CELLULAR AND BIOCHEMICAL CHANGES ASSOCIATED WITH INDUCTION OF THE DEFENSE SYSTEM IN BERANGAN (AAA) BANANA

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CELLULAR AND BIOCHEMICAL CHANGES ASSOCIATED WITH INDUCTION OF THE DEFENSE SYSTEM IN BERANGAN (AAA) BANANA

ABSTRACT

Fusarium infection of bananas is a global problem that threatens the production of bananas. This thesis looks at the effects of the infection upon the Reactive Oxygen Species (ROS) system as well as the induced antioxidant properties and antifungal protein activities in the Berangan fruits at Stage I, V and in infected fruits. ROS assays were divided into two classes: ROS assays and ROS-scavenging assays. Of the ROS assays, lipoxygenase was observed to be higher in the infected samples while peroxidase and polyphenol oxidase were significantly higher in infected fruit samples. Induction of ROS is important for the hypersensitive response to function properly. The ROS-scavenging enzymes, namely ascorbate peroxidase, guaiacol peroxidase and superoxide dismutase, exhibited higher levels in the infected tissue. This is most likely in order to counter the build-up of the ROS enzymes and to prevent further cell death. The increase in ROS-scavenging assays also correlates with higher antioxidant properties as antioxidants play a critical role in regulating the hypersensitive response free radicals. Furthermore, antifungal protein properties were observed to be higher in infected fruit samples.

Keywords: Berangan bananas, PR proteins, Antioxidant properties, Antifungal activity, Reactive Oxygen Species system

PERUBAHAN SEL DAN BIOKIMIA YANG BERKAITAN DENGAN INDUKSI SISTEM PERTAHANAN DALAM PISANG BERANGAN (AAA)

ABSTRAK

Jangkitan pisang oleh *Fusarium* adalah masalah global yang mengancam pengeluaran pisang. Tesis ini melihat kesan jangkitan pada sistem Reactive Oxygen Species (ROS) dan juga sifat-sifat antioksidan teraruh dan aktiviti protein antikulat dalam buah Berangan pada peringkat I, V dan dalam buah-buahan yang dijangkiti. Analisa ROS telah dibahagikan kepada dua kelas: Analisa ROS dan Analisa ROS-memerangkap. Daripada Analisa ROS, lipoxygenase diperhatikan lebih tinggi dalam sampel dijangkiti manakala peroxidase dan polifenol okside adalah lebih tinggi pada sampel buah-buahan yang dijangkiti. Induksi ROS adalah penting untuk tindak balas hipersensitif untuk berfungsi dengan baik. Enzim ROS-memerangkap iaitu askorbat peroxidase, guaiacol peroxidase dan superoxide dismutase, dipamerkan tahap yang lebih tinggi dalam tisu yang dijangkiti. Ini adalah yang paling mungkin untuk menangani pengumpulan enzim ROS dan mencegah kematian sel selanjutnya. Peningkatan dalam analisa ROS-memerangkap juga dikaitkan dengan sifat-sifat antioksidan yang lebih tinggi kerana antioksidan memainkan peranan penting dalam mengawal radikal bebas tindak balas hipersensitif. Lagipun, khasiat protein antikulat diperhatikan lebih tinggi pada sampel buah-buahan yang dijangkiti.

Kata kunci: Pisang Berangan, Protein PR, sifat-sifat antioksidan, aktiviti protein antikulat, Sistem Reactive Oxygen Species

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LIST OF ABBREVIATIONS

°C		Degree Celcius
%		Percentage
µL	:	Microlitres
µg	:	Micrograms
APX	:	Ascorbate Peroxidase
BAEE	:	Benzoyl-L-Arginine Ethyl Ester
BMMV	:	Banana Mild Mosaic Virus
CAT	:	Catalase
DPPH	:	Free Radical Scavenging Activity
FAO	:	Food and Agriculture Organization
FOC	:	*Fusarium oxysporum* f.sp. *cubense*
GAE	:	Gallic Acid Equivalent
GPX	:	Guaicol Peroxidase
HR	:	Hypersensitive Response
INHB	:	Trypsin Inhibitor Solution
LOX	:	Lipoxygenase
mL	:	Mililitres
PCD	:	Programmed Cell Death
POD	:	Peroxidase
PPO	:	Polyphenol Oxidase
PR	:	Pathogenensis-related
ROS	:	Reactive Oxygen Species
SAR	:	Systemic Acquired Resistance
SOD	:	Superoxide Dismutase

TAC : Total Antioxidant Capacity

TFC : Total Flavonoid Content

TLP : Thaumatin-like Proteins

TPC : Total Polyphenol Content

CHAPTER 1: INTRODUCTION

There are four main staple foods cultivated to fulfil the needs of the people, namely wheat, rice, maize and banana. Of the four, bananas serve as a staple food to people across several regions with it being most important to the people in Africa as well as some South American and South East Asian countries. A number of these countries, especially the African countries, are not able to efficiently cultivate other staple foods such as wheat and rice due to the climate and environment. As such, they are largely dependent on cultivation of bananas. In 2014, approximately 114 million tonnes of bananas were produced worldwide (FAO, 2017).

Banana production however, faces a serious problem. Bananas cultivars are produced asexually, meaning there is no genetic variation among the cultivars. This leaves them especially vulnerable to pathogen attacks which cause diseases.

The main pathogen affecting bananas are the fungal pathogens. Fungal pathogens are responsible for a host of rots that plague pre and postharvest bananas. They are known to cause anthracnose, crown rot, stem-end rot and cigar-end rot (Sarkar *et al.,* 2013). In the case of postharvest bananas, these diseases cause the value of the fruit to decrease, affecting the producers and suppliers. The fruits look sickly and unappetizing, resulting in customers not wanting to purchase them and sellers selling them at a loss. Furthermore, should the contaminated fruits be consumed, they could result in adverse health effects.

In the plant kingdom, there are a number of mechanisms to cope with the onset of infection. The most well-known is the plant Hypersensitive Response (HR) which results in apoptosis at the infection site (Walters, 2015). The HR involves many different components such as Reactive Oxygen Species (ROS) and antioxidants.

Another defense mechanism in the plant system is the inducement of the Pathogenesis-Related (PR) proteins which are the proteins responsible for conferring resistance to plants to protect them from pathogen attacks. The PR proteins play a critical role in the plant defense system and the systemic acquired resistance (SAR) of plants (Ahmed *et al.,* 2013). Although both systems are present in bananas as well, bananas still remain susceptible to pathogen attack. As such, this project looked to analyze and assess the activity of the components of the HR and SAR defense systems in healthy and infected Berangan bananas.

For this project, a local banana cultivar, Berangan (AAA), was studied. Berangan is an important cultivar in terms of local consumption as it is the most cultivated and consumed local cultivar.

For chapter three, the activity levels of the ROS proteins were assessed for the cultivar at Stage I and Stage V. The enzymes studied are Peroxidase (POD), Polyphenol Oxidase (PPO), Lipoxygenase (LOX), Ascorbate Peroxidase (APX), Catalase (CAT), Guaicol Peroxidase (GPX) and Superoxide Dismutase (SOD). Infected fingers of the cultivar were also assessed. The purpose behind these assays was to establish the activity levels of the proteins at the different stages of ripening. Considering that banana fruits are more prone to infection following ripening, the principle idea was to establish if there was a decline in the expression of the ROS proteins as the plant ripened. This information would prove useful in tailoring mechanisms to extend the shelf life of the banana fruits.

The second part of the chapter looked at the enzymatic activity in the ROS proteins of infected fruit. The principle idea behind this portion of the experiment was to determine if the ROS protein activity levels played a key role in preventing infection. Seeing as bananas do produce ROS proteins, it was interesting that they were still

insufficient in conferring resistance. This part of the experiment determined if the reason there was a lack of resistance was because the proteins were not being produced.

Chapter four of this study focused on the antioxidant activities of the samples. Antioxidant activities assessed were free radical scavenging activity (DPPH), total polyphenol content (TPC), total antioxidant capacity (TAC) and total flavonoid content (TFC). The antioxidants were studied to see if the antioxidant activities changed through the course of ripening and to ascertain if the changes would correlate with changes seen in the ROS activities. Furthermore, the effects of infection on the antioxidant activity levels were also assessed.

For chapter five, the enzymatic activity levels of the antifungal proteins were assessed. The antifungal proteins assessed were laminarinase, α-amylase and trypsin inhibitors. The purpose behind these assays was to establish the activity levels of the proteins at the two stages of ripening. This would serve useful in determining the antifungal proteins that build up in the fruit. The second part of the chapter looked at the enzymatic activity in the antifungal proteins of infected fruit. As the proteins are antifungal proteins, it is important to know the mechanism that takes place once the fruit is infected by a fungal pathogen. The next part of the chapter focused on the antifungal properties of the proteins. The antifungal properties were determined through the use of a zone of inhibition assay. In the presence of antifungal properties, the fungal strains challenged against the antifungal proteins would not be able to grow and inhibition would be visible. The principle idea behind this experiment was to determine if the antifungal proteins exhibited antifungal properties and if so, how strongly.

Objectives

i. To determine the ROS protein activity of the banana peel and pulp at Stages I,V and infected Stage VII

ii. To determine the antioxidant content and activity of the banana peel and pulp at Stages I, V and infected Stage VII

iii. To determine the antifungal protein activity of the banana peel and pulp at Stages I, V and infected Stage VII

iv. To determine the antifungal properties of the antifungal proteins at $1\times$, $5\times$ and $10\times$ the concentrations in bananas.

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

Bananas and plantains (*Musa* spp.) are the most important food crops in the world. Ranked fourth, behind rice, maize and wheat, they are a staple food crop for the millions inhabiting the regions of Central, East and West Africa, Latin America and the Caribbean. The production of bananas has increased over the last three decades from 90 million tonnes in 1997 to 102 million tonnes in 2002 and to 106 million tonnes in 2011 to 114 million tonnes in 2014 (FAO, 2017). Banana fruit play a critical role in the food security of tropical regions, as the cultivation of bananas is a main source of livelihood for many of the farming communities. The communities earn their living through the trade of bananas both in their country and for export.

Unfortunately, the banana species is not the hardiest of cultivated crops. Due to the nature of how banana crops are cultivated, through asexual reproduction methods, susceptibility to pathogens affects all plants of the same cultivar. Over the last decade, fungal pathogens have proved to be extremely destructive to banana crop production. Fusarium wilt, caused by *Fusarium oxysporum* f. sp. *cubense (Foc),* is a soil-borne fungal pathogen that has severe effects on dessert and cooking bananas. Another major pathogen is *Mycosphaerella fijiensis*. This fungal pathogen is the causative agent for the Black Sigatoka disease. This disease affects nearly all types of banana (Viljoen, 2010). Over the last two to three decades, these pathogens have spread rapidly and now pose a serious and significant threat to the banana production industry. The most widely cultivated and exported banana in the world, the Cavendish cultivar, has been shown to be susceptible to these fungal pathogens. Cavendish became the most exported banana in the world after the Gros Michel cultivar fell prey to an earlier form of Fusarium wilt,

leading to it being completely wiped out (Gauhl, 1994). Cavendish was then cultivated as a replacement that showed resistance to that form of Fusarium wilt.

The dangers that these pathogens pose to banana cultivation has led to increased efforts to introduce resistance to the diseases in the cultivated bananas. In the 90's, efforts focused on breeding programs (Ortiz & Vuylsteke, 1996). Cultivated bananas bred asexually as they are either sterile or seedless. This has led to a lack of genetic diversity within the cultivars. Furthermore, breeding of bananas has significant practical obstacles. The plants take a long time to grow, taking almost 24 months. Following that, they take a further 18 months to fruit. The long-time span makes testing and screening for resistant cultivars extremely time-consuming (Passos *et al.,* 2013).

Advances in science have led to some of these obstacles being surpassed. Earlier methods relied on ploidy manipulations and interspecific hybridization (Rowe & Rosales, 1996; Vuylsteke *et al*., 1997). Nowadays though, genetic engineering is the most widely used method. Genes from resistant species such as onions and dahlias have been introduced into the cultivated bananas so as to confer resistance (Passos *et al.,* 2013). Gene expression studies itself have become important tools in discovering the mechanisms behind the development of disease tolerance (Swarupa *et al.,* 2013). However, more studies on pathogenesis-related (PR) proteins are required to be better able to address the issues of pathogen susceptibility.

2.1.1 Botanical Description of Banana

Musa are giant perennial monocotyledenous herb that originated from Southeast Asia. Banana are under the order Zingiberales, part of the commelinid clade of the monocotyledonous flowering plants. Currently, there are 70 species of banana as recognized by the World Checklist of Selected Plant Families (Royal Botanical Gardens, 2014).

There are two wild type bananas, *Musa acuminata* and *Musa balbisiana*. Hybridization occurred naturally between these two species, with *M. acuminata* contributing the A genome and *M. balbisiana* contributing the B genome, leading to the formation of naturally occurring hybrids (Simmonds, 1962). These hybrids, which are edible, were actively selected by the earlier settlers of the region for cultivation (Kennedy, 2009). The wild, edible banana actively selected by the farmers were the *M. acuminata* Colla which exist in 2 forms, a diploid or a triploid. The diploid, 2n has 22 chromosomes and the triploid, 3n has 33 chromosomes. These cultivars are represented by the symbols AA and AAA respectively.

The other banana wild type parent, *M. balbisiana* Colla, is represented by the symbol BB as it is a diploid. Unlike the *M. acuminata* Colla, *M. balbisiana* Colla is inedible. The fruit of the species is filled with seeds. However, studies have shown that *M. balbisiana* Colla is hardier than *M. acuminata* Colla. As such, it is often hybridized with *M. acuminata* Colla to confer disease resistance to the hybrids (Sadik, 1988).

The hybridization of *M. acuminata* Colla and *M. balbisiana* Colla led to the formation of several additional banana genotypes, namely AB, AAB, and ABB (Perrier *et al.,* 2011). These hybrids are sometimes referred to by a general name, *Musa × paradisiaca* L. Like those of the wild types, the A genome of the hybrids come from *M. acuminata* and the B genome come from *M. balbisiana* (Simmonds, 1962). The main exported banana, the Cavendish cultivars, are AAA species. That means they are pure triploid *acuminata*. Taxonomic classification of the most important plantain/banana cultivated is as follows:

Class	:	Monocotyledonae
Order	:	Scitamineae
Family	:	Musaceae
Genus	:	Musa
Section	:	Eumusa
Species	:	*M. acuminata* (AA)
		M. balbisiana (BB)

2.2 Economic Importance of Bananas

2.2.1 Global: Figure at global production

In the global market, bananas are a very important commodity. The export quantity of bananas globally was estimated to be at 17.5 million tonnes in 2011, up from 14.5 million tonnes in 2001 (FAO, 2013). Bananas are mainly cultivated in developing nations as they require special climate conditions for growth. As it is the case for most tropical products, due to the special climatic conditions needed to grow bananas, they are mainly produced in developing countries.

However, even though banana production is spread across many diverse countries, the core production of bananas takes place in 6 countries, namely India, Ecuador, Brazil, China, Philippines and Indonesia. India, which produces 29.7 million metric tonnes of bananas accounts for nearly 30% of the world's production of bananas. All six countries combined account for nearly 66% of the world's production of bananas. Interestingly, even though India produces 29.7 million metric tonnes of bananas, only a mere 60 000 metric tonnes are exported. The remainder of the bananas produced were

consumed locally (FAO, 2010). The sheer volume of the consumed bananas shows how important it is in the context of feeding the people, especially in well-populated countries such as India, China and Indonesia.

Banana exports in the year 2013 reached 20 million metric tonnes. The largest of the exporting countries was Ecuador, which exported approximately 5.4 million metric tonnes of bananas, about 27% of the world's banana export. The value of the exports of bananas worldwide reached a staggering 9.7 Billion USD. This shows that bananas are economically important and that it has a large impact on the economies of exporters.

2.2.2   Malaysia

In Malaysia, bananas are one of the most important food crops cultivated. Banana plantations in Malaysia was estimated to be at 31, 300 ha, producing 334, 302 metric tonnes of bananas in 2011 (FAO, 2013). About 50% of the banana growing land is cultivated with Berangan and the Cavendish cultivars, and the remaining popular cultivars are Mas, Rastali, Raja, Awak, Pisang Abu, Nangka and Tanduk. Under the National Agricultural Policy, emphasis will be given to this crop as it will be important to the industry. Interestingly though, only approximately 18,814 metric tonnes of banana were exported out of the country in 2013 (FAO, 2017). This indicates that the bananas are important for local consumption. Even so, the value of the exports reached 7 million USD which is a substantial sum.

2.3   Diseases Affecting Banana Production

The key issue affecting banana cultivation is the susceptibility of banana cultivars to pathogens. There are many types of pathogens that prey on bananas. Among them are fungal, bacterial, viral pathogens, nematodes and other pests. The problem is that the banana cultivars are unable to defend themselves adequately or adapt a defense

mechanism that will grant them resistance to these pathogens. In nature, one of the key ways plants develop and pass down defense mechanisms is through sexual reproduction. The combining of genetic material from two different parents creates genetic diversity among the species. This genetic diversity can lead to the development of pathogen resistances and help with the adaptation process. Unfortunately, banana cultivars are sterile. That means that they are unable to breed sexually. As such, they are cultivated asexually. While asexual reproduction enables the sterile species to be cultivated, it comes at a great cost. That being that the offspring are identical to the parent they are cultivated from. As such, if the parent is susceptible to a pathogen, all the cultivated offspring will have that susceptibility with no chance of developing a resistance to it.

This susceptibility threatens the banana species cultivated by farmers. In order to protect their plantations, these farmers turn to the use of pesticides, herbicides and fungicides. Heavy usage of these pesticides, herbicides and fungicides were beneficial in the short-term. This helped decrease the rate of infections and protect the cultivated plants. However, over the years, the very pathogens these chemicals are meant to stop have started to develop a resistance to the chemicals. As it stands, a report shows that pathogen attacks are increasing in spite of the massive chemical applications as pathogens are becoming increasingly resistant (Kema, 2013).

2.3.1 Pre-harvest Disease

Diseases affecting fruits can be divided into two groups, namely pre-harvest and post-harvest diseases. Pre-harvest diseases are caused when the fruit or plant itself is infected before being ready for harvest. Pre-harvest diseases can lead to a loss in production and of quality of the fruit. Furthermore, pre-harvest disease symptoms can

persist and lead to more severe post-harvest diseases, further degrading the quality of the produce (Naqwi, 2004; Timmer, 2005).

In the banana industry, two important pre-harvest diseases are the Fusarium wilt and Black Sigatoka diseases (Thangavelu *et al.,* 2013).

2.3.1.1 Fusarium Wilt (Panama Disease) of Banana

The Fusarium disease is one of the deadliest and most destructive diseases to befall any crop in agricultural history. During the 1950's, the most cultivated banana cultivar was the 'Gros Michel' cultivar. It was planted widely through-out the world. However, the emergence of the Fusarium wilt disease threatened the entire production of the 'Gros Michel' cultivar (Kovacs *et al.,* 2013). The disease spread quickly, fast threatening the world production of banana. It would have resulted in the devastation of the local economies of many banana producers (Ploetz, 1990). In order to prevent this scenario from coming to pass, the 'Gros Michel' cultivar worldwide was replaced with the Cavendish cultivar. Cavendish was resistant to that form of Fusarium wilt and has remained strong for a number of decades.

However, over the last couple of years, the Cavendish cultivar has been suffering infections from a new strain of Fusarium wilt (Chen *et al.,* 2013). The Cavendish has been found to be susceptible to the Rac 4 Fusarium wilt (Ma *et al.,* 2013). As such, history threatens to repeat itself. Once again, the world's plantations, and by association, the banana trade and local economies, are threatened with devastation (Xu *et al.,* 2011).

The Fusarium wilt disease was discovered by Dr Joseph Bancroft in Australia, in 1874 (Monteiro *et al.,* 2003). In 1910, it was discovered that Fusarium wilt was caused by *Fusarium oxysporum* f.sp. *cubense* (FOC), a soil-borne was identified. It is also known as race 4 Fusarium (Lin *et al.,* 2013). The Fusarium wilt disease is a classic

vascular wilt disease. The disease infection begins when the fungus penetrates into the water-conducting xylem vessels. Once through, the fungus produces spores that are then released into the xylem (Saravanan *et al.,* 2003). These spores are carried upwards in the water stream by the flow of water through the xylem. Once the spores reach the perforated vessel end walls that occur at intervals throughout the xylem, the spread of the disease is temporarily halted. The spores will germinate and form hyphae. The hyphae will grow through the perforations found on the vessel end cell walls and produce another batch of spores past it. This process continues until the entirety of the plant xylem system is colonized (Zhang *et al.,* 2013). Colonization occurs quickly and the symptoms are easily observable. The older leaves turn yellow at the margins then die progressively towards the midrib, and the dead leaves hang down as a skirt around the stem. Eventually the whole shoot is killed. If the initial infection is light, and the plant responds rapidly, then the disease can be localized to a few infected vessels. Conversely, if the initial infection is severe and the plant response is slow then the plant dies.

Once the plant is dead, the fungus continues to grow. It penetrates from the xylem into the dead tissue surrounding it. Once complete, it produces many resting spores by the deposition of a thick, melanized wall around individual hyphal compartments. When the plant tissue finally decays, these chlamydospores are returned to the soil (Schippers & Van Eck, 1981). Unsurprisingly, they are near impossible to separate or sterilize from the soil and can survive for decades. Any new bananas planted over that ground will suffer rapidly develop infections. As such, the plantation can now no longer be used for cultivation of banana crops. Currently, studies are underway in order to discover a way to treat infected soil and prevent the spread of Fusarium wilt (Shen *et al.,* 2013).

2.3.1.2 Sigatoka

The Black Sigatoka fungus disease is caused by *Mycosphaerella fijiensis*, an air-borne fungus (Quieroz *et al.,* 2013). This disease has devastated plantations throughout the world since it was first discovered in Honduras, Latin America in the 1960's (Stover, 1974; Gauhl, 1994) and has now spread globally. The *Mycosphaerella* family is the main causative agent of Leaf Spot diseases that affect bananas such as the Black Sigatoka and the Yellow Sigatoka diseases (Arzanlou *et al.,* 2008). The Black Sigatoka disease is a newer variant of the Yellow Sigatoka disease. When the Yellow Sigatoka disease first emerged in 1912, it started a global epidemic, affecting the world's crop of bananas. The Black Sigatoka disease is on the verge of becoming just as deadly.

The Black Sigatoka disease infects a banana cultivar by first attacking the leaves and penetrating into the plant. The leaves turn black from the bottom of the leaf to the midrib and ultimately, wither and die prematurely. Without the leaves, the cultivar is no longer capable of performing photosynthesis. The lack of food means that the plants allocate the food sources to survival instead of the production of fruit, resulting in small bunches which are no longer edible (Liberato *et al.,* 2006).

Reddish brown specks on the lower leaf surface represent the first symptoms of the Black Sigatoka disease. They progressively lengthen and darken into black spots (Bhamare & Kulkarni, 2013). The spots are usually located from the lower leaf surface to the midrib. Premature drying of the leaves then occurs, leading to the death of the leaves. The drying intensifies after flowering, severely affecting the production yield of the banana.

2.3.2 Post-harvest Disease

Post-harvest diseases occur when, following harvest, fruits are exposed to unsuitable conditions which result in the fruit being attacked by fungal pathogens (Sarkar *et al.,* 2013). Most commonly, these diseases are only observable at points of sale or after purchase (Nelson, 2008). Due to the infection of the fruit, the market value is lowered as the fruit no longer looks presentable and appealing to consumers. Furthermore, the nutritional value of the fruit suffers due to the changes in the stored products of the fruit (Sawant & Gawai, 2011).

Improper handling practices during harvesting, transportation, marketing and storage stages are likely to cause damage and deterioration of the fruit. This leads to physical defects which in turn causes pathogen attacks (Sarkar *et al.*, 2013). Pathogens attack fruits due to the fruits being rich in nutrients and having high moisture content (Mehrotra, 1980). The modes of infection vary with different pathogens. Most pathogens require the presence of either a wounding site on the fruit or a natural opening in order for the pathogens to gain entry as they do not have the means to directly penetrate the fruit peel. As such, careful handling practices to ensure no physical damage is done to the fruit can prevent post-harvest infections. However, in the case of fungal pathogens, natural openings or wounding sites are not required as fungal pathogens are capable of penetrating the peel of the fruits. Hence, of the post-harvest pathogens, fungal pathogens are the most prevalent as even careful handling is insufficient to prevent attacks from these pathogens.

In bananas, the most common post-harvest diseases observed are anthracnose, crown-rot and cigar-end rot. The symptoms of anthracnose include blemishes on the peel and the development of brown or black, sunken spots of differing sizes. In some

cases, salmon-colored fungal acervuli can be observed in the spots. Anthracnose can present anywhere on the banana fruit.

Crown rot symptoms develop on the 'crown' – Where the hand and the bunch are severed. A brown to black color will develop on the crown followed by a layer of whitish mold. Fingers may detach prematurely as the mold is capable of penetrating deeply into the neck of the fingers, resulting in the rotting of the tissue and weakening of the hold (Nelson, 2008).

Cigar-end rot occurs around the perianth of the banana fruit. A black rot spreads out from the perianth into the tips of the fruit onwards. The infected portions turn ash-grey, resulting in a cigar like appearance (NHB). These rots are most commonly caused by the fungal pathogens *Collectotrichum* sp. and *Aspergillus niger*.

2.3.2.1 *Colletotrichum* species

The *Collectotrichum* species is one of the main fungal pathogens affecting bananas. The two most common strains of *Collectotrichum* that infect bananas postharvest are *Collectotrichum gloesporioides* and *Collectotrichum musae*. The *Collectotrichum* species has been found to be a key causative agent of anthracnose, crown rot and stem-end rot (Meer *et al.,* 2013). In plantations, they can spread through contaminated runoff rainwater, causing infection either by germinating and forming infection hyphae which colonize the peel and penetrate into the pulp or by entering through wounding site (Chillet *et al.,* 2010).

In 2011, five banana cultivars were sampled and found to be infected with anthracnose. The sampled cultivars were mas, rastali, berangan, awak and nangka. They exhibited classic symptoms of anthracnose infection, namely, brown to black spots that later turned into sunken lesions with orange or salmon colored spores. Using tissue culture and gene sequencing techniques, the causative agent of the anthracnose was

determined to be *Colletotrichum gloeosporioides*. This was the first reported incident of *Colletotrichum gloeosporioides* causing anthracnose in bananas in Malaysia (Zakaria *et al.,* 2013).

Research on 'Embul' bananas carried out in 2013, revealed that *Collectotrichum musae* was the most aggressive fungal pathogen when compared among five fungal species. The *C. musae* was found to cause rot on the crown and stem surface of the bananas (Wijetharam & Sarananda, 2013).

2.3.2.2 *Aspergillus niger*

Aspergillus niger is a filamentous fungus that is present worldwide. It has been isolated from numerous habitats and is capable of fast growth while also being pH tolerant. As such, it is known to be a causative agent for many rot diseases on assorted fruits such as stem-end rot and crown rot Thus, it is recognized as an important spoilage fungi (Pitt & Hocking, 1997; Perfect *et al.,* 2009; Perrone *et al.*, 2007; Gautam *et al.*, 2011). Rot caused by *A. niger* generally occurs during post-harvest storage (Meer *et al.,* 2013). In 2013, analysis of mangoes from the domestic markets of Punjab revealed that *A. niger* was a major post-harvest pathogen that caused stem-end rot of the fruit. It was highly prevalent among all the local markets (Meer *et al.*, 2013).

Research on bananas in India in 2011 had identified *A. niger* as being a major post-harvest pathogen. The research showed that *A. niger* was responsible for the development of rot on bananas kept in storage and their subsequent spoilage. The research also revealed that the nutritional values of the bananas were severely affected by the infection. The infected bananas showed a decrease in the quantity of total soluble sugar, protein, ash, ascorbic acid and mineral elements as compared to controls (Sawant & Gawai, 2011).

2.3.2.3 *Alternaria alterata*

In 2003, research efforts were initiated in America to identify banana cultivars suitable for cultivation in the environment of Georgia. The Tifton Banana Garden was established in Georgia and cultivars have been grown and evaluated there since 2009. However, in 2012, seven of 13 grown cultivars began to exhibit disease symptoms. Light to dark brown spots formed on the axadial leaves of the cultivars. The cultivars themselves displayed on average 35% rate of disease incidences. Through the use of tissue culture techniques, the causative agent was identified to be *Alternaria alternata*. This was the first reported incident of *Alternaria alternata* causing *Alternaria* leaf spot disease on bananas in the US (Parkunan *et al.,* 2013).

2.3.2.4 **Banana Mild Mosaic Virus**

Plantains, part of the banana species, are an important food group. In the Ivory Coast, plantains are the second most consumed food item. The cultivation of plantains itself is a major economic venture among the poor.

In 2011, 10 major plantain-growing regions were investigated for the presence of Banana mild mosaic virus (BMMV) among others. Diseased leaves were collected. Through the use of reverse transcriptase PCR, the diseased leaves were determined to be caused by the BMMV. Although other forms of banana viruses such as Banana Mosaic Virus were previously reported in the Ivory Coast, this was the first reporting of the banana mild mosaic virus in the Ivory Coast (Kouadio *et al.,* 2013).

2.4   **Plant Defense Response**

The plant defense system responds to infection in a myriad of ways. At the onset of infection by pathogens, the plant Hypersensitive Response (HR) is triggered. It serves as the first-line of defense against pathogens. In order to halt the spread of the infection, the HR causes apoptosis to occur in the infected area. Apoptosis is the programmed cell

death (PCD) of plant tissues. This results in an isolation of the pathogen, preventing the pathogen from hijacking the reproductive machinery of surrounding cells and using it spread. The main method of causing apoptosis is through the generation of free radicals. These free radicals go on to attack the target cells, destabilizing the cell membrane and wall, resulting in cell death. The enzymes involved include Reactive Oxygen Species (ROS) and secondary metabolites, among others.

Following the induction of the HR, the systemic acquired resistance (SAR) is induced. SAR results in the induction of phytoalexins, pathogenesis-related (PR) proteins and other immune-related enzymes. The induction of these enzymes increases the readiness of the plant to combat and resist infections following prior infections. The SAR system has been compared to the innate immune system of mammals.

2.4.1 Hypersensitive Response

2.4.1.1 Reactive Oxygen Species (ROS)

ROS are compounds that are produced via oxidative burst following pathogen recognition. Research has shown that the ROS compounds play several important roles in dealing with the infection caused by pathogens. Firstly, they are directly involved in causing the death of both pathogen and host cells though the use of lipid peroxidation and membrane damage (Montillet *et al.*, 2005). Secondly, they strengthen host cell walls by initiating cross-linking of glycoproteins (Lamb & Dixon, 1997, Torres *et al.*, 2006). Last, but not least, they serve as important signal mediators for the host pathogen response (Levine *et al.*, 1994).

The ROS compounds involved in causing host programmed cell death (PCD) and in the killing of the pathogens are the superoxide radicals, O_2^- and its dismutation product, H_2O_2. The main enzymes involved in their generation are peroxidase (POD),

polyphenol oxidase (PPO) and Lipoxygenase (LOX). As part of the HR, these three enzymes react on their respective substrates to produce the ROS compounds.

The biggest problem with the induction of ROS is the fact that it indiscriminately attacks all cells. As such, host cells are also targeted. While this is harnessed in order to cause apoptosis to slow down infection, if left unchecked, it can severely damage the host plant. In order to prevent that, the plant produces ROS enzymes whose function is to scavenge the ROS compounds. These compounds catalyze the breakdown of the ROS compounds to non-harmful elements such as water. Common ROS scavengers are Ascorbate Peroxidase (APX), Catalase (CAT), Guaiacol Peroxidase (GPX) and Superoxide Dismutase (SOD).

(a) ***Peroxidase***

Peroxidases (EC number 1.11.1.x) are a large family of enzymes that play a crucial role in the plant defense system. Peroxidases were first identified as a component of the hypersensitive response in plants. The purpose of the hypersensitive response is to cause localized cell death in areas of the plant that have been infected. Through this, the infection can be contained and prevented from spreading, ultimately leading to the death of the pathogen involved. The mechanism by which this happens is the membrane degradation of the plant cells by the peroxidases. This results in the death of the plant cells through lipid peroxidation (Heath, 2000; Matthews, 2007). This is achieved through the production of active oxygen species such as H_2O_2 and superoxide (Baker *et al.,* 1995; Joseph *et al.,* 1998). The chemical reaction that is typically catalyzed by peroxidases is:

$$ROOR' + \text{electron donor (2 e-)} + 2H+ \rightarrow ROH + R'OH$$
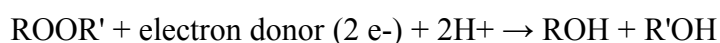
In 2001, a wheat-based heme-peroxidase was successfully isolated and purified. The antifungal properties of the peroxidase were assessed against *Botrytis cinerea*, *Fusarium culmorum* and *Trichoderma viride*. Results showed that the peroxidase was able to inhibit germ tube elongation, effectively preventing the spread of the fungal pathogens (Caruso *et al.*, 2001).

Following the successful isolation of TLPs from French bean legumes, an attempt was made to isolate a peroxidase from the same organism. The peroxidase isolated was found to exhibit antifungal activity against *Coprinus comatus*, *Mycosphaerella arachidicola*, *Fusarium oxysporum* and *Botrytis cinerea* (Ye & Ng, 2002).

Horseradish peroxidase has also been shown to exhibit antifungal activity. When tested against *Pseudocercospora abelmoschi* and *Pseudocercospora cruenta*, the horseradish peroxidase inhibited the fungal growth at high concentrations (Joseph *et al.*, 1998).

(b) *Polyphenol Oxidase*

Polyphenol oxidase (E.C. number 1.14.18.1) is a tetramer that contains four atoms of copper per molecule, and binding sites for two aromatic compounds and oxygen ("Polyphenol Oxidase". Worthington Enzyme Manual). PPO catalyzes the hydroxylation of monophenol molecules to o-diphenols as well as the oxidation of o-diphenols to o-quinones. It also catalyzes the oxidation of tyrosine to o-quinone (Mayer, 2006).

PPO has been shown to be induced upon pathogen challenge. It functions together with POD in order to cause localized cell death to prevent the spread of infection. It has been shown to be upregulated in potato, tomato and hybrid aspen upon pathogen

challenge (Li & Steffens, 2002; Wang & Constabel, 2004; Mahanil *et al.,* 2008; Bhonwong *et al.,* 2009).

(c) Lipoxygenase

Lipoxygenases (EC 1.13.11.x), a family of enzymes containing a non-heme iron group, serve to catalyze the dioxygenation of polyunsaturated fatty acids in lipids. The formula of which is as below:

$$\text{Fatty acid} + O_2 = \text{fatty acid hydroperoxide}$$

Lipoxygenases have been shown to play several roles in the plant defense system. They serve as signal mediators to induce the plant defense response while also being actively involved in apoptosis and the killing of pathogens. In early studies done on wheat cells infected with rust fungi, it was observed that LOX activity was increased in the infected cells. The results showed that the LOX enzymes were induced as part of the HR response and that it was directly involved in the resulting cell necrosis (Campos *et al*, 2008).

More recent studies on the expression of LOX of cucumber during plant-pathogen interactions have also shown that LOX is induced as part of the HR response. The early induction of LOX plays a critical role in the defense of the plant against pathogen attacks.

(d) Ascorbate Peroxidase

Ascorbate peroxidases (E.C. number 1.11.1.11) are responsible for detoxifying peroxides using ascorbate as a substrate. They catalyze the breakdown of peroxides into dehydroascorbate and water as shown below (Raven, 2000).

$$\text{Ascorbate} + \text{Hydrogen peroxide} \rightarrow \text{Dehydroascorbate} + \text{Water}$$

$$C_6H_8O_6 + H_2O_2 \rightarrow C_6H_6O_6 + 2\ H_2O$$
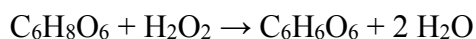
Research has shown that APX increases following the inducement of the plant HR. This is due to its function which enables it to scavenge the hydrogen peroxide present into harmless substances. It is a key component of the ascorbate-gluthathione cycle (Caverzan *et al.*, 2015).

Recent studies of APX activity in rice plants demonstrate that its role is to detoxify toxic substrates thus ensuring that the plant cells are protected from oxidative damage (Wang *et al.*, 2013).

(e) Catalase

Catalase (E.C. number 1.11.1.6) is an enzyme that catalyzes the decomposition of hydrogen peroxide to water and oxygen. It plays a critical role in protecting the cell from oxidative damage due to ROS molecules (Chelikani *et al.*, 2004). Following is the reaction catalyzed by catalase:

$$2\ H_2O_2 \rightarrow 2\ H_2O + O_2$$
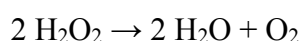
A study of wild and catalase-deficient mutant tobacco plants demonstrated that there were significant increases in the ROS activity of the mutant tobacco. The study clearly highlights the role of catalase in the scavenging of hydrogen peroxide (Mateo, 2004).

(f) Guaiacol Peroxidase

Guaiacol peroxidase (E.C. number 1.11.1.7) is an enzyme that belongs to the larger family of peroxidases. For most peroxides, the optimal substrate is hydrogen peroxide. A typical reaction catalyzed by guaiacol peroxidase is represented by the following equation:

$$ROOR' + \overbrace{2e^-}^{\text{electron donor}} + 2H^+ \xrightarrow{\text{Peroxidase}} ROH + R'OH$$

A recent study reported the upregulation of guaiacol peroxides in dark-germinated mungbean (*Vigna radiata*) to become major scavengers of excess hydrogen peroxide which highlights the role of this enzyme in combating oxidative stress in plants (McCue & Shetty, 2002).

(g) Superoxidase Dismutase

Superoxide dismutase (E.C. number 1.15.1.1) is a metal-containing enzyme that alternately catalyzes the dismutation of the superoxide ($O2^-$) radical into ordinary molecular oxygen (O_2) or hydrogen peroxide (H_2O_2). Superoxide is produced as a by-product of oxygen metabolism and, if not regulated, causes many types of cell damage (Hayyan *et al.*, 2016). Thus, SOD is an important antioxidant defense in nearly all living cells exposed to oxygen. A typical SOD-catalyzed dismutation of superoxide is represented by the following reactions:

$$Cu^{2+}\text{-SOD} + O^{2-} \rightarrow Cu^+\text{-SOD} + O_2$$

$$Cu^+\text{-SOD} + O_2^- + 2H^+ \rightarrow Cu^{2+}\text{-SOD} + H_2O_2$$
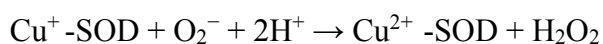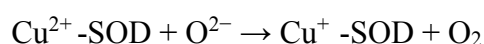
A recent study has reported that in higher plants, the superoxide dismutase enzymes act as antioxidants and protect cellular components from being oxidized by reactive oxygen species (ROS) (Alscher *et al.*, 2002).

2.4.2   Pathogenesis-Related Proteins

There are 17 classes of pathogenesis-related (PR) proteins currently identified (van Loon *et al.*, 2006). PR proteins are defined as 'those proteins that are not or only at

basal concentrations detectable in healthy tissues, but for which accumulation at the protein level has been demonstrated upon pathological conditions and related situations in at least two or more plant–pathogen combinations' (van Loon & van Strien, 1999). The function of these PR proteins is to protect the plants from pathogens and infection. The expressions of these proteins are induced when the plant is subjected to pathogenic attack. The accumulation of the proteins does not just occur only in the infected area but also systematically as they play a key role in the systemic acquired resistance (SAR) of the plant. SAR serves to protect the plant from further pathogenic attack (Antoniw & Pierpoint, 1978; van Loon *et al.*, 1994). PR proteins share an important common feature namely antifungal activity. Certain PR proteins also exhibit activity towards other plant pathogens such as bacteria, insects, nematodes and viruses (van Loon & van Strien, 1999; Selitrennikoff, 2001; Van Loon *et al.*, 1994, Ahmed *et al.*, 2013). A complete list of the PR protein classes is as below (Lu *et al.,* 2006; Sels *et al.*, 2008).

Table 2.1 Main Properties of Classified Families of Pathogenesis-Related (PR) Proteins

Family	Type member	Typical size (kDa)	Properties
PR-1	Tobacco PR-1a	15	Antifungal
PR-2	Tobacco PR-2	30	b-1,3-Glucanase
PR-3	Tobacco P, Q	25–30	Chitinase (class I,II, IV,V,VI,VI)
PR-4	Tobacco 'R'	15–20	Chitinase class I,II
PR-5	Tobacco S	25	Thaumatin-like
PR-6	Tomato Inhibitor I	8	Proteinase-inhibitor
PR-7	Tomato P69	75	Endoproteinase
PR-8	Cucumber chitinase	28	Chitinase class III
PR-9	Tobacco 'lignin-forming peroxidase'	35	Peroxidase
PR-10	Parsley 'PR1'	17	'Ribonuclease-like'
PR-11	Tobacco 'class V' chitinase	40	Chitinase class I
PR-12	Radish Rs-AFP3	5	Defensin
PR-13	Arabidopsis THI2.1	5	Thionin
PR-14	Barley LTP4	9	Lipid-transfer protein
PR-15	Barley OxOa (germin)	20	Oxalate oxidase
PR-16	Barley OxOLP	20	'Oxalate oxidase-like'
PR-17	Tobacco PRp27	27	Unknown

2.4.2.1 Thaumatin-like Proteins

Thaumatin-like proteins (TLP) belong to the PR-5 class of PR proteins. Though PR proteins, TLPs play roles in the development of the plant as well. They have been described to be involved in seed, fruit and flower development besides being a key component of the abiotic and biotic stress response system (Velazhahan *et al.,* 1999;

Anlovar & Dermastia, 2003; Ahmed *et al.*, 2013). The role of TLPs in the plant defense system is as an antifungal protein. TLPs have different mechanisms of conferring antifungal activity however. The most common method documented is through membrane permeabilization and the binding of TLPs to β-1,3-glucanase and the subsequent degradation of the bonds. As β-1,3-glucanase is an integral membrane protein of fungal species, the degradation of the bonds results in the instability of the fungal membrane leading to its eventual death (Vigers *et al.,* 1991; Sakamoto *et al.,* 2006). Besides this, some TLPs are capable of inhibiting the enzymatic activities of important fungal enzymes. Fungal pathogens rely on enzymes such as trypsin to penetrate into the host cell. By inhibiting those activities, the infection can be stopped. Other enzymes that are inhibited by some respective TLPs are xylanases and α-amylase (Fierens *et al.,* 2007; Schimoler-O'Rourke *et al.,* 2001).

Research on TLPs antifungal activity has been extensively conducted. TLPs have been isolated from several different organisms. A TLP isolated from *Solanum nigrum* was found to exhibit antifungal activity towards *Fusarium* and *Collectotrichum in vitro* (Campos *et al.*, 2008; Wang *et al.*, 2013). TLPs, isolated from *Castanea sativa* and *Castanopsis chinensis* have also demonstrated antifungal activity towards *Fusarium* as well as *Trichoderma viride*, *Botrytis cinerea*, *Mycosphaerella arachidicola*, and *Physalospora piricola* (Garcia-Casado *et al.,* 2000; Chu & Ng, 2003; Wang *et al.*, 2013).

From kiwi fruit, a 21 kDa protein was sequenced by Edman degradation and found to be a TLP. Designated kiwi-fruit TLP, it was challenged with *Botrytis cinerea*, *Mycosphaerella arachidicola* and *Coprinus comatus*. The TLP was found to exhibit antifungal activity against *Botrytis cinerea*. However, there were only minimal suppressive effects on the other two strains (Wang & Ng, 2001). In 1999, a TLP was

purified from French bean. When challenged with fungal pathogens, the TLP exhibited antifungal activity against *Fusarium oxysporum*. However, it did not display antifungal activity against *Rhizoctonia solani* (Ye *et al.*, 1999).

Research has also been carried out in over-expressing TLPs in transgenic plants. The over-expression of TLPs in transgenic tobacco and potato plants resulted in the plants being conferred a degree of resistance towards *Phytophthora parasitica* and *Macrophomina phaseolina* infections. Following infection, the symptom development was delayed. However, the symptoms could not be prevented completely; only delayed (Liu *et al.,* 2012; Acharya *et al.,* 2012).

Although the research on banana TLPs are lacking, some interesting findings have been made. A TLP was successfully isolated from Emperor bananas and challenged with *F. oxysporum* and *M. arachidicola*. The TLP not only exhibited antifungal activity; the activity was more potent than TLPs isolated from French beans and kiwi fruits (Vincent *et al.,* 2007; Yasmin & Saleem, 2014). A TLP isolated and purified from Basari bananas has also demonstrated antifungal activity. It was found to inhibit the growth of *F. oxysporum, A. niger*, *A. fumigatus* and *T. viride* at high LC50 values (Yasmin & Saleem, 2014). These findings suggest that there is a need to look into banana TLPs as a source of antifungal activity.

2.4.2.2 Plant Defensins

Plant defensins are small, cysteine-rich proteins belonging to the PR-12 class of PR proteins. They are termed 'plant defensins' as defensins are present in other types of organisms across the different kingdoms. Defensins have been identified in mammals, including humans, as well as in insects. Among the plant species, defensins are highly prevalent and have been isolated from many different species (Lay & Anderson, 2005; van der Weerden & Anderson, 2013). Plant defensins have been demonstrated to have

antifungal activity. However, they have also been observed to exhibit antibacterial activity, zinc tolerance, ion channel blocking and inhibition of protein translation machinery, α-amylases and proteases (Collila *et al.*, 1990; Bloch & Richardson, 1991; Thomma *et al*., 2002; Carvalho & Gomes, 2009; van der Weerden & Anderson, 2013).

Plant defensins have been shown to inhibit the growth of fungi through specific binding to membrane targets. However, the precise mode of action of plant defensins is not yet known. There are two schools of thought as to the mode of action of plant defensins (Thomma *et al.*, 2002). One theory revolves around the idea that plant defensins cause multimeric pores to form on the fungal membranes. Through electrostatic binding, the peptides adhere to the fungal cell surface and subsequently, insert into the energized cell membrane and form multimeric ion-permeable channels (Kagan *et al*., 1990; Cociancich *et al*., 1993; Lehrer *et al*., 1993; Wimley *et al*., 1994; Hristova *et al*., 1996; Maget-Dana & Ptak, 1997; Thomma *et al.*, 2002).

A second model postulates that a mechanism of membrane permeabilization which involves the binding of the peptides onto the anionic lipid head groups of the membrane. This results in the disruption of the integrity of the lipid bilayer, causing pores to open up across the membrane, allowing for the movement of ions and larger molecules across the membrane (Oren & Shai 1998; Shai, 1999; Hoover *et al.,* 2000). Although neither model has been proven conclusively, they both bear similarities in that the mode of action of defensins is centered around ionic activity and membrane instability.

Research done on plant defensins have demonstrated the antifungal activity they possess. In 1999, plant defensins isolated from *Dahlia merckii* where shown to exhibit antifungal activity against *Saccharomyces cerevisiae* (Thevissen *et al.,* 1999).

Transgenic potatoes that had the alfalfa defensin protein introduced into their cells exhibited significant resistance against the fungal pathogen *Verticillium dahlia* in contrast to non-transgenic potatoes (Gao, 2000).

In 2009, a defensin from *Raphanus sativus* was successfully isolated and challenged against *Candida albicans*. The defensin exhibited antifungal activity against the fungal pathogen by causing apoptosis in a metacaspase independent way (Aerts *et al*., 2009).

2.4.2.3 Thionins

Thionins are class 13 PR proteins. First identified and purified in 1968, thionins were the first eukaryotic peptides recognized to play a key role in the plant defense system (Davis *et al*., 1968; Pelegrini & Franco, 2005). Thionins are small basic peptides with a characteristic three-dimensional structure stabilized by six to eight disulfide-linked cysteine residues (Stec, 2006; Ponz *et al*., 1983; Asano *et al*., 2013). The general structure of thionins consist of two small a-helices and two small antiparallel b-sheets which are stabilized by disulfide bridges (Stec, 2006; Abbas *et al*., 2013). The antifungal activity of thionins is due to its actions on fungal membranes. Thionins are known to induce instability and subsequently, the destruction, of fungal membranes. This is achieved by opening pores on the fungal membrane; allowing for the escape of potassium and calcium ions (Pelegrini & Franco, 2005; Oard, 2011; Asano *et al*., 2013).

Thionins were identified before PR proteins and their classifications were officially designated. In 1988, a thionin was isolated and purified from barley leaf. The thionin was found to exhibit antifungal properties. More interestingly at that time was that the expression of the thionin was found to have increased following pathogen attack. That led to the thionin being regarded as a 'naturally occurring, inducible plant protein possibly involved in the mechanism of plant defence' (Bohlmann *et al.,* 1988). That, in essence, was the very definition of a PR protein.

Research on thionins has accelerated in recent times. Thionins are now introduced into plants in order to confer fungal resistance. In 2012, thionins isolated from *Brassicaceae* sp. were introduced into potato plants. The transgenic potatoes were found to exhibit antifungal activity towards *Botrytis cinerea*, having enhanced resistance towards grey mold (Hoshikawa *et al.,* 2012). Also in 2012, sweet potatoes were transformed to contain a thionin isolated from barley. The transformed sweet potatoes were found to exhibit increased resistance towards the black rot disease (Muramoto *et al.,* 2012).

A thionin from *Arabidopsis thaliana* was isolated and tested for antifungal properties. The thionin was found to exhibit antifungal properties against *Fusarium graminearum*. The toxicity of the fruiting bodies produced by the fungal pathogen was suppressed by the thionin (Asano *et al.,* 2013).

CHAPTER 3: REACTIVE OXYGEN SPECIES (ROS) ACTIVITY IN BERANGAN BANANAS

3.1   Introduction

This chapter focuses on determining the activity of ROS found in Berangan bananas. The peel and pulp of healthy Berangan bananas at Stage I and Stage V were sampled as well as the peel and pulp of infected Berangan bananas.

ROS compounds play a crucial role in the hypersensitive response in plants. These compounds are induced when the plant is under attack by pathogens in order to minimize the damage and stem the infection. The ROS compounds cause apoptosis by generating free radicals that proceed to destabilize the cells resulting in cell death. Among the ROS enzymes involved in these processes are peroxidase, poplyphenol oxidase and lipoxygenase.

However, if the apoptosis is not regulated, it could result in significant damages to the healthy plant cells. As such, some ROS compounds serve to scavenge the generated free radicals in order to prevent unrestricted damage. Typically, the radicals are broken down or reduced into non-harmful substances such as water and oxygen. Among the ROS compounds that catalyse these reactions are ascorbate peroxidase, catalase, guaiacol peroxidase and superoxide dismutase.

The aims of this chapter are to:

i. To determine the ROS protein activity of the protein extract of banana peel and pulp at Stages I and V

ii. To determine the ROS protein activity of the protein extract of infected Stage VII banana peel and pulp

3.2 Material and Methods

3.2.1 Preparation of Banana Samples

Berangan bananas were purchased from commercial markets. The bananas were purchased at Stages I and V. Infected bananas were also obtained at the market. Random fingers were taken from the hands of the bananas and the total protein was extracted from the hands.

3.2.2 Preparation of Crude Protein Extract

3.2.2.1 Protein Extraction

Six grams of banana pulp tissue were ground to a fine powder in liquid nitrogen with a mortar and pestle. The powder was suspended in 8 mL of a modified Kanellis *et al.* (1989) protein extraction buffer containing 50 mM Tris-HCl, 0.5 M NaCl, 10 mM 2-mercaptoethanol, 10 µM leupeptin, 1 mM DTT, 1 mM EDTA, 10% glycerol and 0.5% Triton X-100 (tissue to volume ratio = 3:4). The mixture was vortexed thoroughly, left on ice for 10 minutes and then centrifuged at $25,000 \times g$ for 30 minutes at 4 °C in a Sorvall RC5C refrigerated centrifuge. The pellet was discarded and the supernatant was filtered through Mira cloth as crude extract. The crude extract was stored at -20°C until further use.

3.2.3 ROS Assays

3.2.3.1 Peroxidase Assay

(a) Preparation of reagents

0.2M Phosphate buffer pH 6.0

2.43 g of monosodium phosphate and 0.64 g disodium phosphate was dissolved in 100 mL of Sterile Distilled Water.

(b) Assay

This assay was carried out based on the method by Maia *et al.* (2011). In this assay, 2 mL of the 0.2 M Phosphate buffer (pH 6.0) and 0.5% frozen guaiacol was added into a test tube. The test tube was placed into a water bath at 30°C to stabilize its temperature. Following this, 100 µL of the enzyme extract and 100 µL of 0.08% Hydrogen peroxide were added to the tube. The tube was vortexed vigorously and seven absorbance readings at 470 nm were taken at 30 second intervals for a period of three minutes. The results were expressed in enzyme units per minute which is the increase in 0.001 absorbance readings per minute.

3.2.3.2 Polyphenol Oxidase activity

(a) Preparation of reagents

0.2M Cathechol

0.220 g cathechol was dissolved in 10 mL of SDW

(b) Assay

This assay was carried out based on the method by Maia *et al.* (2011). 1.3 mL of 0.2 M Phosphate buffer (pH 6.0) and 1.5mL of 0.2 M Catechol was filled into a test tube. This tube was placed in a water bath at 25°C to stabilize the temperature. Thereafter, 30 µL enzyme extract was added. The tube was vortexed vigorously and seven absorbance readings were taken at 425 nm at 30 second intervals for a period of three minutes. The results were expressed in enzyme units per minute which is the increase in 0.001 absorbance readings per minute.

3.2.3.3 Lipoxygenase Assay

(a) Preparation of reagents

0.2 M Na-Phosphate (pH 6.0)

2.43 g of monosodium phosphate and 0.64 g disodium phosphate was dissolved in 100 mL of SDW.

(b) Assay

This assay was carried out based on the method presented by Bisht *et al.* (2014). The reaction mixture was prepared in a test tube comprising of 2.7 mL of 0.2 M Na-Phosphate (pH 6) and 0.3 mL of linoleic acid. Following that, 25 uL of crude enzyme extract was added to the test tube and mixed by swirling. The absorbance was read at 234 nm for three minutes at one minute intervals. Results were expressed as activity per minute per µL.

3.2.3.4 Ascorbate Peroxidase Assay

(a) Assay

This assay was carried out based on the method presented by Panchuk *et al.* (2002). A reaction mixture of 1 mL containing 25 mM of sodium phosphate (pH 6), 0.1 mM of EDTA, 1 mM of hydrogen peroxide and 0.25 mM of ascorbic acid was prepared. The mixture was mixed by inversion and 200 µL of crude enzyme extract was added. Absorbance readings were taken at 290 nm at 0 minutes and 1 minute.

3.2.3.5 Guaicol Peroxidase Assay

(a) Assay

This assay was carried out based on the method presented by Mika *et al.* (2003). A reaction mixture of 1 mL containing 25 mM of sodium acetate - HCl (pH 5), 8.26 mM of guaicol and 8.8 mM of hydrogen peroxide was prepared. The mixture was mixed by

inversion and 25 µL of crude enzyme extract was added. Absorbance readings were taken at 470 nm at 0 minute, 1 minute and 2 minutes.

3.2.3.6 Catalase Assay

(a) Assay

This assay was carried out based on the method presented by Xu *et al.* (2013). A reaction mixture of 2 mL containing 50 mM of sodium phosphate (pH 6) and 10 mM of hydrogen peroxide was prepared. The mixture was mixed by inversion and 200 µL of crude enzyme extract was added. Absorbance readings were taken at 240 nm at 1 minute intervals for 4 minutes.

3.2.3.7 Superoxidase Dismutase Assay

(a) Assay

This assay was carried out based on the method presented by Xu *et al.* (2013). A reaction mixture of 3 mL containing 50 mM of potassium phosphate (pH 7.8), 13 mM Met, 75 mM NBT, 2 mM riboflavin and 0.1 mM EDTA was prepared. The mixture was mixed by inversion and 100 µL of crude enzyme extract was added. Absorbance readings were taken at 560 nm at 1 minute intervals for 4 minutes.

3.3   Results

Higher peroxidase activity was observed in the peel of the fruit as opposed to the pulp (Figure 3.1). At Stage I, activity was observed to be 213.9 enzyme units per min in the peel and 158.2 enzyme units per min in the pulp. At Stage V, activity was 194.37 enzyme units per min in the peel and 142.4 enzyme units per min in the pulp. Activity in infected samples was observed to be the highest with activity of 238.54 enzyme units per min in the peel and 176.39 enzyme units per min in the pulp.

Figure 3.1: Peroxidase Activity of peel and pulp of Berangan cultivar at Stage I, Stage V, and Infected Stage VII

Higher polyphenol oxidase activity was observed in the peel of the fruit as opposed to the pulp (Figure 3.2). At Stage I, activity was observed to be 261.5 enzyme units per min in the peel and 78.17 enzyme units per min in the pulp. At Stage V, activity was 158.17 enzyme units per min in the peel and 41.72 enzyme units per min in the pulp. Activity in infected samples was observed to be the highest with 281.5 enzyme units per min in the peel and 156.0 enzyme units per min in the pulp.
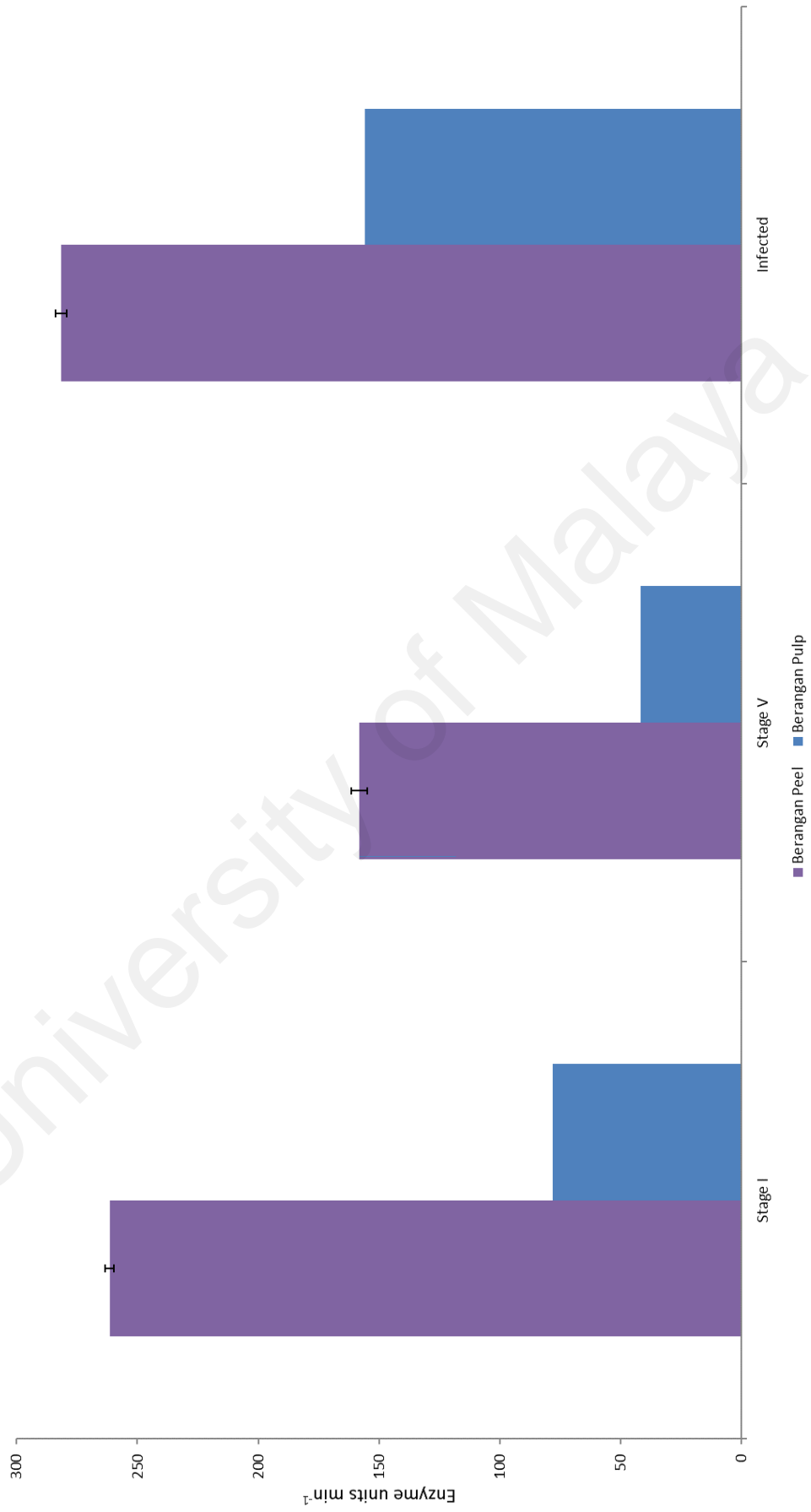
Figure 3.2: Polyphenol Oxidase Activity of peel and pulp of Berangan cultivar at Stage I, Stage V, and Infected Stage VII

Higher lipoxygenase activity was observed in the peel of the fruit as opposed to the pulp (Figure 3.3). At Stage I, activity was observed to be 1.04 enzyme units per min in the peel and 0.14 enzyme units per min in the pulp. At Stage V, activity was 1.09 enzyme units per min in the peel and 0.22 enzyme units per min in the pulp. Activity in infected samples was observed to be the highest with activity of 1.27 enzyme units per min in the peel and 0.25 enzyme units per min in the pulp.
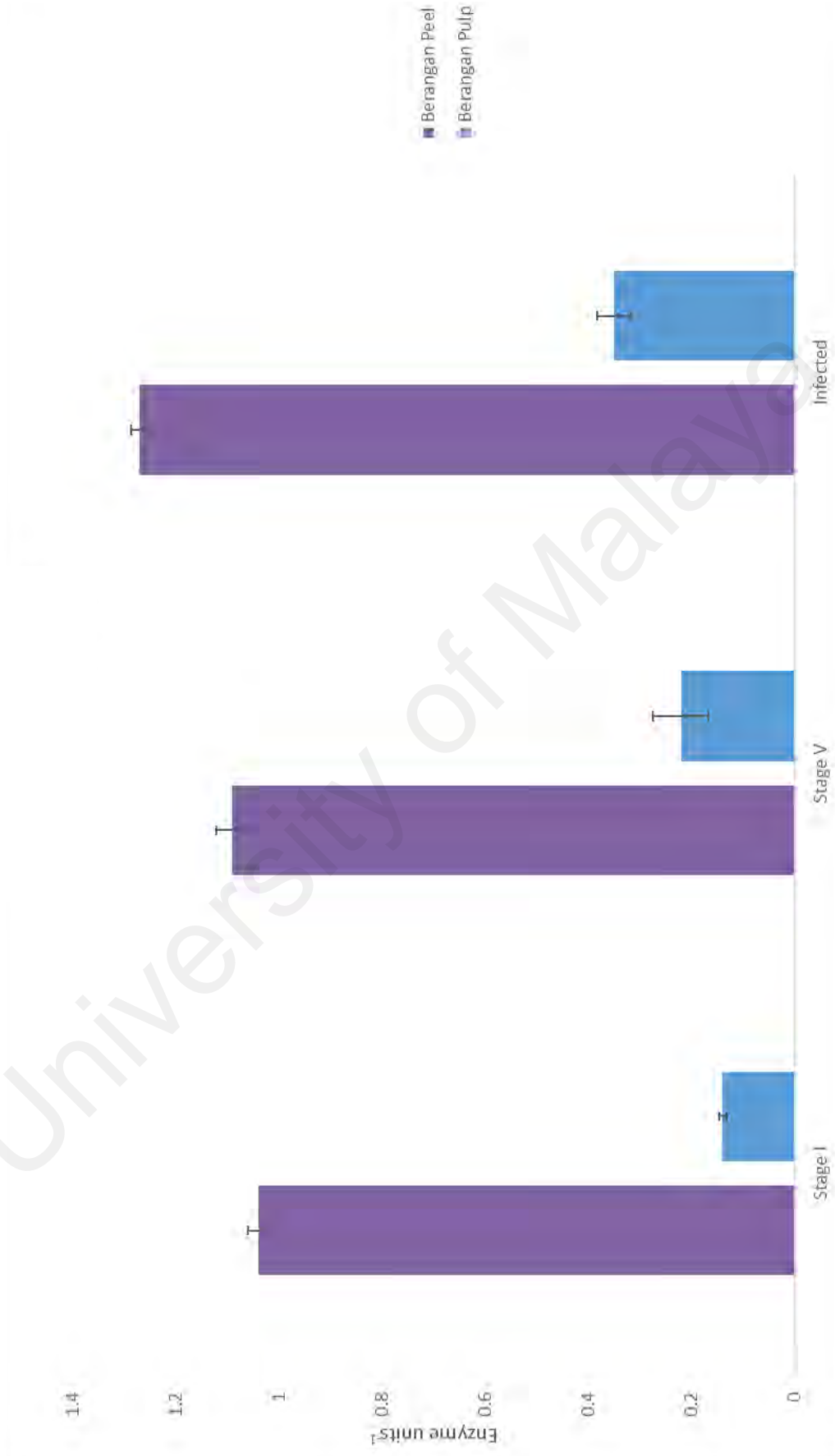
Figure 3.3: Lipoxygenase Activity of peel and pulp of Berangan cultivar at Stage I, Stage V, and Infected Stage VII

Higher ascorbate peroxidase activity was observed in the peel of the fruit as opposed to the pulp (Figure 3.4). At Stage I, activity was observed to be 0.22 enzyme units per min in the peel and 0.07 enzyme units per min in the pulp. At Stage V, activity was 0.29 enzyme units per min in the peel and 0.1 enzyme units per min in the pulp. Activity in infected samples was observed to be the highest with activity of 0.34 enzyme units per min in the peel and 0.31 enzyme units per min in the pulp.
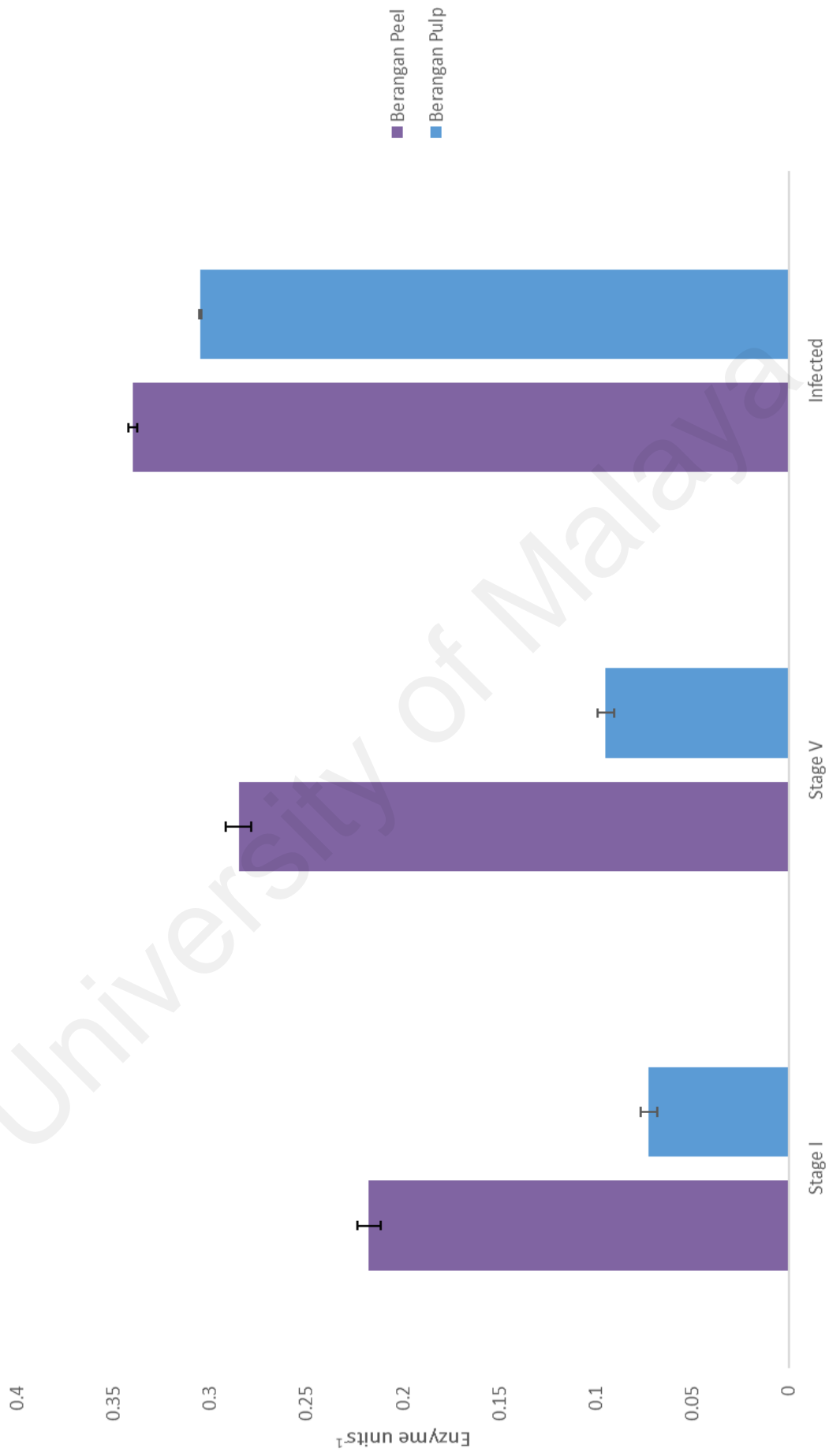
Figure 3.4: Ascorbate Peroxidase Activity of peel and pulp of Berangan cultivar at Stage I, Stage V, and Infected Stage VII

Higher catalase activity was observed in the peel of the fruit as opposed to the pulp (Figure 3.5). At Stage I, activity was observed to be 0.02 enzyme units per min in the peel and 0.017 enzyme units per min in the pulp. At Stage V, activity was 0.01 enzyme units per min in the peel and 0.009 enzyme units per min in the pulp. Activity in infected samples was observed to be the highest with activity of 0.087 enzyme units per min in the peel and 0.071 enzyme units per min in the pulp.
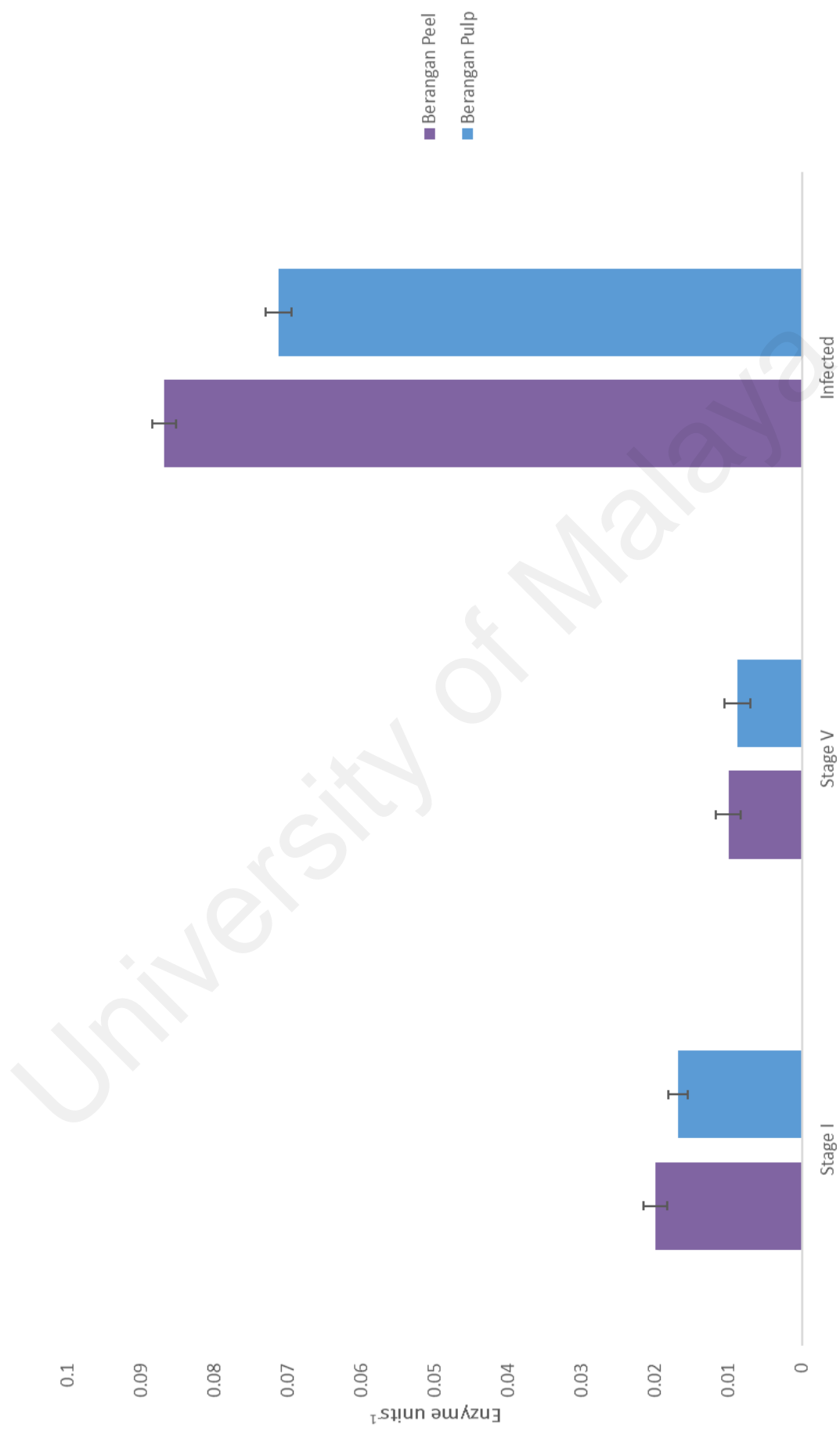
Figure 3.5: Catalase Assay of peel and pulp of Berangan cultivar at Stage I, Stage V, and Infected Stage VII

Higher guaicol peroxidase activity was observed in the peel of the fruit as opposed to the pulp (Figure 3.6). At Stage I, activity was observed to be 0.02 enzyme units per min in the peel and 0.015 enzyme units per min in the pulp. At Stage V, activity was 0.017 enzyme units per min in the peel and 0.014 enzyme units per min in the pulp. Activity in infected samples was observed to be the highest with activity of 0.031 enzyme units per min in the peel and 0.019 enzyme units per min in the pulp.
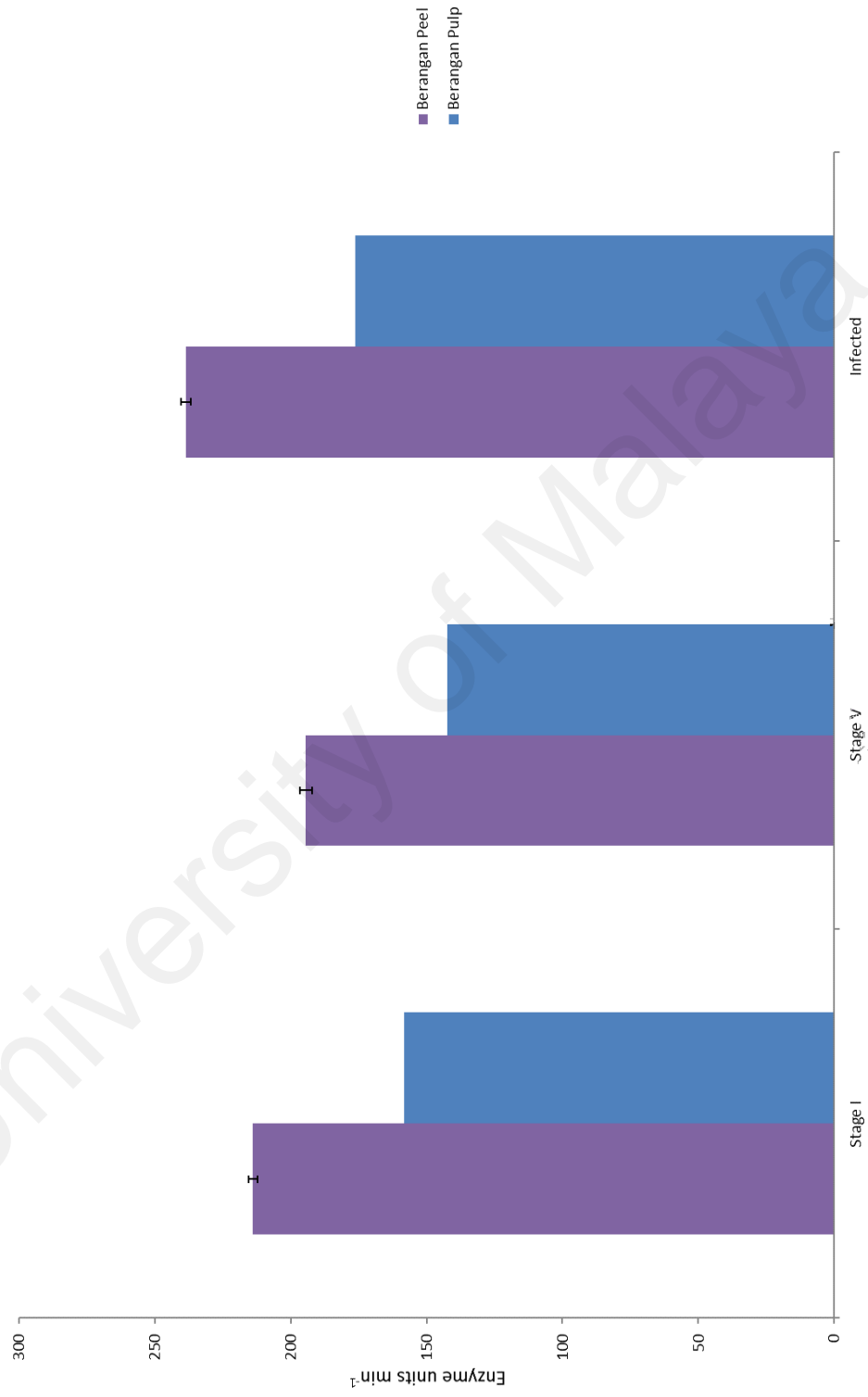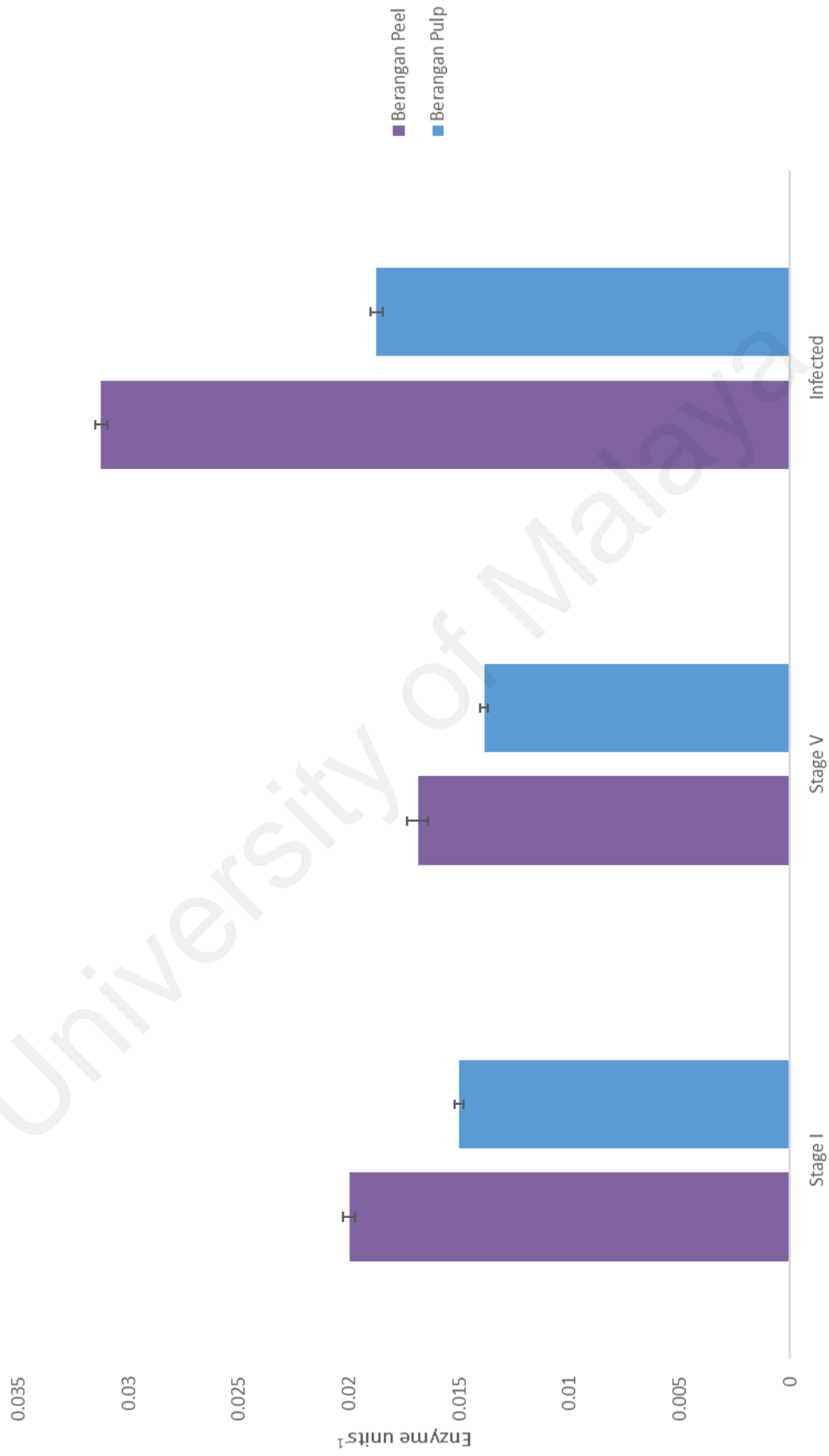
Figure 3.6: Guaicol Peroxidase Activity of peel and pulp of Berangan cultivar at Stage I, Stage V, and Infected Stage VII

Higher superoxide dismutase activity was observed in the peel of the fruit as opposed to the pulp (Figure 3.7). At Stage I, activity was observed to be 0.00145 enzyme units per min in the peel and 0.00095 enzyme units per min in the pulp. At Stage V, activity was 0.00118 enzyme units per min in the peel and 0.00037 enzyme units per min in the pulp. Activity in infected samples was observed to be the highest with activity of 0.00167 enzyme units per min in the peel and 0.00139 enzyme units per min in the pulp.

Figure 3.7: Superoxide Dismutase Activity of peel and pulp of Berangan cultivar at Stage I, Stage V , and Infected Stage VII

3.4   Discussion

A significant decrease is seen in the activity of peroxidase as the fruit ripens in both the peel and pulp. Peroxidases play an important role in the cell wall degradation during the ripening process. Peroxidases cause the destabilising of the cell wall membrane by compromising the integrity of the cell wall. Peroxidases oxidise lignins which are key components of the cell wall, leading to the formation of pores in the cell wall. Due to this, ionic leakage occurs, resulting in the destabilization of the cell wall membrane and subsequently, its degradation (Gandia- Herrero, 2002; O'Brien *et al.*, 2012).

Peroxidase activity was significantly higher in the peel of the fruit at both stages. This could be because plant peroxidases are critical to the plant hypersensitive response (Singh & Upadhyay, 2013). The plant hypersensitive response is one of the first lines of defense in a plant towards pathogen attacks. Seeing as the peel is most susceptible to attack as pathogens must penetrate the peel before reaching the pulp, the high peroxidase activity in the peel could be to serve as a barrier and first line of defense against pathogen attacks.

Significantly higher level of peroxidase activity was observed in the infected samples. This is most likely due to the peroxidase activity being induced by the pathogen attack. Peroxidase is heavily involved in the plant hypersensitive response pathway. The HR effect causes localized cell death to stem the spread of the infection and kill the pathogens involved. Peroxidase activity plays a key role in the cell membrane degradation that causes the plant cells to be destroyed through lipid peroxidation (Heath, 2000; Matthews, 2007).

The polyphenol oxidase activity decreased significantly in the pulp as the fruit ripened. The decrease in the pulp is consistent due to the function of polyphenol oxidase. Polyphenol oxidase is responsible for the breakdown of polyphenols present in

the cell. As the cell membrane is perforated and destabilised by peroxidase, there is a flow of polyphenol oxidases into the cell and of polyphenols out of the cell. The polyphenol oxidases will then react with the polyphenol, leading to their breakdown. This reaction is the cause of enzymatic browning often seen in fruits (Vaughn & Duke, 1984; Cano *et al.,* 1997; Yingsanga *et al.,* 2008).

The decrease seen in the peel of the Berangan cultivar may be because polyphenol oxidase is not considered a key defense protein in the Berangan cultivar. It is possible that other plant pathogenesis- related proteins are expressed instead. As such, the need for polyphenol oxidase is reduced and it is not expressed strongly (Vaughn & Duke, 1984; Kasprzewska, 2003).

The decrease in polyphenol peroxidase activity as the fruit ripens is also similar to the decrease observed in peroxidases. This may be because PPO also plays an active role in the cell wall degradation process. It is responsible for the destabilizing of the cell membrane, leading to ion leakage which eventually causes the breakdown of the cell (Barre *et al.,* 2000; O'Brien *et al.*, 2012).

The infected samples show significantly higher activity than uninfected samples. Similar to POD, PPO plays an important role in the plant hypersensitive response. As such, PPO is induced in order to combat the infection. Its ability to destabilize cell membranes and cause apoptosis is crucial in stemming the tide of the infection. As such, the higher activity observed in the infected samples is likely due to the protein being induced due to the hypersensitive response.

Unlike POD and PPO, the activity of lipoxygenase was higher at the later stages of ripening. Stage V showed significantly higher LOX activity as compared to Stage I. LOX catalyses the oxygenation of polyunsaturated fatty acids and lipids resulting in

unsaturated fatty acid hydroperoxides. This conversion plays an important role in the ripening process as it leads to membrane degradation. As such, the cells are weakened and are ultimately broken down (Brennan & Frenkel, 1977; Brennan *et al*., 1979; Thompson, 1988; Rogiers *et al*., 1998, Yang *et al*., 2012).

The higher activity at Stage V indicates that LOX plays an important role in the fruit softening that occurs following ethylene burst. Fruit softening begins at Stage V which corresponds with the peak in LOX activity. This has been shown in research on other climacteric fruits such as kiwifruit and peaches. The higher activity observed in this study corresponds with the data seen in those studies (Chen *et al*., 1998; Wu *et al*., 1999; Zhang *et al*., 2003).

The infected fruit samples showed the highest LOX activity. That strengthens the inference that LOX plays a significant role in the plant hypersensitive response. LOX functions by catalyzing the addition of molecular oxygen to linoleic acid and linolenic present in plant cells thus producing an unsaturated fatty acid hydroperoxide (Porta & Rocha-Sosa, 2002). The hydroperoxide plays a significant role in destabilising cell membranes and leading to cell death. During pathogen attack, LOX activity plays other important role as well such as the synthesis of signaling compounds to antimicrobial activity. Thus, the significantly high activities of LOX are possibly due to the fact that it plays multiple roles in the plant pathogen response (Creelman & Mullet, 1997; Parchmann *et al*., 1997).

The LOX activity was observed to be highest in the peel of the banana as compared to the pulp. This could be due to the fact that banana peel contains up to $4\times$ the amount of lipid that is contained within the pulp (Goldstein & Wick, 2006; Vilela *et al*., 2014).

Ascorbate peroxidase activity was observed to increase as ripening progresses. Stage V samples demonstrated significantly higher values of APX as compared to Stage I. APX has been shown to play a role in the fruit ripening process. APX scavenges hydrogen peroxide by breaking it down into water and oxygen using ascorbate as a substrate. The main action of peroxidases takes place at the start of the ripening process. By Stage V, the ripening process utilizing the peroxidases has largely been completed. Excess peroxidase at that point would only serve to damage and destroy healthy cells. In order to prevent that, APX activity increases as the plant ripens. This result is related to the reduction in peroxidase activity observed at Stage V earlier. This trend has also been observed with other fruits such as papaya. In papaya, APX was observed to have increased as the fruit ripened (Pandey *et al*., 2013).

Infected samples showed the highest APX activity of all samples. The reason behind the higher activity is due to the induction of the ROS peroxidases. Upon infection, peroxidases are induced in order to combat the infection. They serve to cause localized cell death so as to isolate the infection and prevent it from spreading. However, the peroxidases will indiscriminately kill all cells they come across. As such, the buildup of APX in healthy cells that should not be subjected to apoptosis protects the cells as the APX will scavenge the peroxidases, reducing it to less harmful substances such as water and oxygen. APX activity has been shown to increase in plants infected by pathogens such as apricot, wheat and mung bean (Hernandez *et al*., 2001; Chen *et al*., 2015; Farahani & Taghavi, 2016).

In this study, APX was observed to be higher in the peel of the fruit as compared to the pulp. This is consistent with the POD activity as POD activity was also higher in the peel. As APX works directly on peroxidase, it is logical to find that APX activity is highest where POD activity is. Research on apples has shown similar results with APX

activity being the highest in the apple peel followed by the apple flesh (Patykowski *et al.*, 2007).

Catalase activity was observed to be highest at the initial stages of ripening. The activity at Stage V was significantly lower than the activity at Stage I. Catalase catalyzes the decomposition of hydrogen peroxide to water and oxygen. However, unlike APX which increases as the fruit ripens, CAT decreases. It is highest at the unripe stage. This suggests that the role of CAT in preventing oxidative damage is mainly triggered before the ripening process gets fully underway. This echoes previous research that has shown that CAT activity decreases as the fruit ripens. Researches on oranges and raspberries have both shown that CAT activity decreases as the fruit ripens (Huang *et al.*, 2007; Kivi *et al.*, 2014).

The activity of CAT was observed to be higher in the infected samples. This is in line with APX activity as well as CAT also functions to break down the hydrogen peroxide. Due to the accumulation of hydrogen peroxide in infected tissues, a buildup of hydrogen peroxide scavengers is also induced. CAT plays a critical role in that process as it is among the most efficient decomposers of hydrogen peroxide. Therefore, the increase in CAT in infected samples can be attributed to its role in decomposing the ROS free radicals.

CAT activity was observed to be higher in the peel as compared to the pulp of the fruit. The higher concentration of hydrogen peroxide in the pulp is likely to be the major cause of this as hydrogen peroxide is the substrate that CAT acts on. Thus, the buildup of CAT in the peel is likely because of the role CAT plays in restricting the damage caused by hydrogen peroxide.

In this experiment, guaichol peroxidase activity was observed to be lower at later stages of ripening. Stage V showed significantly lower GPX activity as compared to Stage I. GPX plays a role in the scavenging of POD into harmless compounds such as water and oxygen. It is likely that as POD activity has decreased at Stage V and with the prevalence of other scavengers such as APX, the need for GPX is reduced. In contrast, the high POD activity at Stage I and the lower availability of other scavengers could possibly be the reason for the higher activity of GPX at that stage. The lower activity at Stage V is in line with observations from other fruit models. GPX activity was observed to have decreased during ripening of blackberry and papaya fruits (Wang *et al*., 2001; Pandey *et al*., 2013).

The GPX activity was also observed to be higher in the infected samples as compared to the uninfected samples. This is largely due to the fact that as POD is induced as a defense response to pathogen attack, GPX is induced in order to regulate the POD and prevent unrestricted cell death. GPX works in tandem with the other ROS scavengers in order to ensure that the POD does not destroy healthy cells, causing even more damage to the plant.

GPX activity was observed to be higher in the peel of the banana as compared to the pulp. This is consistent with the trend observed with the previous scavengers where the buildup occurred mainly in the peel of the fruit as it coincided with the accumulation of POD.

Superoxide dismutase activity was observed to be higher at Stage I as compared to Stage V. It can be inferred that the activity decreases following ripening. SOD catalyses the dismutation of superoxide into either oxygen or hydrogen peroxide. Its higher activity at Stage I could be due to the higher activity of plant ROS enzymes that generate the superoxides. As the plant ripens, the activity and concentrations of

superoxides decrease thus reducing the need for SOD. As such, the SOD activity subsequently decreases (Mondal *et al.*, 2004). These results correspond with current literature. SOD has been determined to decrease during the ripening process in other fruits such as in oranges and tomatoes (Mondal *et al.*, 2004; Huang *et al.*, 2007). The results here are in line with those discoveries.

SOD activity was observed to be significantly higher in the infected samples as compared to the healthy samples. As SOD is the sole scavenger of superoxide, it stands to reason that SOD activity increases as the superoxide activity increases. The increase in SOD activity allows it to regulate the superoxide formation so as to ensure that it does not result in unregulated cell death. These results are in line with reported roles of SOD in other fruits. SOD has been shown to increase in infected barley and tobacco plants in order to regulate the superoxides as well as to serve as a signaling mechanism (Bowler *et al.*, 1989; Lightfoot *et al.*, 2016).

As with the previous ROS compounds, SOD was observed to be higher in the peel than in the pulp of the fruit. This is most likely due to the buildup of superoxides in the peel of the fruit as a result of the accumulation of ROS compounds in the peel. Therefore, the buildup of SOD is in order to regulate the superoxides.

3.5 Conclusion

Of the enzymes involved in generating the ROS compounds that led to cell instability and death, POD and PPO had lower activity at Stage V while LOX showed higher activity at Stage V. This infers that POD and PPO play different roles in the ripening process as compared to LOX. These enzymes are involved heavily in the initial cell breakdown before the oxidative burst is triggered. It is possible that the cell breakdown caused by POD and PPO at the start of ripening is crucial for further ripening of the fruit.

By contrast, it can be deduced that the role of LOX becomes more important at the later stages of ripening, especially following oxidative burst. One of the biggest changes that can be observed following the oxidative burst is the significant loss of firmness. It is likely that this is catalyzed by the LOX as it reacts with the lipids in the cell, destabilizing them.

APX was the only ROS scavenger that had higher activity at Stage V as compared to Stage I. It is possible that the APX activity is higher as it ensures that the fruit is kept relatively safe from further cell damage by peroxidases. CAT, GPX and SOD showed higher activity at Stage I and this is likely due to the increased amount of ROS compounds at that stage. These scavengers play a critical role in maintaining the balance so that the fruit is not overly damaged during the ripening process. Furthermore, it is possible that these enzymes are responsible for reducing the activity of POD and PPO as the plant ripens.

The activity of all the ROS compounds was observed to be higher in the infected samples. This is largely due to the HR being stimulated by the pathogen attack resulting in the ROS compounds being induced. The ROS free radical generators are induced in order to stem the infection by generating free radicals to cause localized cell death and to kill the pathogens. The ROS scavenger compounds are induced in order to protect the healthy cells from unregulated cell death.

The activity was also observed to be higher in the peel for all the compounds. The peel serves as a living, physical barrier to the pathogens. Therefore, the first line of defense is in the peel.

CHAPTER 4: ANTIOXIDANT CONTENT AND ACTIVITY IN BERANGAN

BANANAS

4.1 Introduction

Plants are known to be rich in antioxidants as antioxidants are vital to the plant defense system and overall homeostasis. Most known antioxidants have been found to be multifunctional and as such, more than one method of measurement is required in order to obtain an accurate assessment of the antioxidant activity. Among the different functions performed by antioxidants are the scavenging of specific radicals, the chelating of metal ions and the inhibition of lipid peroxidation. The most commonly used methods to assess antioxidant activity is centered around the colorimetric methods (Martinez et al., 2012).

Polyphenols are compounds containing more than one phenol group. These compounds are known to exhibit antioxidant properties. In order to assess polyphenols, the Total Polyphenol Content (TPC) is used. It utilizes the Folin-Ciocalteu reagent. Folin-Ciocalteu reagent contains tungsten and molybdenum oxides which are reduced in the assay to form a blue coloured chromogen under basic conditions. The assay can be measured by an absorption at 745 to 750 nm (Waterhouse, 2002). While the method is simple and sensitive, it is hampered by its lack of specificity as it detects all phenolic groups present (Shahidi & Naczk, 2004).

The radical scavenging ability conferred by antioxidants can be determined using the radical scavenging activity. The assay utilizes the free radical 1, 1 -diphenyl-2-picryllhydrazyl (DPPH). It measures the capacity of the antioxidant to donate hydrogen to the stable free radical DPPH resulting in the formation of diphenylypicrylhydrazine. The free radical is characterized by a deep violet color with an absorption at 517 nm. As

the free radical is converted, the solution changes from violet to yellow. The color change observed exhibits the radical scavenging potential of the sample (Shon *et al*., 2003; Alam *et al*., 2013). While the method is fast and simple, it is hampered by a lack of accuracy as DPPH can be reduced by other reducing agents as well as a sensitivity to the solvent system and pH (Prior *et al*., 2005).

The Total Antioxidant Capacity (TAC) assay is utilized for the quantitative determination of antioxidant capacity. The assay utilizes phosphomolybdenum wherein it evaluates the reduction of Mo(VI) to Mo(V) in an acidic medium. The Mo(V) phosphate complex formed in the reaction is green in colour and can be measured at 695 nm. It is a simple, cheap and effective method of determining antioxidant activity (Prieto *et al*., 1999).

Another group with antioxidant properties is flavonoids. Flavonoids can be assayed using the Total Flavonoid Content (TFC) assay. It utilizes aluminium chloride which forms acid labile complexes with the ortho- dihydroxyl groups in the aromatic ring of flavonoids. This reaction can be measured at 510 nm (Mabry *et al*., 1970).

This chapter focuses on determining the antioxidant activity found in Berangan bananas. The peel and pulp of healthy Berangan bananas at Stage I and Stage V were sampled as well as the peel and pulp of infected Berangan bananas.

The aims of this chapter are to:

i. To determine the antioxidant content of total phenolic and flavonoid in protein extracts of healthy and infected banana peel and pulp at Stages I and V

ii. To determine the antioxidant activities in protein extracts of healthy and infected banana peel and pulp

4.2 Materials and Methods

4.2.1 Preparation of Banana Samples

Berangan bananas were purchased from commercial markets. The bananas were purchased at Stages I and V. Infected bananas were also obtained at the market. Random fingers were taken from the hands of the bananas. The fingers were turned into powder using liquid nitrogen and mortar and pestle extraction. Equal volumes of sample and 80% methanol were added into 50 mL Falcon tubes. The tubes were placed in a shaking incubator (Shel Lab Orbital Shaking Incubator S14, OR, USA) at 250 rpm for 30 minutes at 25 °C and then centrifuged (Beckman J2-MI Centrifuge, California) at 6,500 rpm for 15 minutes at 5 °C. The supernatant of the centrifuged sample was used for the antioxidant analysis.

4.2.2 Total Polyphenol Content (TPC)

4.2.2.1 Preparation of Reagents

20% sodium carbonate

2 g of anhydrous sodium carbonate (BDH) was dissolved in 10 mL of SDW.

Gallic acid standard curve

Stock solution was prepared by dissolving 20 mg of gallic acid (Sigma) in 100 mL of SDW. The different concentrations of gallic acid standards were prepared according to Table 4.1 and the final volume was 2 mL.

Table 4.1: Gallic Acid Standard Preparation

Concentration (mg/100 mL)	0	20	40	60	80	100	120	140
Volume of stock solution (mL)	0	0.2	0.4	0.6	0.8	1.0	1.2	1.4
Volume of SDW (mL)	2.0	1.8	1.6	1.4	1.2	1.0	0.8	0.6

4.2.2.2 Assay

TPC of samples were determined using Folin-Ciocalteu assay (Singleton & Rossi, 1965) modified to a microscale (Bae & Suh, 2007). Sample extract or gallic acid standard solution (10 µL) was added to 790 µL SDW and 50 µL Folin-Ciocalteu reagent (Sigma-Aldrich) in a 1.5 mL microcentrifuge tube and mixed. After 1 minute, 150 µL of 20% sodium carbonate solution was added and the solution was mixed by inverting the tubes. The mixture was allowed to stand at room temperature (25 ± 1 °C) for 120 minutes in the dark. Absorbance was measured at 750 nm (UV-200-RS Spectrophotometer, MRC, Israel) against a prepared blank which contained SDW in place of sample extract. A standard curve of gallic acid ($y=0.000566x$, $r^2=0.9955$) was prepared ranging from 0 to 140 mg/100 mL and results were expressed as milligrams of gallic acid equivalent (GAE) per 100 mL sample extract.

4.2.3 Total Flavonoid Content

4.2.3.1 Preparation of Reagents

<u>5% sodium nitrite</u>

2.5 g of anhydrous sodium nitrite (Systerm) was dissolved in 50 mL of SDW.

<u>10% aluminium chloride</u>

5 g of anhydrous aluminium chloride (Fisher Scientific) was dissolved in 50 mL of SDW.

<u>1N sodium hydroxide</u>

2 g of sodium hydroxide (Merck) was dissolved in 50 mL of SDW.

<u>95% ethanol</u>

95 mL of ethanol was added into 5 mL of SDW.

<u>Cathechin standard curve</u>

Cathechin standard solution was prepared by dissolving 10 mg of (+)-catechin (Sigma-Aldrich) in 10 mL of 95% ethanol. The different concentrations of catechin standards were prepared according to Table 4.2 and the final volume was 1.5 mL.

Table 4.2: Catechin Standard Preparation

Concentration (mg/100 mL)	0	5	10	20	40	60	80	100
Volume of stock solution (mL)	0	0.08	0.2	0.3	0.6	0.9	1.2	1.5
Volume of 95% ethanol (mL)	1.5	1.4	1.3	1.2	0.9	0.6	0.3	0

4.2.3.2 Assay

Total flavonoid content of sample extracts were evaluated using an aluminum chloride colorimetric method described by Sakanaka *et al.* (2005). Sample extract or catechin standard solution (250 µL) was added to 1.25 mL of SDW and 75 µL of a 5% sodium nitrite solution in a test tube and mixed. After 5 minutes of incubation at room temperature (25 ± 1 °C), 150 µL of a 10% aluminum chloride solution was added to the mixture. The mixture was allowed to stand for another 5 minutes and then, 500 µL of a 1 N sodium hydroxide was added. The mixture was made up to 2.5 mL with SDW and vortexed. Absorbance was measured at 510 nm (UC-200-RS Spectrophotometer, MRC, Israel) against a prepared blank containing SDW in place of sample extract. A standard curve of catechin ($y=0.0135x$, $r^2=0.99943$) was prepared ranging from 0 to 100 mg/100 mL and the results were reported as milligrams of catechin equivalent (CE) per 100 mL sample extract.

4.2.4 DPPH Radical Scavenging Assay

4.2.4.1 Preparation of Reagents

<u>80% methanol</u>

80 mL of methanol (Systerm) was added into 20 mL of SDW.

<u>80% methanolic 0.1 mM DPPH solution</u>

3.94 mg of DPPH (Sigma) was dissolved in 100 mL of 80% methanol.

<u>Ascorbic acid standard curve</u>

Stock solutions were prepared by dissolving 100 mg of L-ascorbic acid (Baker analyzed) in 10 mL of SDW. A new stock solution (100 µg/mL) was prepared by diluting 50 µl of this existing stock solution with SDW to a final volume of 5 mL. The different concentrations of ascorbic acid standards were prepared according to Table 4.3 and the final volume was 2 mL.

Table 4.3: Ascorbic Acid Standard Preparation

Concentration (µg/mL)	0	0.5	1.0	2.0	4.0	6.0	8.0	10.0
Volume of new stock solution (mL)	0	0.01	0.02	0.04	0.08	0.12	0.16	0.20
Volume of SDW (mL)	2.00	1.99	1.98	1.96	1.92	1.88	1.84	1.80

4.2.4.2 Assay

This assay is based on the measurement of scavenging ability of antioxidants towards the stable radical DPPH as described by Oyaizu (1986) and Bae and Suh (2007). Sample extracts or ascorbic acid standard solution (500 µL) was added to 1 mL of 80% methanolic 0.1 mM DPPH solution in a 2 mL amber microcentrifuge tube. The mixture was vortexed and incubated in the water bath (Memmert, Germany) at 37 °C for 30 minutes in the dark. Absorbance was measured at 517 nm (UV-200-RS

Spectrophotometer, MRC, Israel) against a prepared blank (80% methanol) and a control containing 80% methanol in place of sample extract. A standard curve of ascorbic acid (y=10.145x, r^2=0.9907) was prepared ranging from 0 to 10 µg/mL and results were reported as mg of ascorbic acid equivalent (AAE) per mL sample extract.

The radical scavenging activity was calculated accordingly:

$$\textbf{\% of DPPH inhibition} = (A_{\textbf{control}} - A_{\textbf{sample}} / A_{\textbf{control}}) \times 100$$

Where,

$A_{control}$ is absorbance reading of the control

A_{sample} is absorbance reading of the sample

4.2.5   Total Antioxidant Activity

4.2.5.1   Preparation of Reagents

<u>0.6 M sulfuric acid</u>

33.33 mL of concentrated (18N) sulfuric acid (Systerm) was made up to 1L with SDW.

<u>28 mM sodium phosphate</u>

0.336 g of sodium phosphate (Sigma) was dissolved in 100 mL of SDW.

<u>4 mM ammonium molybdate</u>

0.494 g of ammonium molybdate (BDH) was dissolved in 100 mL of SDW.

<u>Ascorbic acid standard curve</u>

Stock solutions were prepared by dissolving 100 mg of L-ascorbic acid (Baker analysed) in 10 mL of SDW. The different concentrations of ascorbic acid standards were prepared according to Table 4.4 and the final volume was 2 mL.

Table 4.4: Ascorbic Acid Standard Preparation

Concentration (µg/mL)	0	5	25	50	100	200	400	800
Volume of stock solution (mL)	0	0.001	0.005	0.010	0.020	0.040	0.080	0.160
Volume of SDW (mL)	2.000	1.999	1.995	1.990	1.980	1.960	1.920	1.840

4.2.5.2 Assay

The antioxidant capacity of sample extracts was determined using the phosphomolybdenum method described by Prieto *et al*. (1999). Sample extracts or ascorbic acid standard solution (100 µL) was added to 1 mL of reagent solution in a 1.5 mL microcentrifuge tube. The reagent solution consists of equal volume of 0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate. The tubes were incubated in a water bath (Memmert, Germany) at 95 °C for 90 minutes in the dark. After cooling to room temperature (25 ± 1 °C), absorbance was measured at 695 nm (UV-200-RS Spectrophotometer, MRC, Israel) against a prepared blank where sample extract was replaced with SDW. A standard curve of ascorbic acid ($y=0.0018x$, $r^2=0.9981$) was prepared ranging from 0 to 800 µg/mL and results were reported as mg of ascorbic acid equivalent (AAE) per mL sample extract.

4.3 Results

Higher DPPH activity was observed in the peel of the fruit as opposed to the pulp (Figure 4.1). At Stage I, activity was observed to be 1.197 enzyme units per min in the peel and 0.756 enzyme units per min in the pulp. At Stage V, activity was 1.106 enzyme units per min in the peel and 0.720 enzyme units per min in the pulp. Activity in infected samples was observed to be the highest with activity of 1.567 enzyme units per min in the peel and 1.248 enzyme units per min in the pulp.

Figure 4.1: DPPH Assay of peel and pulp of Berangan cultivar at Stage I, Stage V , and Infected Stage VII

Higher total polyphenol content was observed in the peel of the fruit as opposed to the pulp. At Stage I, activity was observed to be 0.00113 enzyme units per min in the peel and 0.0005 enzyme units per min in the pulp. At stage V, activity was 0.00035 enzyme units per min in the peel and 0.00035 enzyme units per min in the pulp. Activity in infected samples was observed to be the highest with activity of 0.00286 enzyme units per min in the peel and 0.00262 enzyme units per min in the pulp.

Figure 4.2: TPC of peel and pulp of Berangan cultivar at Stage I, Stage V, and Infected Stage VII

Higher total antioxidant capacity was observed in the peel of the fruit as opposed to the pulp. At Stage I, activity was observed to be 0.001523 enzyme units per min in the peel and 0.001321 enzyme units per min in the pulp. At stage V, activity was 0.001439 enzyme units per min in the peel and 0.001228 enzyme units per min in the pulp. Activity in infected samples was observed to be the highest with activity of 0.004069 enzyme units per min in the peel and 0.003175 enzyme units per min in the pulp.
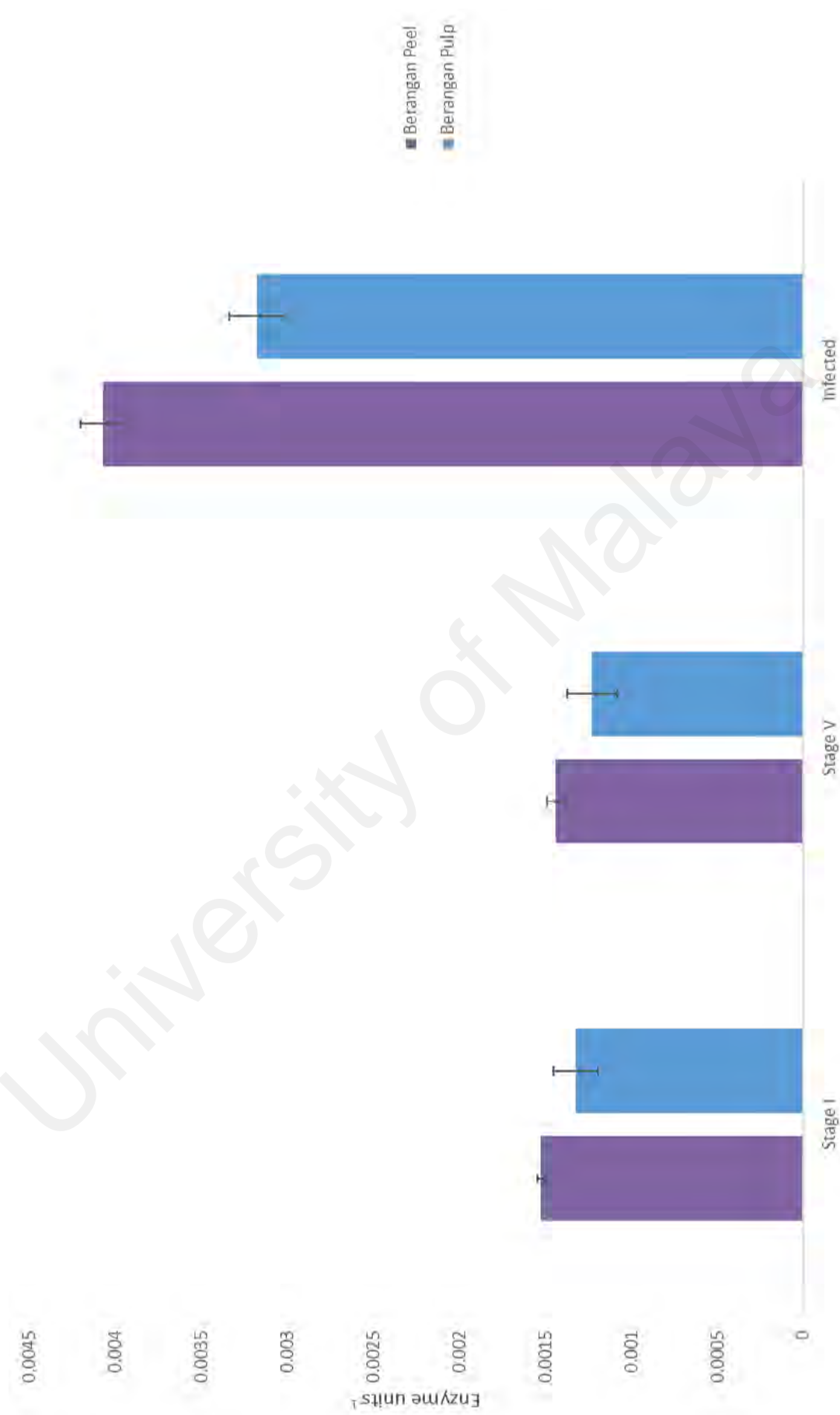
Figure 4.3: TAC of peel and pulp of Berangan cultivar at Stage I, Stage V, and Infected Stage VII

Higher total phenolic content was observed in the peel of the fruit as opposed to the pulp. At Stage I, activity was observed to be 0.001449 enzyme units per min in the peel and 0.000952 enzyme units per min in the pulp. At stage V, activity was 0.001184 enzyme units per min in the peel and 0.000369 enzyme units per min in the pulp. Activity in infected samples was observed to be the highest with activity of 0.001667 enzyme units per min in the peel and 0.001392 enzyme units per min in the pulp.
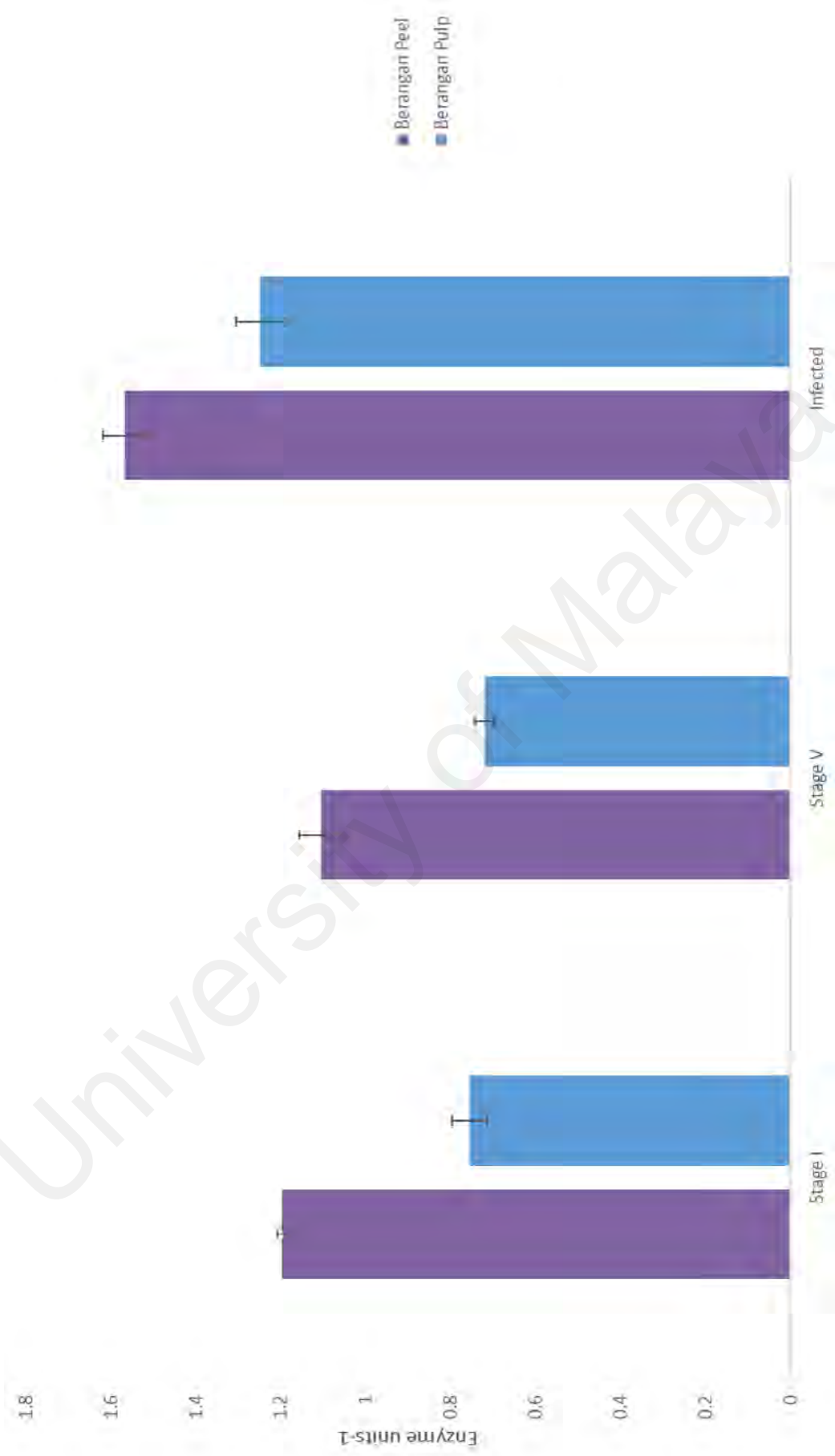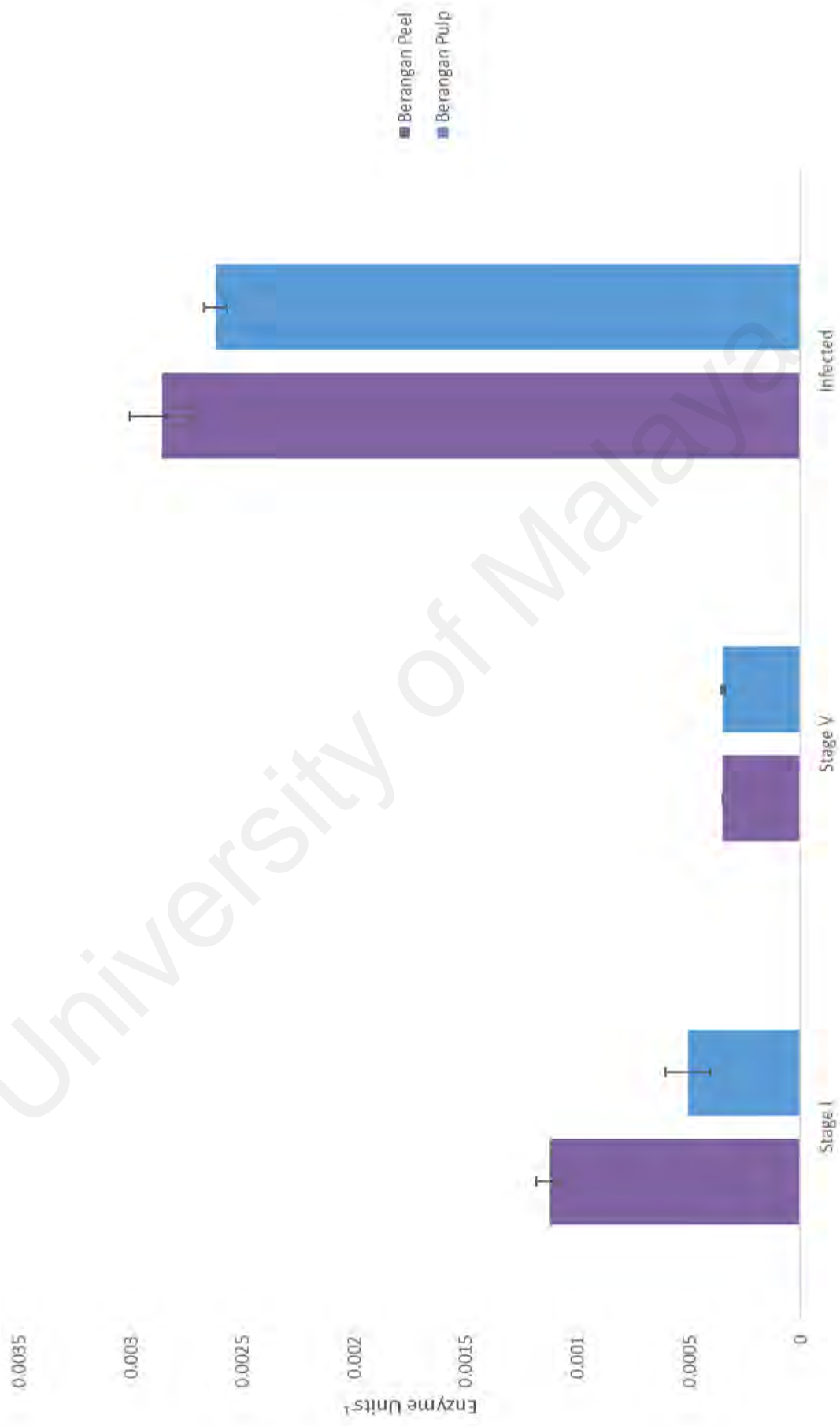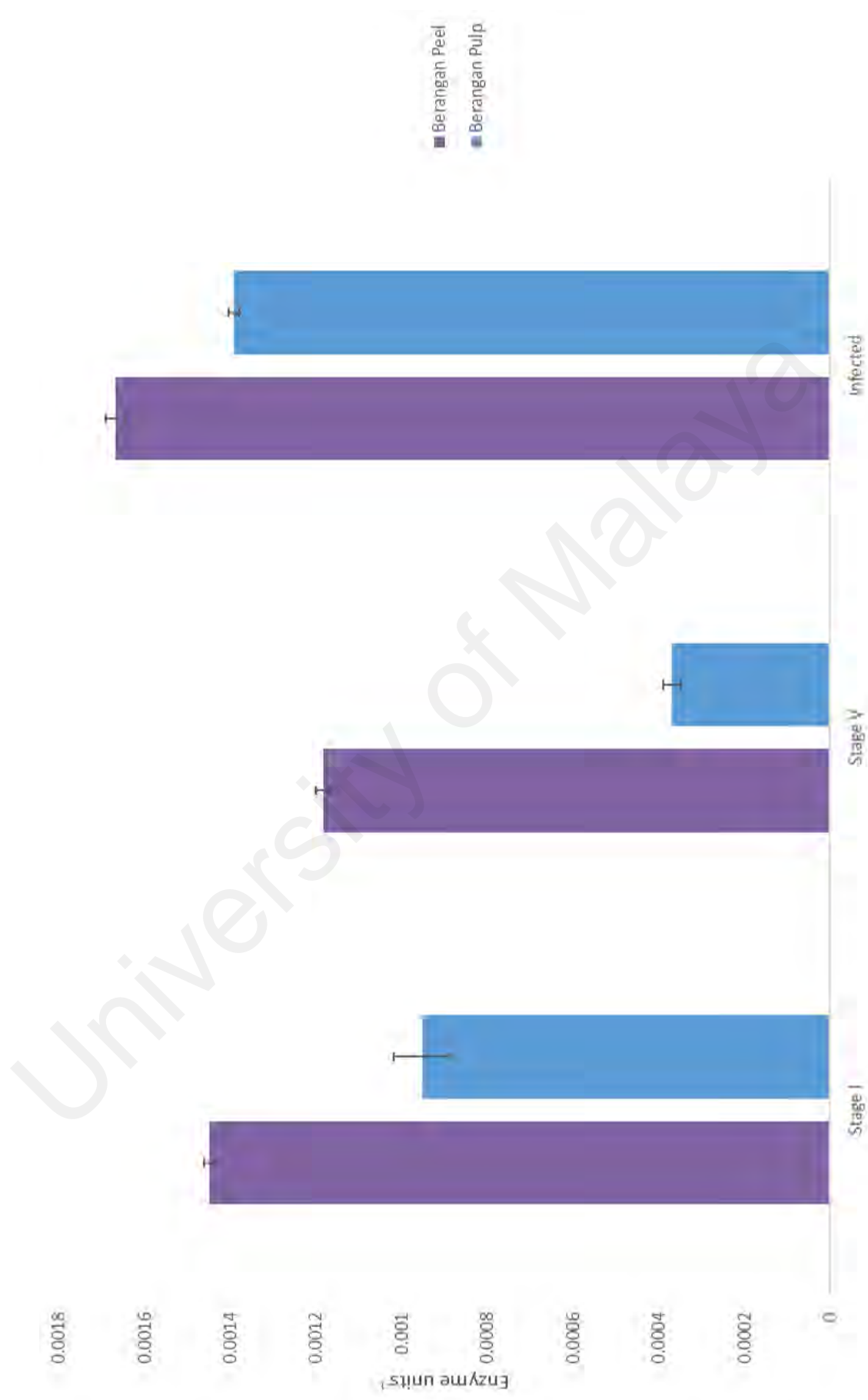
Figure 4.4: TFC of of peel and pulp of Berangan cultivar at Stage I, Stage V, and Infected Stage VII

4.4   Discussion

DPPH free radical scavenging activity was observed to be highest at Stage I of the fruit as compared to Stage V. DPPH is a stable free radical. The assay itself assesses the capacity of the sample extract to donate hydrogen to the free radical, converting it into a stable ion. It is representative of the ability of the antioxidants to convert other free radicals that exist within the plant system. The higher free radical scavenging ability at Stage I is likely related to the increase in ROS enzymes at Stage I. Therefore, the higher free radical scavenging ability is in order to protect the plant and scavenge the free radicals generated by the ROS enzymes. This decrease in free radical scavenging ability at later stages of ripening correlates with reported research. Research on a raspberry native to Korea highlighted that the highest free radical scavenging ability was observed in the unripe fruit. As the fruit ripened, the free radical scavenging ability decreased (Park *et al*., 2008). This is in-line with the results obtained here. Furthermore, research on ripe and unripe fruit juices also produced a similar conclusion. Fruit juice from unripe fruit had higher free radical scavenging ability than fruit juice from ripe fruit (Rekha *et al*., 2012). Research on carob also showed a similar trend with unripe fruit having the highest free radical scavenging activity (Benchikh *et al*., 2014).

The DPPH free radical scavenging activity was noticeably higher in infected samples as compared to healthy samples. When a plant is infected by a pathogen, the HR is stimulated. This results in the induction of ROS and other compounds that serve to limit the spread of infection. This is done by causing apoptosis. The induced enzymes generate free radicals that serve to destabilize the pathogen and plant cells. However, unregulated free radicals are harmful to the plant. Therefore, the antioxidant compounds and free radical scavenging activity becomes important in controlling and minimizing the damage done by the free radicals produced. Hence, the higher free radical scavenging activity observed in the infected samples. This correlates with current

research which shows increases in DPPH free radical scavenging ability following infection of plants. In research carried out on bean leaf, DPPH free radical scavenging activity increased following infection with bean yellow mosaic virus (Radwan *et al.*, 2010). A similar trend was observed in peach trees infected by plum pox virus. In that study, DPPH free radical scavenging activity increased roughly 13% in the infected fruits as compared to healthy fruits (Horsakova *et al.*, 2013).

The peel of the fruit exhibited higher free radical scavenging activity as compared to the pulp at all stages tested. In general, ROS and other free radical generation compounds accumulate in the peel of the fruit. As such, the higher DPPH free radical scavenging activity is in order to scavenge the free radicals present in the peel. Other fruits, such as tomatoes, have been shown to have significantly higher free radical scavenging activity in the peel of the fruit as compared to the pulp (Fuentes *et al.*, 2013).

Total phenolic content was observed to be lower at later stages of ripening. Of the stages assessed, Stage I samples had higher phenolic content as compared to Stage V. This indicates that there is a higher concentration of phenolic compounds present in the fruit samples at the earlier stages of ripening. Polyphenols play many roles in the plant system. They play a major role as antioxidants, protecting the plant from oxidative damage while also being involved in the plant signaling system for ripening and other processes, in the release and suppression of plant growth hormones and in the plant defense system (Lattanzio *et al.*, 2006). The fruit ripening process is a process that is triggered at the earlier stages of ripening. At Stage V, the fruit is nearly fully ripened. As such, the concentration of phenolics required to complete the drive the ripening process is lessened.

Furthermore, phenolic compounds are antioxidants that play a critical role in scavenging the free radicals that are present to prevent oxidative damage. In the plant system, the free radical generating compounds such as the ROS compounds, are present in the plant primarily during the earlier stages of ripening. As such, the higher phenolic content at Stage I correlates with that trend. This is further corroborated by other research. Studies on palm date fruit has shown that the fruit shows significantly higher levels of phenolic content at the earlier stages of ripening. As the fruit ripened, the phenolic content decreased (Eid *et al*., 2013). Studies on olives also showed a similar trend with phenolic content being higher at earlier stages of ripening (Briante *et al*., 2002).

Infected fruit samples showed significantly higher TPC as compared to the healthy fruit samples. This is in-line with earlier results. Polyphenols, being antioxidants in nature, are induced whenever the plant's defense system induced, specifically, to ensure that free radicals generated are controlled and do not harm healthy plant tissues. As such, infections which stimulate the defense system and the ROS compounds result in the induction of the plant antioxidants ie: phenolic compounds. This has been observed in various other fruits as well. Studies on strawberries infected with *Colletotrichum nymphaeae* have shown that the infected strawberry samples show a significantly higher level of phenolic compounds as compared to healthy fruits. The increase in polyphenols is inferred to be induced as part of the defense system of the strawberries (Mikulic-Petkovsek *et al*., 2013). A similar trend was also observed in soybean where infection resulted in increased phenolic activity (John *et al*., 2013).

Phenolic content was observed to be higher in the peel of the fruit as compared to the pulp of the fruit at all stages. The accumulation of phenolics in the peel ties in with earlier results. As the ROS compounds build up in the peel, it can be inferred that the

phenolics also accumulate in the peel to combat it. This has also been shown in other studies. Studies on pomegranate peel, seed and pulp showed that the peel had the highest level of phenolic content (Elfalleh *et al*., 2012).

Total antioxidant capacity was observed to be higher at earlier stages of ripening. Stage I samples showed higher readings for antioxidant capacity as compared to Stage V. The antioxidant capacity is determined by the ability of the samples to reduce Mo(VI) to Mo(V). The higher activity at earlier stages of ripening shows that there is a higher reductive capacity at Stage I. The capacity to reduce free radicals is important to maintain a healthy balance in the fruit. As described earlier, there are higher free radical activity levels at the earlier stages of ripening due to the higher levels of ROS and similar compounds. The successful scavenging of the free radicals generated is critical to ensuring a healthy balance that prevents significant degradation and deterioration of the fruit. As such, there is a need for greater antioxidant capacity at that stage. This has been correlated with various other research. Studies on guava fruit have shown that the unripe guava fruit has the highest antioxidant activity as compared to the semi-ripe and ripe guava fruit. There was a significant decrease in the antioxidant activity as the fruit ripened (Gull *et al*., 2012). This trend was also observed in cashew apples with the fruit having significantly higher antioxidant activity at the earlier stage of ripening (Lopes *et al*., 2012).

The antioxidant activity was observed to be higher in the infected fruit as compared to the healthy. Antioxidants have been shown to be induced as part of the plant defense system. They are believed to play a key role in the plant defense system. The main role they play is in the maintaining of the plant homeostasis. The antioxidants scavenge the free radicals generated by the defense system to prevent unrestricted damage to the plant tissues. As such, they are induced when the free radicals are induced.

The peel samples were observed to have the highest antioxidant capacity at both stages tested. As the peel is the front-line defense, the build-up of ROS compounds occurs mainly in the peel. As such, the antioxidant activity follows suit, accumulating in the peel. This corresponds to other studies. Studies on apples have shown that the peel shows the highest antioxidant capacity among the peel, flesh and seeds. This was shown to be consistent across seven different cultivars. Furthermore, the study showed that certain antioxidants were only found in the peel and not in the flesh of the apple. HPLC studies showed that there were numerous antioxidants present in the peel (Karaman *et al*., 2013). Studies on citrus fruits have also shown that the peels of citrus fruits demonstrate significantly higher antioxidant capacity than the pulp. As the peel serves to protect the fruit from UV radiation among other roles, it is more predisposed to generate biological compounds that serve to reduce the effects of the UV radiation. As such, it can be inferred that the higher antioxidant capacity is in to scavenge free radicals generated by the UV radiation (Ignat *et al*., 2011; de Moraes Barros *et al*., 2012).

Total flavonoid content was observed to be higher at Stage I of ripening as compared to Stage V. Flavonoids are plant secondary metabolites that content ketone-groups such as anthoxanthins. Flavanoids contain many roles in plants. One is to act as pigments that generate the color seen in fruits and flowers, specifically, yellow, red and blue pigmentation. More importantly, they play a critical role in UV filtration and as chemical messengers and physiological regulators (Galeotti *et al*., 2008). The higher levels of TFC in the unripe fruit is due to its role as physiological regulators. The plant physiological balance is affected by the presence of free radicals and free radical generators such as ROS compounds. These compounds affect the physiological balance by causing apoptosis and resulting in cell death. In order to regulate this, there is a need for flavonoids as they scavenge the free radicals produced into harmless forms thus

regulating the plant cell death and protecting the plant tissue. As there are higher levels of free radicals at Stage I of ripening, thus there is a need for higher TFC activity at Stage I. Studies on cumin seeds have shown that the seeds demonstrate higher activity at the earlier stages of ripening, similar to results presented here. The unripe cumin seeds had significantly higher activity when compared to the half-ripe and ripe cumin seeds (Rebey *et al.*, 2014). Studies conducted on palm dates also showed a similar trend with earlier ripening stages showing higher TFC as compared to fully ripe palm dates (Lemine *et al.*, 2014).

The flavonoid content was observed to be higher in the infected tissue samples as compared to the healthy tissue samples. Infection leads to the induction of the plant defense system. Part of that system is the ROS and other free radical generators. The build- up of those compounds result in the accumulation of free radicals. The free radicals serve to limit spread of infection by apoptosis and also by killing the pathogen cells. In order to prevent unrestricted apoptosis and to protect healthy cells, the free radicals need to be scavenged. One of the primary scavengers of free radicals is the flavonoids. Therefore, the flavonoids are induced in the infected tissues in order to scavenge the free radicals.

The flavonoid content was observed to be the highest in the peel of the fruit as compared to the pulp. There are two possible reasons for this: UV filtration and pigmentation. Flavonoids play a critical role in both. Flavonoids filter out the UV radiation that comes with exposure to sunlight. Therefore, there needs to be significant accumulation of flavonoids in the peel of the fruit. Furthermore, as they play a role in the pigmentation of the fruit, there is a need for the flavonoids to accumulate in the peel so as to give the fruit the required pigmentation (Galeotti *et al.*, 2008). This has been observed in other fruits as well. Studies on jujube showed significantly higher flavonoid

content in the peel of the fruit as compared to the pulp. Furthermore, the study also reported that the unripe jujube had the highest flavonoid concentration (Wang *et al*., 2013).

4.5   Conclusion

Results for all the antioxidant assays showed a similar trend. Antioxidant activity was observed to be highest at Stage I of ripening as compared to Stage V. The main reason for this is the accumulation of free radicals is more pronounced at Stage I when compared to Stage V. As the free radicals and free radical generators build up, the plant is exposed to oxidative damage. In order to regulate that and prevent unrestricted damage to the cell, the antioxidants are induced. At later stages of ripening, the free radical accumulation is lower as compared to that of Stage I. Hence, lower antioxidant activity is also reported.

These results show a strong correlation between the free radical accumulation and the antioxidant activity. It can be shown that there is a positive correlation between the accumulation of free radicals and antioxidants. Furthermore, the information is useful in terms of food processing. Foods high in antioxidant activity are a major consideration for health-conscious consumers. Therefore, when using Berangan bananas for food processing, the industry would benefit from using bananas in earlier stages of ripening as they are higher in antioxidant activities.

The increased antioxidant activity in infected fruits highlights the important role that antioxidants play in the plant defense response. The fact that all antioxidant activities increase following infection highlight how important the antioxidants are to combat the infection. It can be inferred that the plant defense system prioritizes maintaining the balance of the system as opposed to allowing unrestricted cell death.

The higher levels of antioxidants in the peel of the fruit strongly correlate to the roles of antioxidants in maintaining balance as well as in UV filtration, preventing oxidative damage. The peel of fruit is clearly the first line of defense and as such, there is a need to strongly protect it.

CHAPTER 5: DETERMINATION OF ANTIFUNGAL PROTEIN ACTIVITY THROUGH THE USE OF DIFFERENT ENZYMATIC ASSAYS

5.1 Introduction

The main pathogens that affect fruits and cause post-harvest diseases are fungal pathogens. Fungal pathogens are responsible for a wide variety of post-harvest diseases such as anthracnose, crown rot, stem-end rot and cigar-end rot. As such, plants have developed defense systems to prevent or resist fungal infections. Antifungal proteins are known to play a key role in these defense systems as fungal pathogens are the primary pathogen infecting fruits.

Bananas however, even with the presence of these antifungal proteins, are still susceptible to pathogen attacks. The fungal pathogen *Fusarium oxysporum* was able to completely wipe out the 'Gros Michel' cultivar in the 1950's leading to the cultivar being replaced by the 'Cavendish' cultivar. However, the Cavendish cultivar is now under threat by a new strain of Fusarium (Chen *et al.,* 2013; Kovacs *et al.,* 2013; Ma *et al.,* 2013). This highlights the susceptible nature of bananas towards fungal pathogens.

Banana fruits are susceptible post-harvest fungal pathogen infections. These infections cause diseases that diminish the quality of the fruit. The external appearance of the fruit is compromised, often bearing brown and black lesions or fungal mold. As such, the fruits can no longer be sold and are discarded or are sold at sub-par prices, leading to losses among the sellers. This affects the banana industry as a whole. Cumulatively, the economic losses are significant.

Furthermore, the nutritional qualities of the fruits are also compromised. The pathogens utilize the nutrients present in the fruit, thereby diminishing them. Considering that fruits are primarily eaten for their health benefits, this is harmful to

consumers. They would be consuming fruits that do not confer the nutrients that they require without being aware of it. Worse, in some cases, consumption of contaminated fruit products can lead to adverse health effects in consumers.

In order to determine antifungal protein activity, enzymatic assays were carried out on Berangan (AAA) bananas. The peel and pulp of the healthy bananas were assayed at Stage I and V. The peel and pulp of infected samples were also assayed.

Antifungal proteins are functional proteins that have enzymatic effects on bananas. They are found to be expressed throughout the plant, including in the peel and pulp of the fruit. Antifungal protein activity is up-regulated in infected tissue and is postulated to play a critical role in the plant defense system (Bol *et al*., 1990; Linthorst, 1991).

The laminarase enzymatic assay was used to determine the β-1,3-glucanase activity. B-1,3-glucanase degrades the cell wall of fungal pathogens, effectively conferring them a form of resistance towards fungal pathogen attacks (Wan *et al.,* 2013). Laminarin, the substrate used in the laminarinase assay contains β-1,3-glucan bonds. When the bonds are hydrolyzed by the protein, the substrate reacts with the colorimetric solution, producing a reading that can be quantified spectrophotometrically.

α-amylase inhibitors are antifungal proteins that have been shown to inhibit α-amylase activity (van der Weerden & Anderson 2013). α-amylase is a well-documented enzyme responsible for the degradation and breakdown of starch into its basic sugars. The sugars produced in the assay then react with the colorimetric agent and are quantified using a spectrophotometer.

Trypsin inhibitors are known to inhibit the activity of trypsin. Tyrpsins are enzymes that hydrolyze benzoyl-L-arginine ethyl ester (BAEE). However, trypsin inhibitors prevent the reaction between trypsin and BAEE from occurring. As the reaction

between trypsin and BAEE can be assayed spectrophotometrically, the inhibition activity of the inhibitor can also be assessed.

In this chapter, the antifungal activities of the antifungal proteins are studied. The method of determining the antifungal properties is through the use of zone of inhibition assays. Should the proteins have sufficient antifungal activity, a zone of inhibition would be observable. However, the lack of a zone of inhibition does not suggest the converse; it does not mean that antifungal activity is non-existent. It could also be that the concentrations of the proteins required to confer antifungal activity are significantly higher than the tested concentrations. Therefore, for this chapter, the antifungal assays were carried out at three different concentrations: 1× the activity assessed in bananas, 5× the activity and 10× the activity.

The aims of this chapter are to:

i. To determine the antifungal protein activity of the protein extracts of the banana peel and pulp at Stages I and V.

ii. To determine the antifungal protein activity of the protein extracts of the infected banana peel and pulp.

iii. To determine the antifungal properties of the proteins at 1× the concentrations in banana.

iv. To determine the antifungal properties of the proteins at significantly higher concentrations: 5× and 10×.

5.2   Materials and Methods

5.2.1   Laminarase Enzymatic Assay

5.2.1.1   Preparation of Reagents

Laminarin solution

1 g of laminarin was dissolved in 100 mL of SDW

100 mM Dinitrosalicylic Acid (DNSA)

2.28 g of DNSA was dissolved in 100 mL of SDW. The mixture was heated in boiling water.

100 mM Sodium Phosphate (pH 6.0)

12 mL of 1 M di-sodium hydrogen orthophosphate and 88 mL of 1M sodium dihydrogen orthophosphate were added into a conical flask and mixed thoroughly.

Table 5.1: Preparation of Reagents Used in Laminarase Enzymatic Assay

	Laminarin	Enzyme Extract	Buffer
Reaction mixture	1mL	1mL	-
Substrate blank	1mL	-	1mL
Enzyme blank	-	1mL	1mL

5.2.1.2   Assay

The Laminarase assay was carried out following the method by Chandran (2008). The prepared solutions were incubated in a water bath for 30 minutes at 37 °C. Subsequently, 3 mL of DNSA reagent was added to all solutions and incubated in a boiling water bath for 10 minutes. Absorbance readings at 540 nm were recorded at the end of incubation with water as the reference blank. Results were expressed in nmoles of glucose liberated per minute based on the standard glucose curve.

5.2.2   α-Amylase Assay

5.2.2.1   Preparation of Reagents

<u>Reagent A</u>

A 100 mL solution containing 20 mM sodium phosphate buffer, 6.7 mM sodium chloride and water was prepared. The pH was adjusted to 6.9 using 1M NaOH and stored at 20°C

<u>Reagent B</u>

A 1.0% (w/v) soluble starch solution was added to 25 mL of Reagent A. The solution was heated in a glass beaker on a hotplate with constant stirring. The solution was brought to boil and maintained at boiling point for 15 minutes. The solution was allowed to cool to room temperature with stirring. The solution was topped up to its original volume of 25 mL using ddH_2O.

<u>Reagent C</u>

12 g of sodium potassium tartrate was dissolved in 8 mL of 2 M NaOH.

<u>Reagent D</u>

96 mM 3,5-Dinitrosalicylic Acid Solution was dissolved in 20 mL of SDW. The solution is heated on a heating/stir plate using constant stirring to dissolve it. The solution was maintained at 45°C to 50°C throughout assay.

<u>Reagent E</u>

With stirring, Reagent C was slowly added to Reagent D. The mixture was diluted to 40 mL with ddH_2O. The solution was stored in an amber bottle at room temperature until further use.

0.2% (w/v) maltose standard solution was dissolved in 10 mL SDW.

Reagent G

A reaction mixture of 100 mL containing 50 mM sodium phosphate, 50 mM sodium chloride, 0.5 mM calcium chloride, 0.1% bovine serum albumin and water was prepared. The pH was adjusted to 6.9 at 20 °C with 1 M NaOH

Reagent H

40 units/mL α-amylase was dissolved in cold SDW. The reagent was prepared immediately before use.

5.2.2.2 Assay

The following reagents were pipetted into a test tube as Table 5.2:

Table 5.2: Preparation of Reagents Used in α-Amylase Assay

	Control (mL)	Sample (mL)
Reagent G	2.0	2.0
Reagent H	0.1	0.1
ddH_2O	0.04	-
Enzyme Extract	-	0.04

This assay was carried out based on the Sigma protocol for α-amylase inhibition assay. The reaction mixtures were mixed by swirling and left to equilibrate at 25 °C for 30 minutes. In another test tube, 1 mL of Reagent B and 0.5 mL of ddH_2O was added and left to equilibrate at 20 °C for 30 minutes. Once equilibrated, 1 mL Reagent E was added to it. 0.5 mL of the control and sample that were left to equilibrate were added to the respective test tubes. All the tubes were capped and boiled for 15 minutes. They were then cooled to room temperature and 9 mL of ddH_2O was added. The mixture was

mixed by inversion and the absorbance reading was taken at 540 nm. Results were recorded in percentage α-amylase activity.

5.2.3 Trypsin Inhibition Assay

5.2.3.1 Preparation of Reagents

Reagent A

0.25 mM N-Benzoyl-L-Arginine Ethyl Ester Solution (BAEE) was prepared in 67 mM sodium phosphate buffer (pH 7.6).

Reagent B

0.036 mL of HCl added to 999.964 mL of SDW.

Reagent C

1 mg/mL trypsin enzyme solution was prepared in 1 mM cold HCl.

Reagent D

1 mg/mL trypsin inhibitor solution (INHB) prepared immediately before use in cold 67 mM sodium phosphate buffer (pH 7.6).

5.2.3.2 Assay

The reagents were pipetted into test tubes as Table 5.3 to make up the enzyme solution. The samples were mixed by inversion and allowed to stand for only 5 minutes.

Table 5.3: Preparation of the Enzyme Solution

	Enzyme Blank (mL)	Inhibitor (mL)	Sample (mL)
Reagent B	9.5	9.4	9.4
Reagent C	0.5	0.1	0.1
Reagent D	-	0.5	-
Enzyme Extract	-	-	0.5

The reagents were pipetted into cuvettes as Table 5.4 as the substrate solution:

Table 5.4: Preparation of the Substrate Solution

	Enzyme Blank (mL)	Inhibitor (mL)	Sample (mL)	Blank (mL)
Reagent A	3	3	3	3
Reagent B	0.1	0.1	0.1	0.2

The assay was carried out based on the Sigma protocol for Trypsin inhibition assay. The solutions were mixed by inversion and allowed to equilibrate to 25°C. The absorbance reading at 253 nm was monitored until constant. Once constant, 0.1 mL of the respective enzyme solution was added to the substrate solution. The solutions were immediately mixed by inversion and the increase in the absorbance readings at 253 nm was recorded for after five minutes. Results were expressed in percentage inhibition of trypsin activity.

5.2.4   Preparation of Fungal Colonies in Broth Media

5.2.4.1   Preparation of Reagents

<u>Growth Broth</u>

20 g of malt extract and 5 g of peptone was dissolved in 1 L of SDW. The broth was adjusted to pH 6.8 using 1 M NaOH

5.2.4.2 Preparation of Fungal Colonies

A 100 mL of media was poured into a 500 mL flask and the flask was stopped using cotton wrapped in gauze. Aluminium foil was then used to cover the flasks and the flasks were autoclaved. Following autoclaving, the flasks were left to cool to room temperature.

The flasks were then inoculated with *Fusarium oxysporum*. Inoculation was done using an adapted streaking method. The adaptation was that instead of streaking onto a plate, the inoculation loops were dipped into the broth and the fungal matter mixed in. Following inoculation, the flasks were placed on a shaker for 72 hours at room temperature and with a rotation of 100 rpm.

5.2.5 Laminarinase Antifungal Assay

Laminarinase was prepared in three concentrations of 1×, 5× and 10×. Spread plating was carried out using the inoculated broth as inoculum. For spread plating, a total of 1 mL of the growth media was pipetted onto five different points of the plate. A clean L-shaped glass rod was prepared by wiping it down with ethanol. The glass rod was then used to spread the inoculum to cover the entire plate. Once done, the glass rod was again wiped down with ethanol and placed into a beaker containing ethanol.

Before the spread plating, 1 cm diameter disc were cut out from filter paper and subjected to UV irradiation to sterilize them. Following spread plating, a disc was taken using clean, ethanol sterilized and flamed forceps and placed approximately in the centre of the plate. 500 µL of the respective laminarinase solution concentration was pipetted onto the disc. The plate was then sealed using parafilm and incubated at room temperature for 72 hours. The plates were then observed for the formation of a zone of inhibition of fungal growth.

5.2.6 α-Amylase Antifungal Assay

α-amylase was prepared in three concentrations of 1×, 5× and 10×. Spread plating was carried out using the inoculated broth as inoculum. For spread plating, a total of 1 mL of the growth media was pipetted onto five different points of the plate. A clean L-shaped glass rod was prepared by wiping it down with ethanol. The glass rod was then

used to spread the inoculum to cover the entire plate. Once done, the glass rod was again wiped down with ethanol and placed into a beaker containing ethanol.

Before the spread plating, 1 cm diameter disc were cut out from filter paper and subjected to UV irradiation to sterilize them. Following spread plating, a disc was taken using clean, ethanol sterilized and flamed forceps and placed approximately in the center of the plate. 500 µL of the respective a-amylase solution concentration was pipetted onto the disc. The disc was then sealed using parafilm and incubated at room temperature for 72 hours. The plates were then observed for the formation of a zone of inhibition of fungal growth.

5.2.7   Trypsin inhibitor Antifungal Assay

Trypsin inhibitor was prepared in three concentrations of $1\times 5\times$ and $10\times$. Spread plating was carried out using the inoculated broth as inoculum. For spread plating, a total of 1 mL of the growth media was pipetted onto five different points of the plate. A clean L-shaped glass rod was prepared by wiping it down with ethanol. The glass rod was then used to spread the inoculum to cover the entire plate. Once done, the glass rod was again wiped down with ethanol and placed into a beaker containing ethanol.

Before the spread plating, 1 cm diameter disc were cut out from filter paper and subjected to UV irradiation to sterilize them. Following spread plating, a disc was taken using clean, ethanol sterilized and flamed forceps and placed approximately in the center of the plate. 500 µL of the respective Trypsin inhibitor solution concentration was pipetted onto the disc. The disc was then sealed using parafilm and incubated at room temperature for 72 hours. The plates were then observed for the formation of a zone of inhibition of fungal growth.

5.3   Results

Higher laminarinase activity was observed in the pulp of the fruit as opposed to the peel (Figure 5.1). Activity is defined as nmoles of glucose liberated per minute. At Stage I, activity was observed to be 0.367452 nmole of glucose liberated min^{-1} in the peel and 0.718599 nmole of glucose liberated min^{-1} in the pulp. At stage V, activity was 0.25316 nmole of glucose liberated min^{-1} in the peel and 0.654214 nmole of glucose liberated min^{-1} in the pulp. Activity in infected samples was observed to be the highest with activity of 0.312586 nmole of glucose liberated min^{-1} in the peel and 0.743941 nmole of glucose liberated min-1 in the pulp.
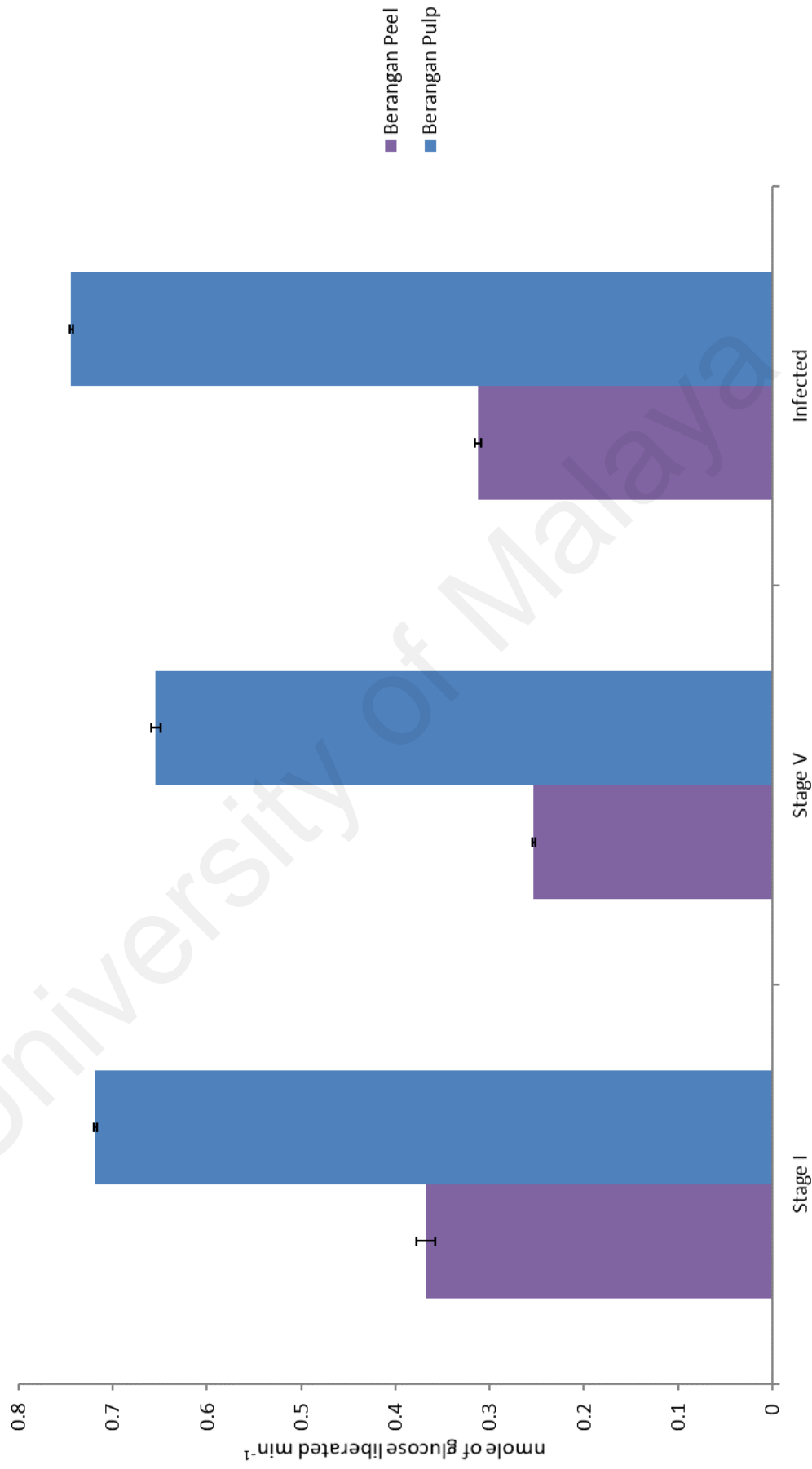
Figure 5.1: Laminarinase Enzymatic Assay of peel and pulp of Berangan cultivar at Stage I, Stage V, and Infected Stage VII

Higher a-amylase activity was observed in the pulp of the fruit as opposed to the peel (Figure 5.2). Activity is defined as percentage activity of sample extract compared to a-amylase enzyme. At Stage I, activity was observed to be 90.90% of a-amylase activity and 125.81% of a-amylase activity in the pulp. At stage V, activity was 128.00% of a-amylase activity in the peel and 184.00% of a-amylase activity in the pulp. Activity in infected samples was observed to be the highest with activity of 179.06% of a-amylase activity in the peel and 250.90% of a-amylase activity in the pulp.
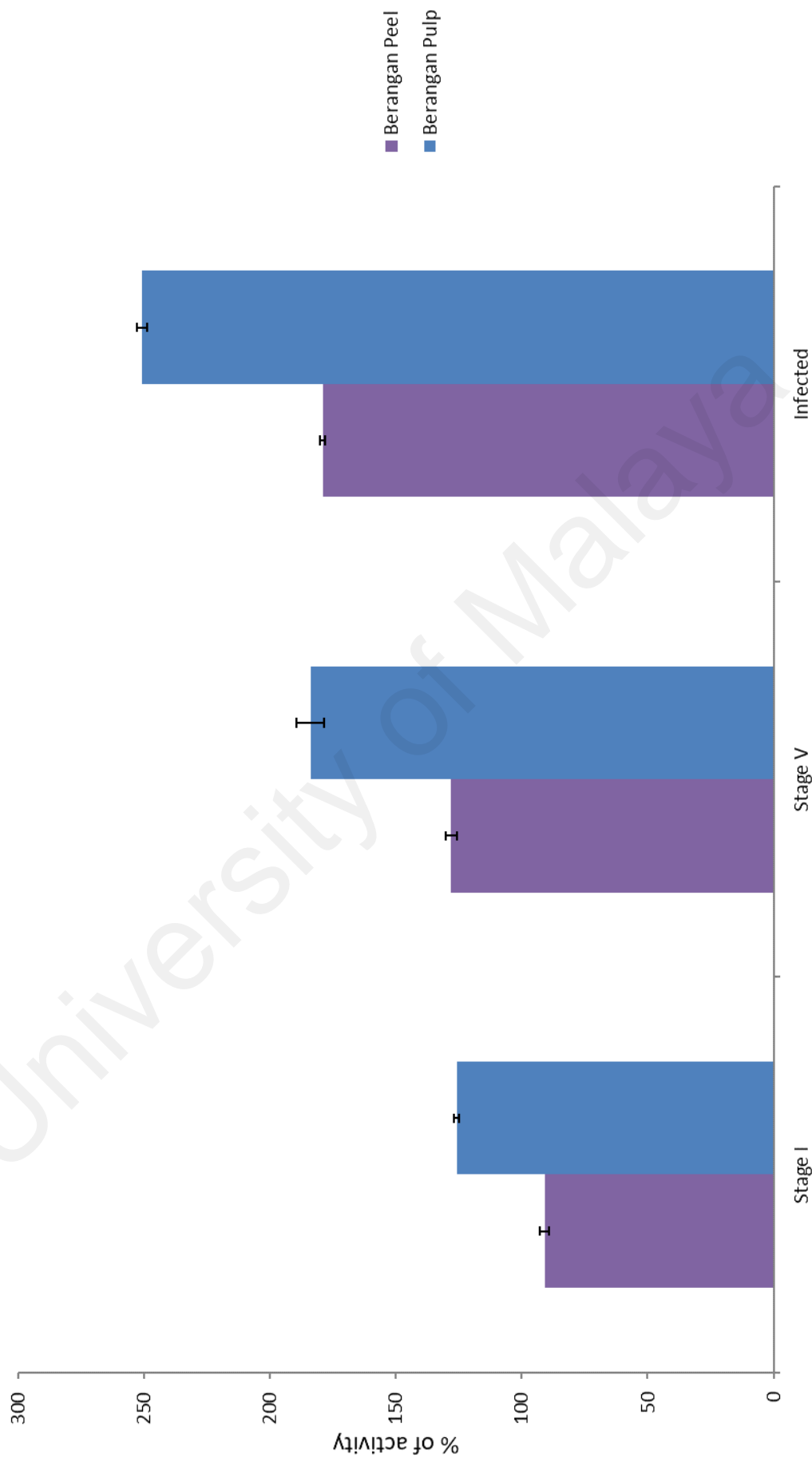
Figure 5.2: α-amylase Inhibition Enzymatic Assay of peel and pulp of Berangan cultivar at Stage I, Stage V, and Infected Stage VII

Higher trypsin inhibitor activity was observed in the pulp of the fruit as opposed to the peel (Figure 5.3). Activity is defined as percentage activity of sample extract compared to trypsin inhibitors. At Stage I, activity was observed to be 8.902% and 12.764% in the pulp. At stage V, activity was 17.844% in the peel and 21.376% in the pulp. Activity in infected samples was observed to be the highest with activity of 30.188% in the peel and 36.544% in the pulp.
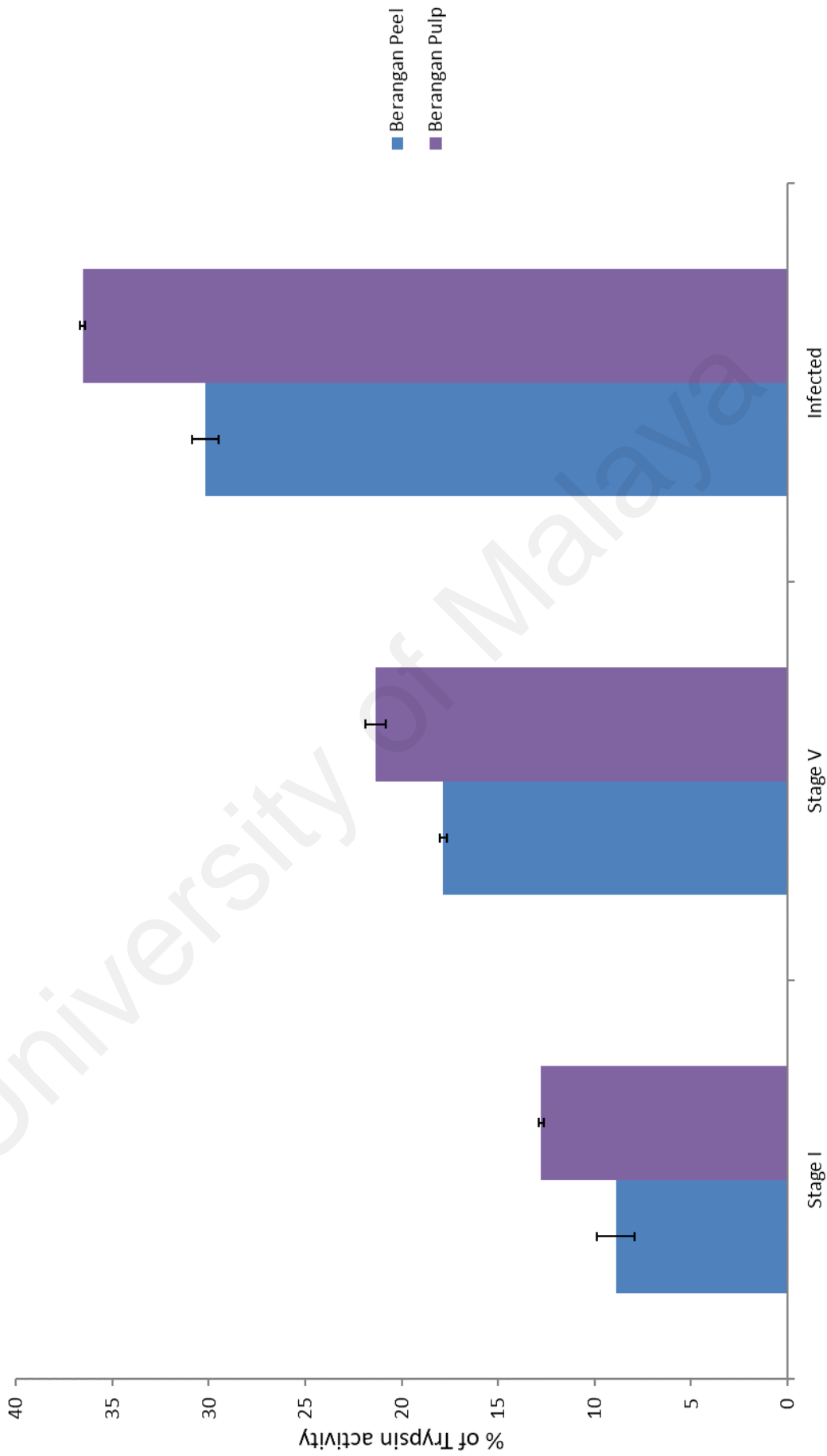
Figure 5.3: Trypsin Inhibitor Enzymatic Assay of peel and pulp of Berangan cultivar at Stage I, Stage V, and Infected Stage VII

5.3.1 Laminarinase Antifungal Assay

No zones of inhibition were observed for 1×, 5×, and 10× concentrations of Laminarinase.

Table 5.5: Laminarinase Inhibition of Fungal Strains at Different Concentrations

Concentration	Inhibition
1×	None
5×	None
10×	None

5.3.2 α-Amylase Antifungal Assay

No zones of inhibition were observed for 1×, 5×, and 10× concentrations of α-Amylase.

Table 5.6: α-Amylase Inhibition of Fungal Strains at Different Concentrations

Concentration	Inhibition
1×	None
5×	None
10×	None

5.3.3 Trypsin Inhibitor Antifungal Assay

No zones of inhibition were observed for 1×, 5×, and 10× concentrations of Trypsin Inhibitor.

Table 5.7: Trypsin Inhibitor Inhibition of Fungal Strains at Different Concentrations

Concentration	Inhibition
1×	None
5×	None
10×	None

5.4   Discussion

In the Laminarinase enzymatic assay, the higher levels of enzymatic activity in the unripe fruit may be due in part to the presence of enzymes that degrade the β-1,3-glucan bond. This is an important reaction during the ripening process as has been found to be related to the cell wall degradation and fruit softening that occurs during ripening (Bennett & Labavitch, 2008; Choudhury et al., 2010). Considering that the unripe fruit has a stronger cell wall and is firmer than the ripe fruit (Dadzie & Orchard, 1997), the higher activity in the unripe fruit is in order to degrade the cell wall. As the ripening process continues, lower levels of the laminarinase activity are needed as the firmness of the pulp has decreased significantly (Dadzie & Orchard, 1997).

The enzymatic activity is significantly higher in the pulp of the fruit as compared to the peel. This could be due to the fact that the pulp is richer in sugar than the peel as sugar is a substrate of enzymes exhibiting β-1,3-glucanase activity (Peumans et al., 2000). As such, the presence of the enzymes in the pulp is to aid in the breakdown of sugar. The decrease of the enzymatic activity in the pulp as it ripens might also be due to the fact that starch content decreases throughout the ripening process. The lower starch content means less enzymes are needed for the reaction (Marriot et al., 1981; Prabha & Bhagyalakshmi, 1998).

In this study, the infected tissue had higher laminarinase enzymatic activity as compared to the healthy samples. A significantly higher increase was observed in the pulp as compared to the peel. This suggests that during infections, the pulp automatically increases production of the enzymes that confer laminarase activity in preparation for a possible invasion by pathogens (Choudhury et al., 2010). The lower increase in the peel also suggests that these enzymes do not function in a preventive capacity but rather as a 'cure' towards the pathogens. Another possible explanation for

the difference of the increase in the expression level in the peel and pulp of the fruit is that the enzymes responsible are produced in the pulp and then exported to the peel. As such, the build-up occurs quicker in the pulp than the peel. Furthermore, the lower expression levels in the uninfected fruit indicate that a pathogen attack is required to induce the expression of the proteins that catalyse this enzymatic reaction. Should these enzymes be responsible for preventing the infection, it is likely that they would be constitutively expressed and have higher levels of expression in the healthy fruit. As that is not the case, it lends credence to the theory that these enzymes function as a direct response to pathogen attack. Research has shown that antifungal proteins that confer laminarinase activity such as thaumatin-like proteins are induced by pathogenic attack (Pritsch *et al.,* 2000; Hanselle *et al.,* 2001; Monteiro *et al.,* 2003). It is therefore likely that the increase in expression of protein in the infected banana fruit is due to the pathogen attack.

α-amylase enzymatic activity was observed to increase throughout the ripening process. The activity was also seen to be higher in the pulp as compared to the peel. It is known that α-amylase hydrolyses starch, leading to its breakdown into basic sugars. As the fruit ripens, α-amylase activity increases as it is needed to breakdown the starch present in the fruit. The absence of starch buildup from the peel of the fruit indicates that α-amylase activity is not required in the peel. However, the presence of α-amylase activity indicates that it plays another role in the peel. It may serve to degrade other proteins that pathogens may use to wound and invade the fruit (Mao & Kinsella, 1981; Garcia & Lajolo, 1988).

A higher α-amylase activity is observed in infected fruit samples as compared to healthy samples. The highest activity was observed in the pulp of the fruit. Similar to

the laminarinase activity, α-amylase activity is routinely related to the breakdown of starch and other polysaccharides (Mao & Kinsella, 1981; Garcia & Lajolo, 1988).

The activity of α-amylase was observed to be higher in infected fruits as compared to the uninfected fruit samples. This is in-line with expected results. Plant defensins, an antifungal protein, inhibits a-amylase activity (Thomma *et al.*, 2002). The lower α-amylase activity in the uninfected fruit indicates the higher expression of plant defensins as compared to the infected fruits. It is entirely likely that the lower levels of plant defensins in the infected fruit contributed to the plant succumbing to the pathogen attack, resulting in infection.

Results in the study showed that trypsin activity decreased as the fruit ripened. This indicates the presence of trypsin inhibitors within the peel and pulp of the fruit itself. The activity was also observed to be higher in the pulp of the fruit as compared to the peel of the fruit. This could be because trypsin activity occurs mostly in the pulp and not the peel as the function of trypsin is to degrade proteins (Rawlings & Barrett, 1994). The higher inhibitory effect observed in the ripened stages of the cultivars is consistent with published results. Studies by Rao showed that trypsin inhibitory effect was stronger in ripened Bontha, Poovan, Nendran, Cavendish and Rastali bananas as opposed to their unripened counterparts (Rao, 1991).

Trypsin activity was observed to decrease in the presence of an infection. This decrease was observed in both the peel and pulp tissues. This could be due to the increased expression of plant antifungal proteins. Thionins in particular are known to inhibit trypsin activity. As the function of trypsin is to hydrolyze proteins, it may be actively employed by pathogens to better attack the fruit of the plant (Rawlings & Barrett, 1994). The inhibition of trypsin is vital to minimizing the damage done to the fruit. Research has shown that trypsin inhibitors accumulate at wound sites of plant

species (Walker-Simmons & Ryan, 1977). This reinforces the postulated hypothesis that trypsin inhibitors such as thionins are only induced after the plant has suffered a pathogen attack.

Laminarinases are proteins that exhibit β-1,3-glucanase activity as a mechanism of antifungal activity. In the antifungal assay, three concentrations of Laminarinase were used. The base concentration of 1× was determined based on the enzymatic activity levels obtained in 5.3.1. The other two concentrations used were 5× and 10× of the base. The reasoning behind this was to analyse if significantly higher concentrations of the protein were sufficient to confer antifungal properties.

All three concentrations were found to not inhibit the growth of any of the fungal strains. This is interesting as research has shown the success of β-1,3-glucanase isolated from various sources in conferring antifungal activity. The item of note then is that research generally utilizes extremely high concentrations of purified β-1,3-glucanase; concentrations as high as 50 μM (Vincent *et al.* 2007; Yasmin & Saleem, 2014). However, in the base banana, the concentration of β-1,3-glucanase activity was found to be only 0.0008 μM. As such, it is clear that the much lower concentrations present in the fruit are insufficient to confer resistance.

As the initial concentration is low, the significant increase of 5× and 10× was also found to be insufficient. This indicates that for any transgenic over-expression of β-1,3-glucanase to work, the expression has to increase by a value much greater than 10×. Based on literature, an increase of at least 1000-fold looks likely to be a good start point (Liu *et al.,* 2012; Acharya *et al.,* 2012).

For the α-amylase inhibitors assay, the concentrations used was obtained from section 5.3.2. All three fungal strains were not inhibited by the 1×, 5× or 10×

concentrations used. α-amylase is used to hydrolyze starch to produce sugar for the growth and development of the fungal strains. The presence of inhibitors is meant to prevent fungal pathogens from obtaining those sugars in order to grow. A reason that no inhibition was observed could be because the concentration used was insufficient. In literature, purified defensins, which are α-amylase inhibitors, are assayed at high concentrations (Thevissen *et al.,* 1999; Gao *et al.,* 2000; Aerts *et al.,* 2009). However, in the plant, the α-amylase inhibitors are not present in such concentrated forms hence leading to the low base values. As such, those values are insufficient to prevent fungal growth. Furthermore, since the base values are low, the 10-fold increase was also insufficient to confer resistance.

For the trypsin inhibitors antifungal assay, the concentrations used were obtained from section 5.3.3. Trypsin is a fungal pathogen protein used to penetrate into plant tissue. Similar to α-amylase inhibitors, trypsin inhibitors are not present in the plant in high quantities. As such, the base value and it's 5× and 10× values are insufficient to confer fungal resistance. Due to this, no zones of inhibitions were observed on any of the fungal strains in this study similar to those reported by Asano *et al.* (2013) and Muramoto *et al.* (2012).

5.5   Conclusion

The enzymatic activities vary based on maturity of the fruit. This shows that the enzymes responsible for the activities are expressed at different strengths through-out the ripening process. Further studies on the expression of the enzymes that affect the enzymatic activities would be beneficial to understanding how to delay the ripening process, which can then result in shelf-life extension of the fruit. Furthermore, the difference in expression of enzymes can be inferred from the difference in the enzymatic activity of infected and healthy banana tissues. These warrants further study

as understanding the plant defense response can help augment existing procedures to protect fruit crops from pathogens.

CHAPTER 6: GENERAL DISCUSSION

The purpose of this research project was to study the Pathogenesis Related proteins present in local banana cultivars. Due to the susceptibility of the cultivars to fungal pathogen, there is a great focus on PR proteins as a solution to the problem. Much research has been conducted on the introduction of PR proteins from other organisms into banana species and the over-expression of the PR proteins. This project looked at five PR proteins that have been shown to possess antifungal activity.

In Chapter 3, the enzymatic assays of the PR proteins were studied. Several interesting conclusions could be drawn from that. Firstly, the activity of the PR proteins in most of the cultivars decreased towards the end of the ripening process. This indicates that the PR proteins themselves were not as expressed towards the end of the ripening. This was most evident when looking at the expression of defensins. The enzymatic activity of defensins decreased across all cultivars. The decrease signals that the defensins were no longer heavily expressed. The same could be seen in the activity of Peroxidases and to an extent, in TLPs and PPOs.

These decreases could explain the increased susceptibility of post-harvest bananas towards fungal pathogens. Essentially, without a defense mechanism in place, the fungal pathogens are easily able to penetrate into the fruit and infect it. The low concentrations of PR proteins are unable to fight the infection and prevent. This also explains why ripe bananas succumb to infection far more easily than unripe bananas. The high concentrations of PR proteins in the ripe bananas protect the fruit from the fungal pathogens.

The question that begs asking from this is: why does the expression of the PR proteins decrease? This is especially important as should the reasoning behind this be

found, it could play a key role in conferring pathogen resistance to the plant. The PR proteins present in the banana are able to confer it resistance when sufficiently expressed. Therefore, if a mechanism could be discovered to reduce prevent the decrease of the expression of the PR protein, it is possible to generate that resistance even in the post-harvest bananas. The only PR protein studied that did not follow that pattern was the thionins. The enzymatic activity of the thionins was highest at Stage VII. This indicates that the expression of thionins is increased as the fruit ripens.

When the enzymatic activities of the infected fruit were assessed, TLPs, Peroxidases, PPOs and Thionins showed increased activity in the infected fruit as compared to the uninfected Stage VII. This clearly shows that the activities and expressions of the PR proteins are inducible upon pathogen attack (Bohlmann *et al., 1988;* van Loon & van Strien, 1999). As such, when the banana is under attack by fungal pathogens, the PR protein expression increases tremendously as the PR proteins are induced as part of the plant defence mechanism to respond to the fungal infection in order to negate the infection (van Loon *et al.,* 2006).

The question that then arises is that if the PR proteins have been induced to such a degree, why does the infection persist? According to literature, the antifungal properties of the PR proteins should then clear up the infection and prevent it from getting worse (Asano *et al.,* 2013; Joseph *et al.,* 1998; Wang *et al.,* 2013; Yasmin & Saleem, 2014). However, once a fungal infection such as anthracnose caused by *Collectotrichum musae* or crown rot caused by *Aspergillus niger* infects a banana fruit, the infection does not clear up nor does it slow down completely.

To answer this question, an antifungal assay highlighted in Chapter 5 was run on the PR proteins using the enzymatic activity levels obtained in Chapter 4. The results from the antifungal assays showed that none of the PR proteins displayed any sort of fungal

growth inhibition based on the base concentrations obtained. Further assays were run using 5-fold and 10-fold concentrations and the results remain unchanged. What this effectively demonstrated was that the PR proteins were unable to inhibit fungal growth.

This clearly explains why even though the PR proteins are induced, the expression levels of the induced PR proteins are still woefully insufficient when it comes to inhibiting or destroying the fungal pathogens. As such, the fungal infections continue to persist on the fruit once the fruit has been infected.

Taken together, this indicates that the main role of the PR proteins is not to inhibit fungal growth or to 'cure' it but rather to prevent it all together. In order to do that, the expression of the PR proteins need to be maintained. Constitutive expression of the PR proteins could be a viable method of ensuring that the fruits do not get infected even post-harvest.

6.1   Conclusion

This study has shown that there is a significant decrease in PR protein expression of most PR proteins at the latter stages of ripening. This is an area that needs to be looked into as preventing that decrease-in expression could potentially ensure that pathogen resistance can be maintained, even post-harvest.

As the activity of thionins continuously increases and is highest at the Stage VII, it is possible that the mechanism that controls thionin expression could be useful in increasing expression of the other PR proteins at the later stages of ripening. The fact that thionins are continuously expressed shows that there is a mechanism already in place. The key then becomes coupling that mechanism with other PR proteins.

Lastly, the antifungal assays have shown that PR proteins are unable to effectively stop a fungal infection of the fruits. In order for the PR proteins to work effectively in stopping an infection, the proteins need to be over-expressed many times over.

However, the lack of infection on unripe bananas indicates that even at those concentrations, the proteins may be able to prevent fungal pathogens from infecting the plant.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

PUBLICATION 1

Anthony, K. K., George, D. S., Baldev Singh, H. K., Fung, S. M., Santhirasegaram, V., Razali, Z., & Somasundram, C. (2017). Reactive oxygen species activity and antioxidant properties of Fusarium infected bananas. *Journal of Phytopathology, 165*(4), 213-222. (ISI Cited, Q3).

PUBLICATION 2

Anthony, K. K., George, D. S., Razali, Z., & Somasundram, C. (2015). Changes in cell-wall degradation, cell membrane integrity and antifungal protein activity during ripening of Mas bananas. *Acta Horticulturae* (SCOPUS Cited).

PUBLICATION 3

Anthony, K. K., George, D. S., Razali, Z., & Somasundram, C. (2015). Enzymatic activity of pathogenesis-related (PR) proteins of five local banana cultivars during ripening. *Acta Horticulturae* (SCOPUS Cited).