage (Burdon, 2015). Mahlaba et al. (2021) also determined that a minimum harvest and export value should be ≥ 6.2 °Brix for gold kiwifruit produced in South Africa. Thus, harvesting at the optimum maturity stage minimizes the loss of bioactive compounds. In spite of this, South African gold kiwifruits destined for export, are not harvested at a singular maturity stage but harvested at the mid (≥ 6.2 °Brix) and late (≥ 8.5 °Brix) maturity stages (Mahlaba et al., 2021; Nkonyane et al., 2022). However, these authors demonstrated that late harvested kiwifruit experience a substantial decrease in quality during storage. Therefore, it is essential to employ postharvest treatments that can maintain bioactive compounds of kiwifruit harvested at various maturity stages.

Past research has investigated the impact of various postharvest treatments such as 1-methylcyclopropene (Lim et al., 2016; Xu et al., 2021; Zhang et al., 2021), edible coatings (Allegra et al., 2016; Hu et al., 2019; Kumarihami et al., 2022), ozone (Goffi et al., 2019), UV-C (Hu et al., 2022) and heat treatment (Shahkoomahally and Ramezanian, 2015; Chiabrando et al., 2018) on the maintenance of bioactive compounds in kiwifruit. Notably, these studies predominantly focused on kiwifruit harvested at only one maturity stage. To the best of our ability, we have not found studies investigating the efficacy of postharvest treatments on optimizing bioactive compounds of gold kiwifruit harvested at different maturity stages.

Considering this gap in literature and the growing demand for high-quality kiwifruit, it is essential to explore a postharvest treatment that can optimize the bioactive compounds of kiwifruit harvested at various maturity stages. Hexanal is a naturally occurring volatile compound, specifically a six-carbon aldehyde formed from linoleic acid via the lipoxygenase pathway during lipid peroxidation in plants (Paliyath and Subramanian, 2008). Enhanced freshness formulation (EFF) is a formulation that comprises of 1% hexanal, 1% geraniol, 1% α-tocopherol and 1% ascorbic acid (Cheema et al., 2014). EFF has the capacity to optimize phenolics and ascorbic acid of various fruit (Cheema et al., 2014; Gill et al., 2015; Jincy et al., 2017). Hexanal's capacity to reduce the loss in bioactive compounds could serve as a potential tool of optimizing quality and storability of South African grown gold kiwifruit. South African gold kiwifruits are harvested at both the mid and late maturity stage. However, literature has illustrated that late harvested experience a substantial decrease in quality. Considering that the export of South African grown gold kiwifruit is expected to rise given the growing demand from international markets, this makes it necessary to devise a postharvest strategy that can maintain optimum quality of kiwifruit harvested at both maturity stages.

Therefore, the current study was conducted to evaluate the efficacy of EFF to optimize bioactive compounds (phenolic and flavonoid compounds, ascorbic acid and carotenoids) of gold kiwifruit (cv. 'Y368') harvested at two maturity stages. Furthermore, the activity of enzymes, phenylalanine ammonia-lyase, tyrosine ammonia-lyase, polyphenol oxidase and ascorbate oxidase, involved in the synthesis and oxidation of these bioactive compounds (phenolic and flavonoid compounds, ascorbic acid and carotenoids) were investigated.

2.12. Materials and methods

2.12.1. Fruit source

Kiwifruit (*Actinidia chinensis*) cv. 'Y368' were harvested from Roselands farm, a commercial kiwifruit farm located in the Richmond area (Latitude: 29.9033°S, Longitude: 30.2397°E), KwaZulu-Natal Province, South Africa. Kiwifruits were harvested when the total soluble solid content was greater than 6.2 °Brix (Mahlaba et al., 2021), at two maturity stages namely, maturity stage one (M1) and maturity stage two (M2) with 7 and 9 °Brix, respectively. Harvested fruit were immediately transported in a ventilated vehicle to the Postharvest Laboratory of the University of KwaZulu-Natal, where kiwifruit without blemishes, decay or physical damage were graded and selected for uniformity in size, then assigned to the respective postharvest treatments.

2.12.2. Preparation of postharvest treatments and storage

The enhanced freshness formulation (EFF) was prepared by making a stock formulation comprising of 1 % (v/v) hexanal, 1 % (v/v) geraniol, 1 % (w/v) α-tocopherol, 1 % (w/v) ascorbic acid, 0.1 % (w/v) cinnamic acid and 10 % (v/v) Tween 20 dissolved in ethanol (10 % v/v) (Cheema et al., 2014). A 0.01 and 0.02 % (v/v) hexanal concentrations were prepared by mixing the stock solution in 100 L and 50 L of distilled water, respectively. Kiwifruit were immersed in 0.01 and 0.02 % (v/v) EFF solution for 2.5 min, then air dried at room temperature (Cheema et al., 2014). Control fruit were left untreated. Each treatment consisted of three replicates (n = 3); each replicate had three fruit per storage interval for each maturity stage. The kiwifruits were stored for 8 weeks in a cold room with temperature set at 0 °C and relative humidity at 90%, then transferred to 20 °C for one week to stimulate shelf life.

2.12.3. Total Phenolic Content (TPC)

The total phenolic content was determined using a method described by Singleton et al. (1999). Kiwifruit pulp (1 g) was homogenized with 5 mL of 80 % methanol. Thereafter, 2.6 mL of distilled water and 200 µL of Folin-Ciocalteu's phenol reagent was added to 200 µL of the

methanolic extracts. After six minutes, 2 mL of 7 % (w/v) sodium carbonate was added to the reaction mixture. The absorbance was measured at 750 nm after 90 min using a Shimadzu UV spectrophotometer ((Model UV-1800 240V, Kyoto, Japan). The total phenolic content was expressed as mg gallic acid equivalents GAE g^{-1} fresh weight (FW) of kiwifruit.

2.12.4. Total Flavonoid Content (TFC)

Distilled water (3.2 mL) and 150 µL of 5% (w/v) sodium nitrite was added to 500 µL of methanolic extract. After five minutes, 150 µL of 10% (w/v) aluminium chloride was added. After six minutes, 1 mL of 1M sodium hydroxide was added and the absorbance recorded at 510 nm. The total flavonoid content was expressed as mg catechin equivalents CE g^{-1} fresh FW of kiwifruit.

2.12.5. Ascorbic Acid (AA)

A method described by Malik and Singh. (2005) and modified by Goffi et al. (2019), was used to determine the ascorbic acid content of kiwifruit. Ascorbic acid was extracted from 1 g of kiwifruit pulp using 4 mL of 16 % (v/v) metaphosphoric acid, containing 0.18 % (w/v) disodium ethylenediaminetetraacetic acid and centrifuged at 1100 x g for ten minutes at 4 °C. The assay mixture contained 200 µL supernatant, Folin's reagent (1 : 5 v/v) and 0.3% (v/v) metaphosphoric acid in a final volume of 2 mL. The absorbance was measured at 760nm, using ascorbic acid as standard and expressed as mg g^{-1} FW.

2.12.6. Total carotenoids (TC)

Total carotenoid content was determined using a method adapted from Yan et al. (2015) and modified by Xia et al. (2020). Five mL of acetone containing 0.1% butylated hydroxytoluene was used to extract carotenoids from one gram of kiwifruit pulp. After centrifugation at 10 000 x g for ten minutes at 4 °C, the supernatant was filtered through the Whatman filter paper No. 42. The total carotenoid content was determined by measuring the absorbance of the extract at 450 nm and expressed as µg g^{-1} FW.

2.12.7. DPPH Radical-Scavenging Activity (DPPH)

DPPH was assessed using a protocol described by Brand-Williams et al. (1995). Thus, 6 x 10^{-5} mol/L DPPH was dissolved in methanol (1 L) and 3.9 mL of this solution was added to 100 µL of methanolic extracts. After 30 minutes at room temperature, the absorbance of the reaction mixture was measured at 517 nm. The DPPH radical scavenging activity was quantified using Eq. 1 and expressed as percentage DPPH radical-scavenging activity (DPPH %)

$$(\%) = [Absorbance(control) - Absorbance(sample)] / (Absorbance(control)) \times 100 \quad 1$$

2.12.8. Ferric-Reducing Antioxidant Power (FRAP)

The FRAP was assessed using a previously reported protocol (Benzie and Strain, 1996). The FRAP working solution was prepared daily by mixing 300 mM acetate buffer (pH 3.6), 10 mM TPTZ solution in 40 mM HCl, and 20 mM $FeCl_3$ solution at a ratio of 10:1:1. The freshly solution was warmed at 30 °C before usage. Then, 100 µL of each kiwifruit pulp extract was mixed with 3 mL of FRAP solution and incubated at 37 °C for four minutes. Thereafter, the absorbance was recorded at 593 nm. A calibration curve was prepared using ferrous sulphate. The findings were expressed as µM g^{-1} FW.

2.12.9. Phenylalanine Ammonia Lyase (PAL) and Tyrosine Ammonia Lyase (TAL) extraction

The enzymatic activity of PAL (EC 4.3.1.5) and TAL (EC 4.3.1.25) was evaluated using a method described by Khan et al. (2003). Extraction was carried out by homogenising one gram of kiwifruit pulp with 10 mL of ice-cool Tris-HCl buffer (50 mM, pH 8.5) comprising of 5% (w/v) polyvinylpolypyrrolidone (PVPP) and 14.4 mM β-mercaptoethanol. After centrifugation at 10 000 g at 4°C for 20 minutes, the enzyme activity of PAL and TAL was quantified using the recovered supernatant. Bradford assay (1976) was used to determine the protein concentration.

2.12.9.1. Phenylalanine Ammonia Lyase (PAL)

The activity of PAL was quantified using an assay mixture containing 800 µL of Tris-HCl buffer (0.5 mM, pH 8.0), 100 µL 6 µM of L-phenylalanine, and 100 µL of enzyme extract. After incubation at 40 °C for an hour, the reaction was stopped by adding 100 µL of 5 N HCl to the mixture. The enzyme activity was quantified using the absorbance measured at 290 nm. The activity was expressed as U g^{-1} of protein, where a unit of enzyme activity was defined as the amount of enzyme that produces 1 nmole of cinnamic acid min^{-1}.

2.12.9.2. Tyrosine Ammonia Lyase (TAL)

The activity of TAL was quantified using an assay mixture containing 800 µL of Tris-HCl buffer (0.5 mM, pH 8.0), 100 µL of 5.5 µM of L-tyrosine and 100 µL of enzyme extract. After incubation at 40 °C for an hour, the reaction was stopped by adding 100 µL of 5 N HCl to the mixture. The enzyme activity was quantified using the absorbance measured at 333 nm. The

activity was expressed as U g^{-1} of protein, where a unit of enzyme activity was defined as the amount of enzyme that produces 1 nmole of coumaric acid min^{-1}.

2.13. 2.10. Ascorbate oxidase (AO)

The enzyme activity of ascorbate oxidase (EC 1.10.3.3) was assayed using a previously described protocol by Jiang et al. (2018). AO was extracted by homogenising one gram of pulp with 3 mL potassium phosphate buffer (50 mM, pH 7.5) comprising of 1 mL of potassium phosphate buffer (100 mM, pH 6.8), 0.1mM ethylenediaminetetraacetic acid (EDTA), 0.3% (w/v) Triton X-100, 4% (w/v) polyvinyl-polypyrrolidone (PVPP), and 0.1mM ascorbic acid. After centrifugation at 16 000 g for 15 minutes, the absorbance was measured. The enzyme activity was expressed as U g^{-1} of protein and defined as the amount of enzyme causing an increase of 0.01 in the absorbance per min at 265 nm at 25 °C. The Bradford assay (1976) was used to determine the protein concentration.

6.3.2.11. Polyphenol oxidase activity (PPO)

A protocol by Adiletta et al. (2019) was used to assay PPO (EC 1.14.18.1) activity. One gram of pulp was homogenised in sodium phosphate buffer (100 mM, pH 6.4) comprising of 0.05 g of PVPP. After centrifugation at 10 000 g for 30 minutes at 4 °C, 100 µL of the enzyme extract was added to a buffered substrate (500 mM catechol in 100 mM sodium phosphate buffer, pH 6.4) and the increase in absorbance was monitored at 398 nm. PPO activity was expressed as U g^{-1} of protein. The Bradford assay (1976) was used to determine the protein concentration.

6.2.12. Statistical analysis

All experiments were performed using a completely randomized factorial design. The experiment comprised of three factors: treatments, fruit maturity stage and storage period. Statistical analysis was performed in R software version 4.3.1 (R Development Core Team, 2023). Data were expressed as mean values ± SE and were subjected to analysis of variance (ANOVA) with a 5 % level of significance. A Pearson correlation test was used to correlate the measured parameters.

6.3. Results and discussion

2.13.1. Total phenolic (TPC) and flavonoid content (TFC)

The interaction among treatment, maturity stage and storage time had no significant ($p > 0.05$) effect on the TPC. The results illustrate that treatments significantly influenced ($p < 0.001$) the TPC (Figure 6.1 A), with treated fruit registering substantially greater TPC compared to the control. The TPC was 0.88, 1.17 and 1.29 mg GAE g^{-1} FW for the control, 0.01 and the 0.02

% EFF treatment level, respectively. The interaction between treatment and storage time had no significant effect ($p > 0.05$) on TPC. However, the interaction between maturity stage and storage time (Figure 6.1 B) had a significant ($p < 0.001$) effect on TPC, with M2 registering significantly higher TPC than M1. Kiwifruit at both maturity stages exhibited an increase in TPC, reaching maximum values of 1.21, and 1.58 mg GAE g^{-1} FW for M1 and M2, respectively, at six weeks of storage. At the end of storage, TPC for M1 and M2 was 1.06 and 1.19 mg GAE g^{-1} FW, respectively.

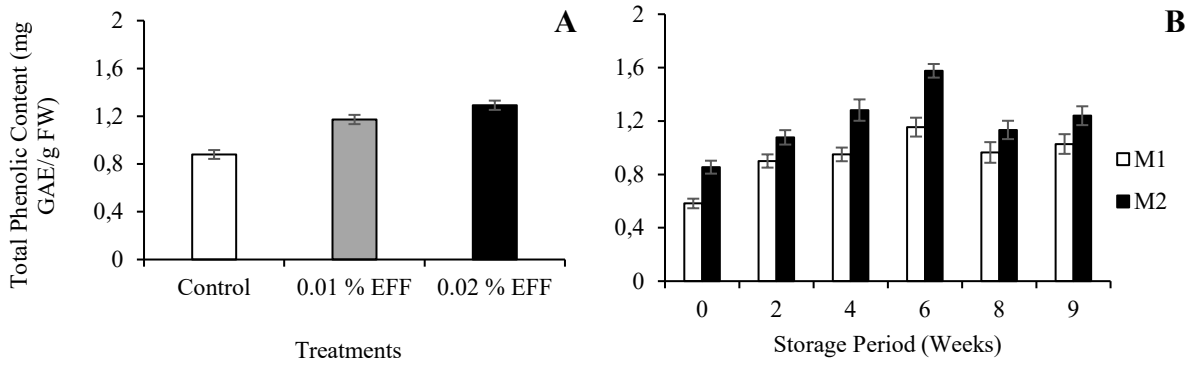
The interaction among treatment, maturity stage and storage time had no significant ($p > 0.05$) effect on the TFC. The interaction between treatment and storage time had no significant effect ($p > 0.05$) on TFC. However, the combined effect of treatment and maturity stage (Figure 6.1 C) significantly ($p < 0.001$) influenced the TFC. Treated fruit exhibited significantly higher TFC compared to the control. Maturity stage influenced the TFC, with M2 exhibiting significantly greater TFC relative to M1. Significant differences among treated fruit were only observed at M2, with 0.02 % EFF registering higher TFC. At M1, no significant differences among treated fruit were observed. In addition, the TFC significantly ($p < 0.001$) increased throughout the storage period (Figure 6.1 D).

The findings of this study illustrated that storage duration affects phenolic content of Y368 kiwifruit. This is characterised by a significant decrease in TPC after six weeks of storage. These findings corroborate those of Lim and Eom. (2018); Goffi et al. (2019) and Jeong et al. (2020), who demonstrated that TPC in kiwifruit decreased during cold storage. Furthermore, Gullo et al. (2016) and Nkonyane et al. (2022), attained comparable findings to the present study. These authors illustrated that kiwifruit exhibited an increase in TPC when transferred to ambient conditions. Results obtained from this study demonstrated that EFF-treated kiwifruit had substantially higher TPC and TFC during storage. The findings attained are in alignment to those reported by Gill et al. (2015), Sharma et al. (2023) and Öz et al. (2023), who demonstrated that EFF effectively maintained higher phenolic content of guava, jujube and persimmon fruit, respectively. Secondary metabolites such as phenolic and flavonoid compounds are crucial for the maintenance of fruit quality, as they delay senescence induced by oxidative degradation, by functioning as antioxidants that scavenge free radicals (Gulcin, 2020). Therefore, the efficacy of EFF to enhance TPC and TFC may contribute to optimised quality and storability of kiwifruit during storage.

2.13.2. Ascorbic acid (AA)

The interaction among treatment, maturity stage and storage time had no significant ($p > 0.05$) effect on AA. The interaction between treatment and storage time had no significant effect ($p > 0.05$) on AA. The treatments had a significant effect ($p < 0.001$) on AA content (Figure 6.1 E), with the 0.02 % treated fruit registering higher AA content, followed by 0.01 % treated fruit. AA content for the control, 0.01 and 0.02 % EFF treatment level was 2.72, 3.12 and 3.34 mg g^{-1} FW. These findings indicate that EFF treatment application effectively retained AA content throughout the storage period. Our results corroborate those reported by Cheema et al. (2014), Gill et al. (2015), Jincy et al. (2017), Silué et al. (2022) and Sharma et al. (2023) who illustrated that EFF retained ascorbic acid of tomato, guava, mango and jujube fruit, respectively. The maturity stage had a significant effect ($p < 0.001$) on AA content (Figure 6.1 F), with M2 (3.53 mg g^{-1} FW) exhibiting a greater AA content than M1 (2.54 mg g^{-1} FW).

Findings from the present study are in alignment with those of Lim and Eom. (2018), who illustrated that AA content increased with an advancement in maturity. In plants, AA preserves cell integrity by reducing hydrogen peroxide and reacting rapidly with radical species (Dumanović et al., 2021). Thus, it provides DNA and lipids with protection from oxidative damage (Meitha et al., 2020). The loss of AA in kiwifruit during storage is estimated to be between 50 – 70% (Sharma et al., 2015). AA undergoes oxidative loss as it is converted to dehydroascorbic acid by the enzymatic action of ascorbate oxidase (Chatzopoulou et al., 2020). EFF has been reported to delay the loss of AA in guava fruit by reducing the oxidation process (Gill et al., 2015). Therefore, the findings from the present study show that EFF has the potential to preserve the nutritional value of kiwifruit by delaying the loss of AA during storage.



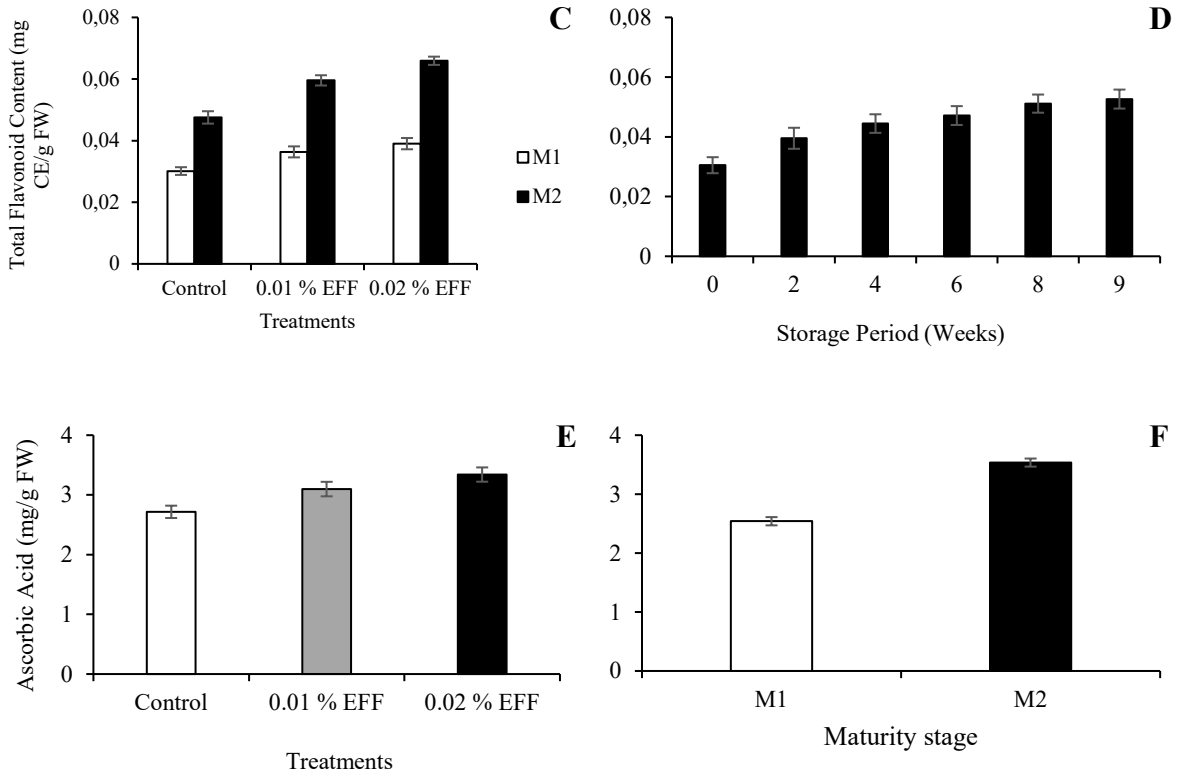


Figure 6.1. Effect of treatments (A) and the effect of interaction between maturity stage and storage time (B) on the total phenolic content of kiwifruit. Effect of interaction between treatments and maturity stage (C) and the effect of storage time (D) on the total flavonoid content of kiwifruit. Effect of treatments (E) and maturity stage (F) on ascorbic acid content of kiwifruit. Values are the means ± SE (n = 3).

2.13.3. Total carotenoids (TC)

The interaction among treatment, maturity stage and storage time had no significant ($p > 0.05$) effect on TC. Treated kiwifruit exhibited significantly ($p < 0.01$) lower TC, compared to the control (Figure 6.2 A). The TC was registered at 5, 4.78 and 4.71 µg g^{-1} FW for the control, 0.01 and 0.02% treatment level, respectively. The interaction between treatment and storage time had no significant effect ($p > 0.05$) on TC. The interaction between maturity stage and storage period significantly ($p < 0.05$) influenced the TC content (Figure 6.2 B). The TC significantly decreased after four weeks of storage for kiwifruit harvested at M1. TC significantly decreased after two weeks for kiwifruit harvested at M2. Throughout the storage period, kiwifruit harvested at M2 exhibited greater TC relative to M1.

The antioxidant properties of carotenoids provide protection from certain kinds of cancer. EFF treated fruit exhibited less carotenoid content relative to the untreated kiwifruit. Similarly, Tiwari and Paliyath. (2011) and Dek et al. (2018) showed that EFF lowered the accumulation

of carotenoids in tomato fruit. This may be indicative of better-quality maintenance due to a slower rate of ripening (Cheema et al., 2018). Storage temperature has a strong influence on carotenoid metabolism (Matsumoto et al., 2009). Exposing kiwifruit to low temperatures slows down the accumulation of carotenoids, whereas high temperatures enhance the accumulation of carotenoids (Xia et al., 2020). Low storage temperatures upregulate the expression of cleavage dioxygenase and 9-cis-epoxycarotenoid dioxygenase involved in the degradation of carotenoids, resulting in a decrease in carotenoid content (Xia et al., 2020).

2.13.4. Antioxidant capacity

The interaction between treatment, maturity stage and storage time had no significant ($p > 0.05$) effect on DPPH. The interaction between treatment and storage time had no significant effect ($p > 0.05$) on DPPH. Treatments had a significant effect ($p < 0.001$) on DPPH scavenging activity (Figure 6.2 C). Treated fruit had higher DPPH scavenging activity relative to the control. The maturity stage had a significant ($p < 0.01$) effect on DPPH scavenging activity (Figure 6.2 D). Kiwifruit harvested at M2 exhibited greater DPPH scavenging activity compared to kiwifruit harvested at M1. The interaction among treatment, maturity stage and storage time had no significant ($p > 0.05$) effect on the Ferric Reducing Antioxidant Power (FRAP). The interaction between treatment and storage time had no significant effect ($p > 0.05$) on FRAP. Treatments had a significant effect ($p < 0.001$) on FRAP (Figure 6.2 E). The control, 0.01 % and 0.02 % EFF treatment level had 0.68, 0.87 and 0.95 µmol Fe (II) g^{-1} FW, respectively. The interaction between maturity stage and storage time had a significant effect ($p < 0.05$) on the FRAP (Figure 6.2 F), with M2 registering significantly greater FRAP relative to M1. The FRAP significantly increased and decreased during cold storage, followed by an increase during shelf life. The findings are comparable with those attained by Jincy et al. (2017) and Öz et al. (2023), where hexanal treated mango and persimmon fruit had higher antioxidant capacity.

Bioactive compounds such as ascorbic acid, polyphenols and flavonoids in plants have a significant influence on the antioxidant capacity (He et al., 2019). Thus, DPPH and FRAP assays were performed to assess the antioxidant capacity of these bioactive compounds. EFF treatment application substantially ($p < 0.05$) increased the antioxidant capacities, which is in accordance with results attained by Jincy et al. (2017), for EFF-treated mango fruit. Therefore, the enhanced antioxidant capacity exhibited by EFF-treated kiwifruit may be owed to a higher content of bioactive compounds in treated fruit (Figure 6.1). Furthermore, the findings of this study show that the maturity stage significantly influenced the antioxidant capacity of kiwifruit

(Figure 6.2 D, F). Kiwifruit harvested at M2 exhibited greater antioxidant capacity than kiwifruit harvested at M1. Considering that bioactive compounds influence antioxidant capacity, these findings indicate that the higher antioxidant capacities of kiwifruit harvested at M2 is attributed to their higher concentration of bioactive compounds (Figure 6.1). These results are in alignment with those of Lim and Eom (2018), who demonstrated that kiwifruit harvested at later maturity stages exhibited a greater concentration of bioactive compounds. The findings obtained in the current study, indicate that EFF treatment effectively optimised the bioactive compounds and antioxidant capacity in kiwifruit at both maturity stages.

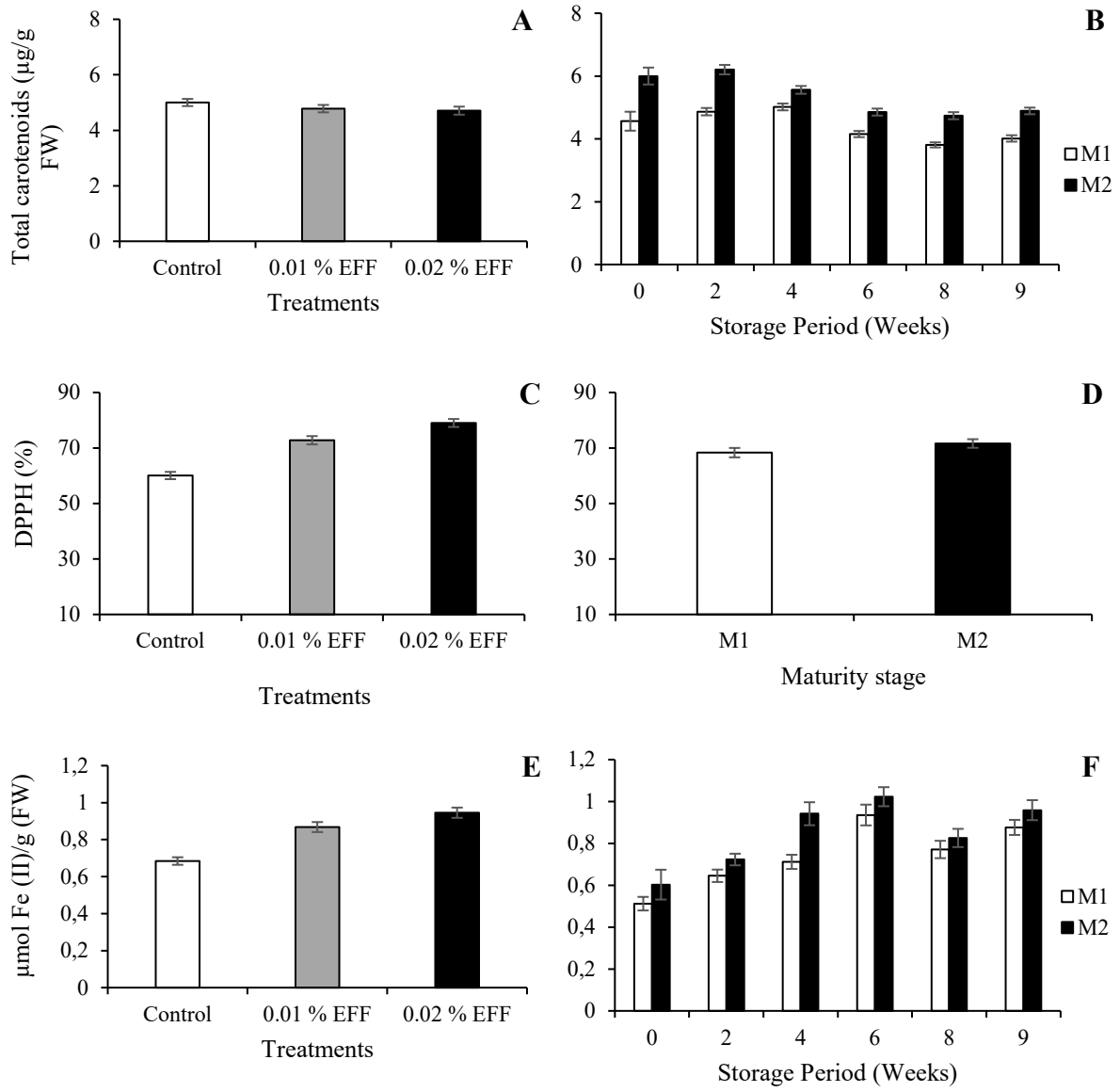


Figure 6.2. Effect of treatments (A) and the effect of interaction between maturity stage and storage time (B) on the total carotenoid content of kiwifruit. Effect of treatments (C) and maturity stage (D) on DPPH scavenging activity of kiwifruit. Effect of treatments (E) and the

effect of interaction between maturity stage and storage time (F) on FRAP of kiwifruit. Values are the means ± SE (n = 3).

2.13.5. Phenylalanine Ammonia-Lyase (PAL) and Tyrosine Ammonia-Lyase (TAL)

The interaction among treatment, maturity stage and storage time had no significant ($p > 0.05$) effect on PAL activity. The interaction between treatment and storage time had no significant effect ($p > 0.05$) on PAL. Treatments significantly ($p < 0.001$) influenced the PAL enzyme activity (Figure 6.3 A). The control, 0.01 % and 0.02 % EFF treatment level registered 1.11, 1.32 and 1.41 U g^{-1} FW, respectively. The maturity stage had a significant ($p < 0.001$) effect on PAL activity (Figure 6.3 B), with kiwifruit harvested at M2 (1.46 U g^{-1} FW) exhibiting greater PAL activity compared to kiwifruit harvested at M1 (1.08 U g^{-1} FW). The interaction among treatment, maturity stage and storage time had no significant ($p > 0.05$) effect on TAL. The interaction between treatment and storage time had no significant effect ($p > 0.05$) on TAL. The treatments significantly ($p < 0.001$) influenced TAL activity (Figure 6.3 C), with treated fruit exhibiting higher TAL enzyme activity relative to the control. A significant difference was not observed among treated fruit. The interaction between maturity stage and storage period had a significant effect ($p < 0.001$) on the TAL activity (Figure 6.3 D).

Results obtained from this study are in accordance with those reported by Bai et al. (2014) and Dhakshinamoorthy et al. (2020), who illustrated that hexanal treatment enhanced the PAL activity of oriental sweet melon and banana fruit, respectively. To the best of our ability, we have not found research studies reporting on the effect of hexanal or EFF on TAL enzyme activity. The phenylpropanoid biosynthesis pathway is a metabolic pathway in plants that utilities phenylalanine and in some instances tyrosine to synthesize secondary metabolites (de Barros Rates and Cesarino, 2023). This pathway is governed by the enzymatic action of PAL, which is involved in the synthesis of phenolic compounds such as phenolic acids and flavonoids (Barros et al., 2015; Bian et al., 2020). Similar to PAL, TAL is an enzyme in the phenylpropanoid biosynthesis pathway involved in the conversion of tyrosine to *p*-coumarate, and it also facilitates the production of secondary metabolites (Yadav et al., 2020).

The findings in this study suggest that EFF influenced the phenylpropanoid biosynthesis pathway by enhancing PAL and TAL enzyme activities in treated kiwifruit. Considering that PAL and TAL participate in the synthesis of phenolic compounds, the higher TPC and TFC (Figure 6.1 A and C) in EFF-treated kiwifruit, may be attributed to the ability of EFF to enhance

PAL and TAL enzyme activities. Previous research studies have shown that hexanal upregulates the expression of genes that encode for PAL enzyme activity (Padmanabhan et al., 2020; Hyun et al., 2022). Thus, we hypothesize that the enhanced enzyme activity of PAL in EFF-treated kiwifruit may be due to the upregulation of PAL genes in response to EFF treatment. However, further research investigating the response of gene expression to EFF-treated kiwifruit is warranted to strengthen this argument.

2.13.6. Ascorbate oxidase (AO)

The interaction between treatment, maturity stage and storage time had a significant ($p < 0.001$) effect on AO enzyme activity (Figure 6.3 E and F). Kiwifruit harvested at M1 had higher AO activity (control = 9.93 U g^{-1} FW, 0.01 % EFF = 6.11 U g^{-1} FW, 0.02 % EFF = 4.21 U g^{-1} FW) in comparison to kiwifruit harvested at M2 (control = 9.69 U g^{-1} FW, 0.01 % EFF = 4.50 U g^{-1} FW, 0.02 % EFF = 3.91 U g^{-1} FW) at week nine of storage. The treatments significantly ($p < 0.001$) influenced the AO activity, with the treated fruit exhibiting significantly lower AO enzyme activity compared to the control. Considering that AO is involved in the degradation of ascorbic acid (Chatzopoulou et al., 2020), the efficacy of EFF to optimise retention of ascorbic acid (Figure 6.1 E) may be attributed to its capacity to suppress the enzymatic activity of AO. A qRT-PCR analysis conducted by El Kayal et al. (2017) demonstrated that EFF treatment application downregulated the gene expression of AO in strawberry fruit. Thus, we hypothesize that the mechanism in which EFF suppresses the enzymatic activity of AO is due to its ability to downregulate the gene expression of AO, which in turn, leads to a reduction in the oxidation of ascorbic acid.

6.3.6.7. Polyphenol oxidase activity (PPO)

The interaction among treatment, maturity stage and storage time had no significant ($p > 0.05$) effect on PPO enzyme activity. The interaction between treatment and storage time had no significant effect ($p > 0.05$) on PPO. The treatments significantly ($p < 0.001$) influenced the PPO activity (Figure 6.3 G). The control had the highest PPO activity, followed by the 0.01 % and 0.02 % treatment level. The interaction between maturity stage and storage period had a significant effect ($p < 0.01$) on the PPO enzyme activity (Figure 6.3 H). Kiwifruit harvested at M2 exhibited significantly higher PPO activity than those harvested at M1. PPO was 0.13 and 0.16 U g^{-1} FW for M1 and M2 at week 9 of storage, respectively.

The findings of the present study are in accordance with those reported by Kaur et al. (2019) and Sharma et al. (2023), who demonstrated that EFF suppressed the PPO enzyme activity in

oriental sweet melons, grape berries and jujube fruit, respectively. PPO is a terminal oxidase that utilises polyphenols as substrates, which leads to internal browning of fruit. Furthermore, the enzymatic activity of PPO can be inhibited by aliphatic alcohols (Valero et al., 1990). Past research has demonstrated that hexanal in fruit tissues is converted to hexanol which is an aliphatic alcohol (Sharma et al., 2008). The lower PPO enzyme activity exhibited in treated fruit may be attributed to the conversion of hexanal to hexanol in kiwifruit tissues after EFF treatment application. To the best of our abilities, we have not found studies investigating the capability of hexanol to inhibit PPO enzyme activity. Therefore, further research must be conducted to examine if the hexanol volatile compound has an inhibitory effect on the enzymatic activity of PPO.

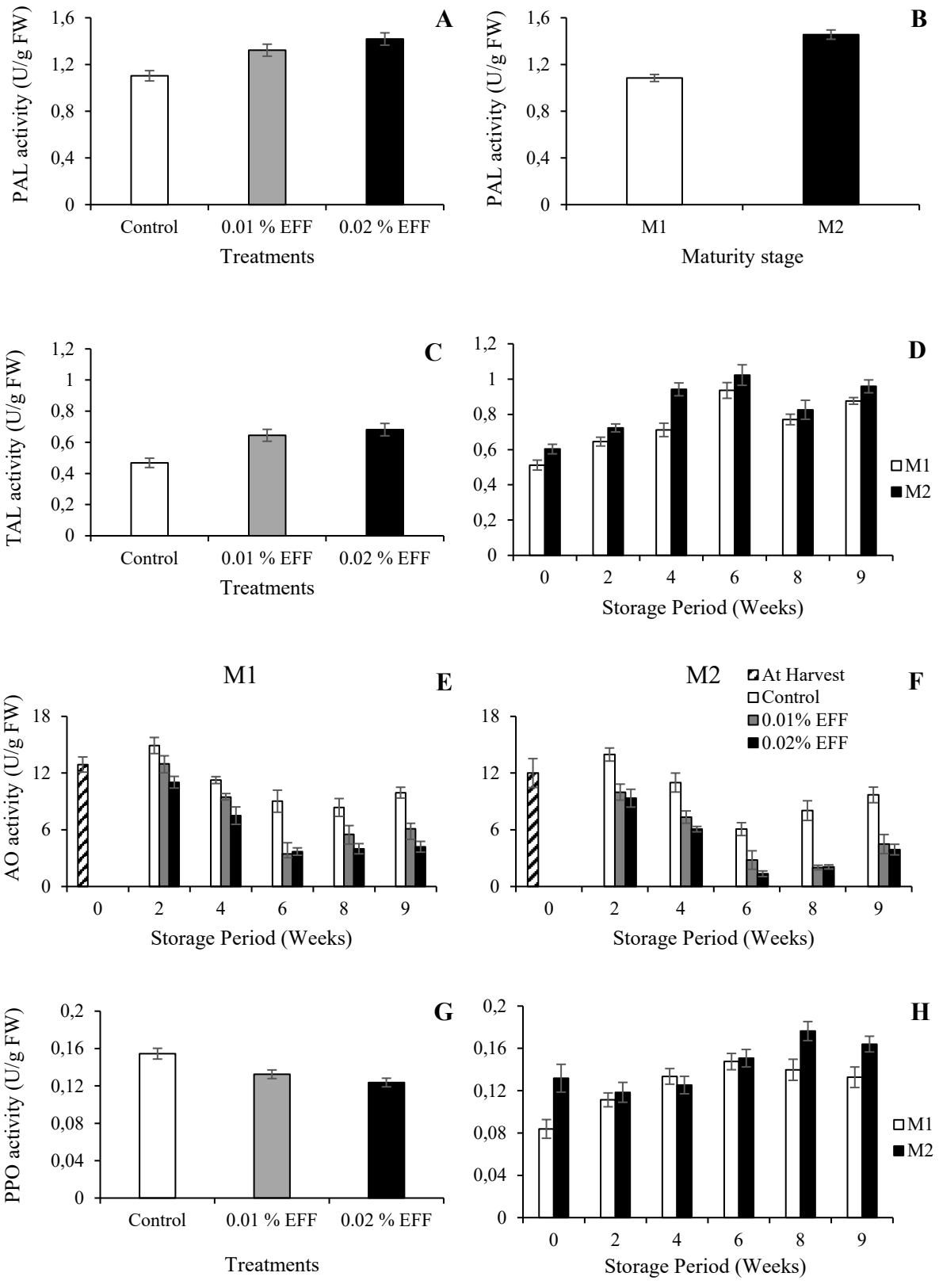


Figure 6.3. Effect of treatments (A) and maturity stage (B) on PAL activity of kiwifruit. Effect of treatments (C) and the effect of interaction between maturity stage and storage time (D) on TAL activity of kiwifruit. The effect of interaction among treatments, maturity stage and

storage time on AO activity of kiwifruit harvested at M1 (E) and M2 (F). Effect of treatments (G) and the effect of interaction between maturity stage and storage time (H) on PPO activity of kiwifruit. Values are the means ± SE (n = 3).

6.3.6.8. Pearson correlation coefficient

A correlation analysis was done to examine the relationships between bioactive compounds on antioxidant capacity and the enzymes (Figure 6.4). The DPPH scavenging activity exhibited a significant and positive correlation with TPC ($r = 0.67$; $p < 0.001$), TFC ($r = 0.62$; $p < 0.001$) and AA ($r = 0.38$; $p < 0.001$). FRAP showed a significant and positive correlation with TPC ($r = 0.81$; $p < 0.001$), TFC ($r = 0.67$; $p < 0.001$) and AA ($r = 0.50$; $p < 0.001$). The correlation between PAL activity, TPC and TFC was 0.78 ($p < 0.001$) and 0.76 ($p < 0.001$), respectively. This indicates that TPC and TFC is strongly correlated with PAL enzyme activity. TAL activity exhibited a strong positive correlation with TPC ($r = 0.71$; $p < 0.001$) and a weak correlation with TFC ($r = 0.39$; $p < 0.001$). AA content and AO enzyme activity exhibited a significant but moderately negative correlation ($r = -0.44$; $p < 0.001$).

The results from the correlation analysis show that bioactive compounds have a direct influence on the antioxidant capacity of kiwifruit, which is in alignment with findings reported by He et al. (2019). Furthermore, the correlation analysis indicates that the accumulation of phenolic compounds is associated with the enzymatic activity of PAL and TAL. These findings are corroborated by those reported by Khan et al. (2003). Furthermore, the inverse relationship between AA and AO suggests that AO has an influence in the degradation of AA (Jiang et al., 2018). These findings indicate that the bioactive compounds in kiwifruit can be optimised by enhancing the activities of enzymes involved in the synthesis of phenolic compounds and suppressing the enzyme (AO) involved in the degradation of AA. Therefore, the efficacy of EFF to optimise the bioactive compounds in kiwifruit, could be attributed to its capacity to enhance and suppress the activity of enzymes involved in the synthesis and oxidation of these bioactive compounds. Which in turn improves kiwifruit quality and storability.

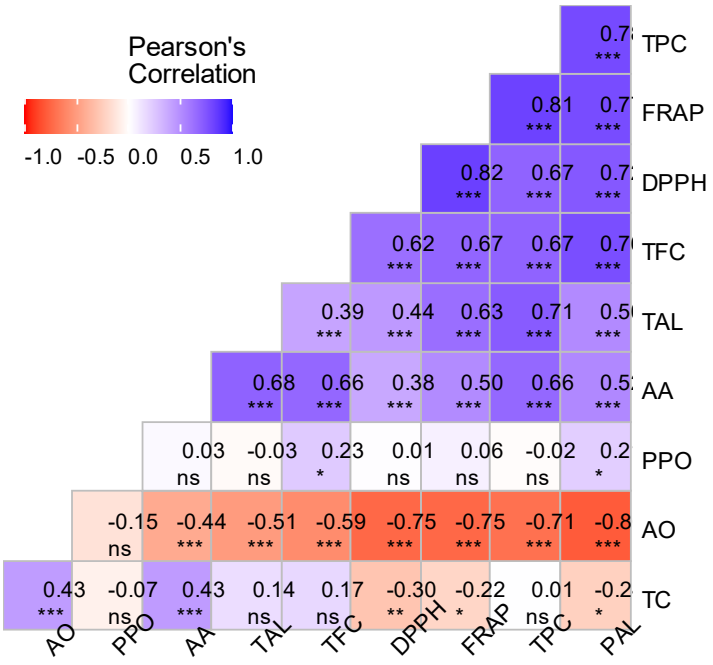


Figure 6.4. Pearson correlation matrix between total phenolic content (TPC), total flavonoid content (TFC), ascorbic acid (AA), total carotenoids (TC), DPPH, Ferric-Reducing Antioxidant Power (FRAP), phenylalanine ammonia lyase (PAL), tyrosine ammonia lyase (TAL), ascorbate oxidase (AO) and polyphenol oxidase (PPO). The correlation coefficients are proportional to the colour intensity. Positive correlation is displayed in blue and negative in red. n.s. = not significant.

6.4. Conclusion

The findings from the present study showed that EFF effectively optimised the antioxidant capacity in kiwifruit by regulating the action of enzymes involved in both the synthesis and oxidation of bioactive compounds. Therefore, it can be concluded that EFF has the potential to optimise the functional quality of South African grown gold kiwifruit, harvested at the mid and late maturity stage. The present study primarily examined the effect of EFF on the activity of enzymes involved in both the synthesis and oxidation of bioactive compounds. However, transcriptome analysis is needed in order to gain an in-depth understanding of the underlying mechanism on how EFF influences these enzymes. Therefore, further research using RNA Sequencing is warranted to provide a comprehensive understanding of the mechanism in which EFF optimizes the synthesis of bioactive compounds. Furthermore, assessing the inhibitory

effects of the hexanol volatile compound against PPO activity, may elucidate how hexanal application suppresses the action of this enzyme.

6.5. References

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Chapter Seven

General discussion and conclusions

7.1. Introduction

Rapid softening and fungal decay are the main limiting factors that reduce the storage life of kiwifruit (Cha et al., 2019; Li et al., 2020). Cold storage is the main preservation strategy used to optimise the storage life of kiwifruit during export (Choi et al., 2022). However, cold storage is not enough to optimise the storability of kiwifruit, as they still experience rapid softening at low temperatures (Mitalo et al., 2019; Xu et al., 2023). The susceptibility of kiwifruit to fungal decay increases as they soften, which makes firmness a critical parameter that determines the storability and marketability of kiwifruit (Ge et al., 2020). Therefore, rapid softening is a significant problem that results in postharvest and economic losses for the kiwifruit industry (Ma et al., 2023).

The application of agrochemicals is an effective strategy to control fungal decay, however, stringent maximum residue limit (MRL) policies put kiwifruit growers under pressure to find alternative treatments. In addition to firmness, kiwifruits are appreciated for their high antioxidant content, and extended storage results in a loss of antioxidants (Choi et al., 2022). These factors necessitate the need to explore a treatment that can reduce softening, fungal decay and maintain antioxidants of kiwifruit during storage. Understanding the mechanisms that cause fruit softening can facilitate in the development of an effective treatment for firmness retention. Therefore, this dissertation investigated the influence of enhanced freshness formulation (EFF) on factors that contribute to fruit softening.

The first research objective (Chapter 3) assessed the effect of EFF on the softening process by evaluating the treatment's impact on the activities of cell wall degrading enzymes. The second research objective (Chapter 4) bridged the gap between softening and the cellular processes by examining the influence of EFF on the integrity of the cell membrane. The third research objective (Chapter 5) evaluated the inhibitory effects of EFF on fungal growth, which contributes to premature softening and loss of marketability. The fourth research objective (Chapter 6) investigated the capacity of EFF to facilitate the retention of antioxidants in order to optimise the nutritional value of kiwifruit. In addition, firmness and antioxidant retention of kiwifruits is influenced by harvest maturity. Therefore, the study further evaluated the response of fruit maturity to EFF treatment application.

7.2. Efficacy of eco-friendly treatments in delaying fruit softening: A review

Fruit firmness is a commercially important quality attribute, which governs the postharvest life of fresh produce. Fruits should be harvested at the optimal maturity stage in order to optimise their storability during transit. Kiwifruits harvested at an early stage do not develop adequately whereas fruits harvested late deteriorate quickly (Wang et al., 2020). In spite of this, South African grown kiwifruits that are destined for export are still harvested at the optimal and late maturity stages (Mahlaba et al., 2021). Therefore, finding a strategy that can delay the softening and deterioration process of kiwifruits at different maturity stages is undeniably important. The extensive review of the literature revealed that there is a lack of studies investigating the efficacy of eco-friendly treatments in suppressing softening of fruit harvested at different maturity stages. Furthermore, these treatments have limitations when it comes to implementation within the agricultural industry. The major limitation of adopting technologies such as Ozone, UV-C and heat treatments includes high levels of capital investment added and trained labour (Mahajan et al., 2014).

Edible coatings are a cost-effective alternative, however, inefficiencies in their performance (Fagundes et al., 2014; Poverenov et al., 2014; Hira et al., 2022) can hinder their adoption within the industry. Hexanal appeared to be the most economically and practically feasible treatment, owed to its versatility. Hexanal can be applied at the preharvest and postharvest stage, as a spray, dip or vapour treatment. This makes hexanal appropriate for farmers who cannot afford high levels of capital investment or packhouse facilities. South African grown gold kiwifruits destined for export are harvested at both the mid and late maturity stages and experience rapid softening during transit. This prompted the current study to investigate the efficacy of hexanal (active ingredient of EFF) to delay the softening process in kiwifruits harvested at two maturity stages.

7.3. Mechanism of enhanced freshness formulation in delaying softening whilst optimising quality of gold kiwifruit harvested at two maturity stages

The results from the first objective (Chapter 3) showed that untreated kiwifruit lost approximately 76% of their initial firmness. However, the application of EFF treatment (0.02% EFF) effectively improved firmness retention of kiwifruits by approximately 15%. The results are in accordance with those reported by Cheema et al. (2018), who showed that EFF optimised firmness retention of bell peppers. These findings demonstrate the significance of incorporating

postharvest treatments with cold storage to delay rapid softening of kiwifruit. Furthermore, the softening rate of kiwifruits increased with an advancement in fruit maturity, which corroborates the findings reported by Tilahun et al. (2020). The higher softening rates of late-harvested kiwifruits were effectively reduced by the 0.02% EFF treatment. This shows that the efficacy of EFF to delay the softening of late-harvested kiwifruit is dose dependant. Therefore, the firmness retention of late-harvested kiwifruits destined for export can be improved by increasing the dosage of EFF treatment.

7.4. Enhanced freshness formulation preserves membrane integrity of kiwifruit by regulating reactive oxygen species

The second objective (Chapter 4) revealed that EFF treatment reduced membrane deterioration which resulted in significantly firmer kiwifruits. These findings are corroborated by those reported by Jincy et al. (2017), who illustrated that hexanal treatment preserved the membrane integrity of mango fruit. The mechanism in which EFF maintained membrane integrity involved the suppression of PLD activity and the enhancement of antioxidant enzymes which scavenge reactive oxygen species (ROS). The findings in this study support the theory that late harvested kiwifruits exhibit greater membrane deterioration and senescence than those harvested at mid maturity (Wang et al., 2020). EFF effectively alleviated the higher membrane deterioration of late harvested kiwifruits. This further demonstrates the potential of EFF in delaying membrane deterioration which contributes to the softening of kiwifruits, harvested at both the mid and late maturity stage.

7.5. Preserving gold kiwifruit postharvest quality: Efficacy and mechanism of enhanced freshness formulation to suppress *Botrytis cinerea*

Considering that fungal decay accelerates fruit softening and senescence, the third objective (Chapter 5) investigated the effect of EFF to suppress fungal growth of *Botrytis cinerea*. The results revealed that EFF increased disease resistance to *Botrytis cinerea* by enhancing the activities of defence-related enzymes (phenylalanine ammonia lyase, guaiacol peroxidase, β-1,3-glucanase and chitinase) and enhancing the synthesis of secondary metabolites (phenolic and flavonoid compounds). These findings agree with those reported by Seethapathy et al. (2016), who showed that hexanal treatment induced disease resistance by enhancing the activities of defence-related enzymes in mango fruit. The findings of the present study showed that EFF's versatility and simplicity as a dip treatment method make it accessible without

specialized equipment, whilst revealing the mechanism behind EFF's antifungal action. These findings advance our understanding of EFF's role in disease control, offering practical implications for postharvest management. These results suggest that EFF has the potential to reduce postharvest losses and improve the marketability of kiwifruit in an eco-friendly manner. Therefore, EFF can serve as an alternative to agrochemicals for controlling fungal decay of kiwifruits. This can improve the market participation of kiwifruit growers who export their produce to international markets with stringent MRL policies.

7.6. Mechanism of enhanced freshness formulation in optimising antioxidant retention of gold kiwifruit (*Actinidia chinensis*) harvested at two maturity stages

Gold kiwifruits are extremely perishable and storing them for extended storage periods leads to the loss of antioxidants (Kumarihami et al., 2020). Therefore, the fourth objective (Chapter 6) assessed the efficacy and mechanism of EFF in improving antioxidant retention of kiwifruit. The results showed that EFF treatment optimised antioxidant retention by enhancing and suppressing the activities of enzymes involved in the synthesis and oxidation of bioactive compounds, respectively. The results are in alignment with those reported by Gill et al. (2015), who demonstrated that EFF-treated guava fruit maintained significantly higher contents of antioxidants. These findings suggest that EFF has the potential to optimise the nutritional value of kiwifruits during export.

7.7. Conclusions

The outcomes of this study demonstrated that EFF application delayed the softening of kiwifruits during cold storage. Furthermore, the findings provide new insights into the interaction between postharvest treatment application and fruit maturity. The higher softening rates exhibited by late harvested kiwifruit were effectively suppressed by increasing the concentration of EFF. This illustrates the potential of EFF to optimise the storability of late-harvested kiwifruit which are more susceptible to softening during extended storage periods. These positive results can benefit the kiwifruit industry by reducing the postharvest and economic losses of kiwifruit attributed to rapid softening. The outcomes of this study contribute to the literature by providing a postharvest strategy that can improve the firmness retention and storability of late harvested kiwifruit which are susceptible to softening and deterioration.

7.8. Contributions of the study

7.8.1 Industry

The findings of this dissertation highlight a practical solution for reducing postharvest and economic losses of kiwifruit attributable to rapid softening. This has the potential to improve the welfare of stakeholders in the kiwifruit industry by maximizing the profitability of kiwifruit production. The efficacy of EFF to optimise firmness and antioxidant retention of kiwifruit harvested at both the mid and late maturity stages, offers an effective approach for enhancing preservation strategies for extended storage. The efficacy of EFF dip treatment in suppressing the fungal decay in kiwifruit offers growers an eco-friendly and cost-effective alternative to fungicides.

This has the potential to reduce reliance on agrochemicals and alleviate the pressure enforced by countries with stringent MRL policies. Thus, further increasing the market participation of kiwifruit growers. Majority of studies investigating the inhibitory effects of hexanal on fungal growth focused on the vapour application approach. Studies investigating the effectiveness of the EFF dip treatment approach are limited. The efficacy of EFF dip treatment to suppress the fungal growth of *Botrytis cinerea* in this study may increase confidence in the credibility and reliability of this treatment approach.

7.8.2 Science community

Prior work has primarily focused on determining the optimum maturity stage to optimise the storability of kiwifruit. However, there is a need to provide effective preservation strategies for regions where kiwifruits are harvested at different maturity stages, such as South Africa for example. This research fills this knowledge gap by adding to the scientific understanding of postharvest management. The findings of this study provide a foundation for future research in terms of further investigating more mechanisms related to firmness and antioxidant retention in kiwifruit or other horticultural produce. Previous studies have predominantly focused on inhibiting fungal decay using hexanal as a vapour treatment. The results of this study have demonstrated the efficacy of EFF dip treatment in suppressing fungal growth in kiwifruit. This outcome contributes to the literature by providing insights to an alternative application approach that can be further used to investigate the inhibitory effects of EFF on wide range of pathogens and horticultural produce.

7.8.3. Limitations of the study

The present study primarily focused on the effect of EFF on the mechanisms that regulate fruit softening, disease resistance and antioxidant retention at the enzymatic level and not the genomic level. Even though the findings of this study elucidated the mode of action of EFF, the manner by which EFF triggers these enzymes remains unclear. Furthermore, ethylene production initiates the mechanisms behind fruit softening by regulating the action of cell wall degrading enzymes and production of ROS. Thus, clarification on whether the suppression of cell wall degrading enzymes in kiwifruit is due to the inhibition of ethylene production in response to EFF treatment is needed. Regardless of these limitations, the findings of this study yielded positive results that have the potential to solve the challenges experienced by the kiwifruit industry. Furthermore, unveiling the mechanism by which EFF regulated the softening process and synthesis of antioxidants at the enzymatic level, provides a comprehensive understanding of EFF's capacity to optimise firmness and antioxidant retention in kiwifruits.

7.8.4. Recommendations for future research

Based on the findings and limitations of this study, the following recommendations are made for future research:

- The activity of cell wall degrading enzymes is regulated by ethylene production. Therefore, future research investigating the influence of EFF on the ethylene biosynthesis is needed to gain further understanding on the mechanism of EFF in delaying softening of gold kiwifruit.
- Transcriptome analysis on the gene expression of enzymes involved in fruit softening, disease resistance and the antioxidant system should be conducted to unveil the manner in which EFF regulates these enzymes.
- In order to instil confidence in the adoption of this treatment, practical applications need to be evaluated to assess the economic costs and efficacy of EFF treatment on a commercial scale.

7.9. References

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