

Postharvest Application of Polyamines Influence Ripening Related Changes and Ameliorate Chilling Stress in Carambola during Cold Storage



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ABSTRACT

Malaysian carambola (*Averrhoa carambola* L.) B10 variety is one of the promising exotic tropical fruit traded in the global market. The alarming demand for carambola was stimulated by its unique shape and flavour, increasing health consciousness and great potential in fresh-cut industry. Nevertheless, carambola is a highly perishable commodity that greatly impairs its postharvest quality and long term storability. Therefore, investigations were carried out to verify the potential of putrescine (PUT), spermidine (SPD) and spermine (SPM) as a postharvest dip treatment for maintaining quality, reducing chilling injury (CI) and extending the storage life of carambola.

Carambola at Index II was dipped in different concentrations (0.0, 0.1, 1.0 and 2.0 mM) of PUT, SPD and SPM, and then stored at 3°C (± 0.5), with 85-90% RH. Physicochemical changes and fruit quality attributes were made at harvest, 7 day intervals for 28 days of cold storage plus 6 days at 22 $\pm$ 1°C for shelf life. PUT treatment effectively lowered the weight loss, maintained peel color (L^* , C^* , h°), retained higher firmness, reduced the rate of respiration and ethylene emission, suppressed the degradation of soluble solid content (SSC) and titrable acidity (TA) throughout shelf life. Moreover, PUT-treated fruit substantially suppressed the advancement of dominant softening enzymes in carambola such as β -galactosidase (β -gal), polygalacturonase (PG) and pectin methylesterase (PME). The softening enzymes activity trends in PUT-treated fruit were concomitant with less apparent changes in flesh firmness.

Accordingly, the applied PUT treatment significantly reduced CI symptoms in carambola. Lipid peroxidation levels (MDA) and membrane leakage activity were declined in fruit treated with PAs, and the reduction was more distinct in fruit treated with

PUT. This study demonstrates that the influence of PUT on postharvest quality and protection of cell membrane against chilling damage was more profound at the highest concentration (2.0 mM). Ascorbic acid (AA), total antioxidant (TAA) and total phenolic contents (TPC) were enhanced to greater levels in PAs-treated fruit, while control fruit experienced significant losses of these bioactive compounds throughout chilling storage. Highest SPM concentration (2.0 mM) exhibited the best results on increasing TAA through an increment of TPC. On the other hand, AA content was increased with the lower concentration of SPD (1.0 mM).

Exogenously supplied PUT induced tremendous endogenous PAs accumulation in carambola tissue, particularly free PUT and SPD concentrations. Such responses suggest that PUT may stabilize and protect cellular membrane integrity against chilling stress. In general, the increment of endogenous PAs with PUT treatment induced stress tolerance mechanisms in carambola during cold storage. An attempt to identify unknown genes sequence of SAMDC, ADC, ACS and ACO in carambola was carried out. In line with this, an efficient extraction protocol of RNA isolation from carambola mesocarp tissue was optimized. CODEHOP features were exploited to design degenerate oligonucleotide primers and RT-PCR approach was utilized to attest degenerate primer specificity. Phylogenetic trees revealed the sequenced products of SAMDC, ADC, ACS and ACO in carambola exhibited close evolutionary relationship with protein sequences present in muskmelon, peach, tobacco and sweet oranges respectively.

These present findings throw light on the potential of PAs particularly PUT at a dose of 2.0 mM for 5 min could be an effective means for maintaining postharvest quality and protection against CI, with a net increase in shelf-life of B10 carambola.

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DECLARATION

I hereby declare that the thesis is based on my original work except for the quotations and citations, which have been duly acknowledged. I also declare that it has not been previously submitted or concurrently submitted for any other degree at the University of Nottingham or other institutions.

Azhane Ahmad
Date: 19th May 2017

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CHAPTER ONE

INTRODUCTION

1.1 Introduction

Carambola or starfruit (*Averrhoa carambola* L.) is a tropical fruit with sweet and tart flavour. This exotic, five-pointed shape fruit is classified as a late-ripening fruit with a relatively low physiological activity (Kader, 2002; Ali et al., 2004b; Thompson, 2008). High quality carambola cultivated in Malaysia has attracted global demand due to its unique taste and intrinsic economic value as an impressive source of essential antioxidants, vitamins and minerals for human consumption (Mahmood, 2007; Bicknell, 2009; MOA, 2012).

Carambola is a highly perishable crop mainly due to the progression of deterioration such as softening, senescence, chilling disorders and pathogen susceptibility during postharvest storage (O'Hare, 1993; Perez-Tello et al., 2001; Warren and Sargent, 2011). These perishable natures impair its appearance, preservation and health-enhancing properties. Eventually, these physiological disorders will greatly affect fruit postharvest quality, limit its marketability and lead to serious reputation consequences on the agroindustry. Therefore, it is imperative to find an alternative postharvest application that can extend the shelf life of carambola without having to compromise its keeping quality.

Serious efforts have been put in to develop an effective practice that reduced carambola postharvest losses. Postharvest treatment with vapour heat, hot water and forced hot air on carambola was unable to tolerate heat treatments without sustained serious damage (Hallman, 1991; Miller and McDonald, 1997). Modified atmosphere packaging

(MAP) with polyethylene (PE) followed by cold storage at 10°C effectively delay water loss and softening rate, however, fruit are susceptible to chilling injury like symptoms when held for 40 days (Ali et al., 2004b).

Postharvest carnauba wax coating in carambola which has successfully reduced moisture loss, but the treatment had no significance changes on physicochemical qualities, sensory texture or flavour (Miller and McDonald, 1993). Using wax may slow down the respiration rate, thus reduced the weight loss in carambola however, wax application inhibited normal ripening and resulted in the development of off-flavour (Warren, 2009). Also, further research using an edible coating of chitosan-palm stearin slowed down physiological changes, however, drawbacks such as inconsistent results and incompatibility of chitosan-stearin ratios hinder the effectiveness of coating film-forming properties (Nurul Hanani et al., 2012).

Later, application of plant modulators such as methyl jasmonate (MeJa) and salicylic acid (SA) has been utilized to improve postharvest quality in carambola. Both treatments significantly increased titrable acidity (TA), soluble solid content (SSC) and ascorbic acid (AA) content together with delayed water loss. However, there were no significant effects of MeJa and SA treatment on colour changes and firmness in carambola (Mustafa, 2015).

This study was carried out to understand the roles of polyamines (PAs) to exert their antisenescence effects for improvement of carambola postharvest quality during cold storage and shelf life. To the best of our knowledge, there is no information about the influence of PAs application on the carambola postharvest physiology and biochemistry. Therefore, such information is needed in order to prevent or possibly to control crop loss that occurred throughout all parts of the supply chain from harvesting in the field to the

human consumption.

Polyamines (PAs); the diamine, putrescine and the tri- and tetraamine, spermidine and spermine, respectively, are plant growth regulators and are ubiquitous in all kingdoms of life (Valero et al., 2002; Valero and Serrano et al., 2010). PAs are naturally occurring molecules and present endogenously in the plant tissue (Valero and Serrano et al., 2010). The free form of PAs has been pictured as an antisenescence agent due to its antagonistic effect to ripening (Liu et al., 2006; Shiri et al., 2013). These polycationic compounds have direct implications on various aspects of the postharvest biology of crops including quality, storage life, senescence, chilling and other stresses, and disease development (Valero, 2002).

Ethylene and PAs are known to have interrelationship where the balance between these two growth modulators is a decisive factor in either to retard the onset of ripening and senescence or to accelerate and triggering the processes (Liu et al., 2006; Valero and Serrano et al., 2010). The crosstalk between PAs and ethylene has been seen as a competitive biosynthesis mechanism with key enzyme S-adenosylmethionine (SAM) as a metabolic shift that will determine which pathway will become operative (Bassard et al., 2010; Handa and Mattoo, 2010).

Nowadays, an increasing public concern about the negative impacts of hazardous agrochemical residues on human health and the environment. Therefore, it has become necessary to search an alternative approach with no toxicological effects on human and subsequently reduce postharvest losses in carambola. The huge demand for safe, fresh carambola with high marketable quality has prompted this study to investigate the effects

of PAs as a potential postharvest treatment on fruit quality, shelf life and provide resistance to chilling stress. The study was undertaken with the following objectives:

1.2 Objectives

1. To evaluate the effect of polyamines application on physicochemical changes and quality improvement in carambola during cold storage.
2. To assess the effectiveness of polyamines treatment in reducing chilling injury, lipid peroxidation and membrane ion leakage in carambola during cold storage.
3. To examine the effect of polyamines treatment in regulating softening enzymes activity in carambola during cold storage.
4. To evaluate the efficacy of polyamines treatment on bioactive compounds with antioxidant activity in carambola expose to cold storage.
5. To study the changes in endogenous free polyamines level prior to exogenous application of polyamines and cold exposure in carambola.
6. To attain partial fragments of the genes encoding polyamines and ethylene biosynthetic enzymes in carambola with degenerate primers.

CHAPTER TWO

LITERATURE REVIEW

2.1 Carambola

Carambola or starfruit (*Averrhoa carambola* L.) is undoubtedly one of the unique creations of nature. Carambola fruit is native to Southeast Asia (Warren and Sargent, 2011; Paull and Duarte, 2012). The fruit is commonly cultivated and widely found in tropical and warmer subtropical countries (Rathod, 2011; Paull and Chen, 2014). In Asian countries, the fruit has many different names such as ‘belimbing’ (Indo-Malay), ‘caramba’, ‘yang-tao’ (Chinese), ‘ma fueng’ (Thai), ‘fuang’ (Laos) and ‘khe’ (Vietnamese) (Paull and Duarte, 2012). The term starfruit, however, is the most popular name which is derived from the fact that each slice of the fruit resembles five-pointed star shape when sliced transversely (Warren and Sargent, 2011). In general, carambola is categorized as slow-ripening fruit with long storage life as compared to a banana, mango and papaya (Chin et al., 1999; Ali et al., 2004a) but easily damage both before and after harvest (O’Hare, 1993).

2.1.1 Origin and distribution

As the plant originated from Southeast Asia, it thrives well in the humid countries such as Indochina (Vietnam, Cambodia and Laos), Malaysia and Indonesia. The fruit was probably introduced to Hawaii sometime after 1789, Florida in the late 1880s and was appearing in the Australia in the 19th century (Paull & Duarte, 2012). The major producing countries are Malaysia, Thailand, Indonesia and Taiwan (Rathod, 2011). Carambola has been grown since 1937 and large scale of commercial plantation began in 1983, with largest growing

areas are in Johor, Selangor, Kedah, Perak, Melaka and Negeri Sembilan (Rahman et al., 2007).

Carambola tree is categorized as an intermediate juvenile period (2-3 years), that is, the period between planting and first fruition. Carambola was considered as a long-term crop with a life span about 40 years (Yaacob and Subhadrabandhan, 1995). Many superior carambola tree clones have been registered and propagated, with a total of 19 clones were registered under the Department of Agriculture, however only clones B10, B17 and B2 were widely planted (Rahman et al., 2007).

Two clones namely, B10 and B17 were selected by the local growers and widely cultivated due to their performance, and since then became the recommended clones in cultivation (Chin et al., 1996). B2 clones mainly for tree pollination (Warren and Sargent, 2011). The B17 sweet variety of carambola was found to be most popular among local consumers and B10 variety was found to be more suitable for European markets prior to sweet-sour taste (Chin et al., 1996).

2.1.2 Physiology and characteristics

The fruit belongs to the Oxalidaceae family (Rathod, 2011) and has an ovoid shape with four to six very marked ribs. The closest family is the wood sorrels (*Oxalis* sp.), a type of weed and bilimbi (*Averrhoa bilimbi* L.), also recognized as the tree cucumber (Warren and Sargent 2011). The fruit length is usually 5-18 cm and 3-6 cm across, depending on the cultivar (Paull and Duarte, 2012). The surface of the fruit is shiny, smooth with a waxy cuticle and the thin, translucent skin is strongly bonded to the flesh. Whilst, the texture of the young fruit is firm with dark green skin and when ripe, skin color turns to light yellow to orange (overripe) with softer texture (Cecilia, 2008).

The flesh is light yellow to yellow, crisp and juicy. Its light-brown seeds are edible as well (Warren and Sargent 2011). Depending on the varieties and ripeness stage, carambola taste may vary between sour-tart and mildly sweet (Cecilia, 2008). Sugar content in carambola do not increase after picking therefore fruit should be harvested when the furrow between the ribs turned from green to yellow, while the tips of the fins (fondly known as the ribs) remain light green for optimum sweetness and flavour (O'Hare, 1993; Warren et al., 2007).

Carambola is classified as non-climacteric (Kader, 2002; Thompson, 2008) as the fruit showing little or no activity in ethylene emission and respiration rate throughout ripening (Lam and Wan, 1987; O'Hare, 1993). This non-climacteric fruit prior to lack the auto-catalytic ethylene response, exhibited a low rate of less than $3 \mu\text{l C}_2\text{H}_4 \text{ kg}^{-1} \text{ h}^{-1}$ at 20°C depending on ripeness stage (Paull and Chen, 2014). Higher rates of ethylene production occurred when fruit is considered overripe and tend to be related to tissue senescence or microbial decay (O'Hare, 1993). Respiration rates of carambola were recorded as 37 to 92 $\text{mg kg}^{-1} \text{ h}^{-1}$ at 20°C depending on maturity stage at harvest and varieties (Paull and Chen, 2014).

2.1.3 Economic importance and market value

Carambola is listed as one of the most popular and economically attractive tropical fruits with Malaysia being a major exporter. In 2015, the export value of Malaysian exotic tropical fruit to Europe worth an export value of RM 43.1 million compared to RM 41.4 million in 2014. Among the favoured fruit are carambola, jackfruit and mangosteen. The main buyers of Malaysia's carambola are Netherlands, Sweden, Norway, Finland, France,

Belgium and Germany, together these countries represent 85% of the total exports (Malaysiakini, 2017).

This upward trend is expected to continue because of increased consumer awareness of the high nutritional value of carambola, improved quality and aggressive promotional efforts taken by the private and public sectors (Bicknell, 2009). There is also a high demand using carambola in food preparation and serves as an excellent garnish and unripe fruit can be used as vegetables (Rathod, 2011). Thus, based on the two factors aforementioned, it can be deduced that the demand for carambola has increased at an alarming pace.

Malaysia has an advantage over other competitors such as Taiwan and Israel, which can only cultivate carambola seasonally in summer, in that Malaysia's tropical climate allows for the production of the fruit all year around (Yaacob and Subhadrabandhan, 1995). Carambola plantations in Malaysia faced many challenges such as land issues, lack of foreign investment and required high labor. However, there has been positive feedback due to increasing demand for carambola markets in Europe and the Middle East and changing in government attitudes to emphasize agriculture as a new prominence in the Malaysia's economy (Bicknell, 2009).

2.1.4 Consumption trends, nutritional values and significance to human health

Exotic fruit like carambola is well known for health benefits. Being rich in antioxidants such as vitamin C and phytonutrient polyphenolic compounds (Shui and Leong, 2004), carambola is known to have higher antioxidant content as compared to guava, orange, mango and lemon (Leong and Shui, 2002). It has been reported that carambola contained abundant phenolic compounds and provides between 131 - 221 mg per 100 g fresh fruit (Lim et al., 2007). Some of the important polyphenolic flavonoids present are

proanthocyanidins, epicatechin and gallic acid. Nonetheless, 100 g of fresh fruit contains a good quantity of water soluble vitamin C between 4 - 34 mg (Shui and Leong, 2006). 70% of the total antioxidants in carambola are generously present in tissues (Shui and Leong, 2006), in spite of the juice content represent more 90% of the total fresh weight (Warren and Sargent, 2011). The fruit is enriched with minerals such as sodium, potassium, magnesium and phosphorus (Rathod, 2011) and contained groups of amino acids such as serine, glutamic acid and alanine (Shui and Leong, 2006). Carambola is high in water content with over 90%, constitute of 7% carbohydrates and 1% protein. It also carries good amount 1.3 - 2.8% of dietary fiber (Paull and Duarte, 2012).

During ripening, carambola showed an increase in ascorbic acid contents and distinct changes in the chemical compositions. As ripening continues, the fresh fruit may have 8% total sugars, 11.2% total soluble content, 0.25% acidity with a pH of 4.5 (Tongchitpakdee, 2012). Development of orange-yellow pigment of carambola was contributed by the presence of carotenoids (Gross, 1983) and the total carotenoid content was 22 µg per gram fresh weight (Tongchitpakdee, 2012).

The taste of the fruit is contributed by the presence of oxalic acid and tannins. The impressive content of oxalic acid (5.8 mg per gram fresh weight) is the main responsible for the tart taste of the fruit. Carambola also has low malic acid content (0.4 mg per gram fresh weight) (Paull and Duarte, 2012). The aroma and taste of high quality Malaysian carambola were pictured as a mix of peach-apricot-plum like (Cecilia, 2008).

Nowadays, consumption of vegetables and fruits has been closely correlated with a prevention of harmful and deleterious diseases indicated by the activities of free radicals cause oxidative damage to tissue (Díaz-Mula et al., 2009). Antioxidant compounds are

claimed can hinder or delay the oxidation of an oxidisable substrate in a chain reaction and effectively scavenge free radicals therefore seem to be very important in the prevention of that disease (Leong and Shui, 2002; Díaz-Mula et al., 2009).

Owing to the unique flavour and shape, carambola have a great potential as a fresh-cut product use in salads and as garnishes for salads, drinks and cocktails (Teixeira et al., 2007). In Malaysia, the fruit is either eaten raw or served as juice beverages and the juice has a pleasant, slightly sweet and refreshing flavour that quenches thirst. Around the world the fruit is eaten raw or cooked into curries, stew or pudding, salted and turn into pickles or used to make wine. Some prefer to eat carambola in the form of fruit preserves and jams, and in Asian countries carambola was mixed into their main dishes such as boiled with shrimp (Thailand) or stewed with fish (China) (Warren and Sargent, 2011).

As far as its health-giving properties are well known, carambola has various therapeutic reputations around the world (Rathod, 2011). The people of India used ripe fruit to stop hemorrhaging, to alleviate bleeding hemorrhoids and the juice or the sliced and dried fruit was used to treat fevers. It also administered to relieve diarrhea, biliousness and even used as a hangover cure. Carambola often made into a salve to cure eye afflictions (Tongchitpakdee, 2012). In Brazil, the fruit is believed to treat bladder complaints, kidney sufferers such as diuretics and is employed in the treatments of eczema.

In China, carambola is consumed to relieve fever and increase the salivary secretion (Tongchitpakdee, 2012). However, carambola is not recommended to be consumed by a patient with kidney problems as the fruit contain caramboxin and oxalic acid (Garcia-Cairasco et al., 2013). There is evidence that indicates that these compounds believed to have a deleterious effect on renal function (Warren and Sargent, 2011).

2.2 Pre- and postharvest management of carambola

2.2.1 Preharvest factors affecting quality and current practices

2.2.1.1 Physical damage

Strong and dry wind movement may cause wind-scar damage when fruit swing and rub branches and against each other fruit while still attached to the parent tree. Cultivars with marked rib edges are vulnerable to marking and scarring damage than fruit with rounded edges (Paull and Duarte, 2012). The slight damage, scratch and cuts of the fruit are the most decisive factor of postharvest rejections and at the retail level. Scars will develop on the skin surface and rib edges lowering the market quality (Warren and Sargent, 2011).

Fruit harvested at the or later than the one-quarter yellow ripeness yields optimal sugar content when allowed to ripen on the tree however, fruit which is highly susceptible to wind damage (Paull and Duarte, 2012). In Florida, the use of windbreaks and wind screen either temporary or permanent prevented wind damage from occurring while the fruit remains on the tree. Windbreaks or wind-barriers usually consist of multicropping systems with other fruit trees. Meanwhile, wind screens are usually made of cables and poles that hold and support shade cloth (Warren and Sargent, 2011; Paull and Duarte, 2012).

2.2.1.2 Preharvest infection

Preharvest infection may occur through several attempts, such as direct penetration of the skin, infection through natural openings of the produce and infection through existing damage (Adaskaveg et al., 2002). Several pathogenic fungi were identified from carambola namely anthracnose (*Colletotrichum* sp.), sooty mold (*Gloeodes* sp.) and stem end rots (*Cercospora* sp., *Phyllosticta* sp., *Gloesporium* sp., *Phomopsis* sp. and *Corynespora cassiicola*,) (O'Hare, 1993; Paull and Duarte, 2012). *Cercospora* sp. was reported to cause stem end rots in Malaysian carambola B10 (Wan and Lam, 1984). Some fungi able to

initiate an infection during the flowering phase and on developing fruit, the infection is arrested and remains quiescent until the conditions become favorable for growth such as fruit ripening and tissue senescence (Adaskaveg et al., 2002).

Leaf spot fungi caused by *Cercospora averrhoa* are commonly observed in nutritionally deficient or environmentally stressed trees. The disease caused a total drop of tree leaves and fruit (Mossler and Crane, 2002). Pathogenic microorganisms, usually present on dead plant tissue or associated with the soil, infect commodity through surface injuries and require favorable weather conditions at the time of maturation and ripening of the commodity, to cause major loss (Adaskaveg et al., 2002).

In Southeast Asia, pest problem is major concern reducing yield and fruit quality in the field. The carambola tree is affected by pests such as *Dacus dorsalis* known as Oriental fruit flies (Green, 1987). Due to fruit flies, some countries such as the USA and Japan have strict regulation concerning quarantine requirements for carambola export from tropical countries (Thompson, 2008; Tongchitpakdee, 2012). Moth (*Othreis* sp.) caused damage to mature fruit, feed on the juice which can lead to subsequent rotting and fruit drop prematurely (O'Hare, 1993). A number of scale insects such as mealy bugs, fruit borers, scales, beetles and thrips can attack the carambola tree in the field. This is particularly caused an infection of fungi on branch, fruit and flowers and eventually leads to soft rot of the fruit (Mossler and Crane, 2002; Paull and Duarte, 2012).

Disinfestation is required by many importing countries, apart from the usage of known control measures such as pesticide and insecticide (Mossler and Crane, 2002), disinfestation methods such as heat treatment (water/air) (Hallman 1991; 1992), cooling methods (Miller and McDonald, 2000) as well irradiation treatment (Paull and Chen, 2004)

has been recommended. In Malaysia, farmers adapted to use a conventional method like bagging their fruit while it's developed on the tree. Not only being protected from insect infestation and wind damage but also fruit tends to be cleaner (Green, 1987).

2.2.2 Postharvest factors affecting quality and current practices

2.2.2.1 Harvesting, transport and handling management

Carambola harvesting is carried out entirely by hand to reduce physical damage (Thompson, 2008). The fruit should be handled carefully in the field and carried to the packing house as soon as possible (Paull and Duarte, 2012). Depending on the market destination and the time needed to reach consumers, carambola for export is ideally harvested at the light green or one-quarter yellow stage. Fruit at this stage is firmer, less susceptible to mechanical damage during handling and long shelf life (Campbell et al., 1987). Carambola for local market can be more fully coloured but will typically have a short market life.

Physical injuries such as scrapes, cuts and impact bruising can easily occur during harvesting, accumulation in the field and packing. Defective fruit will start to deteriorate and prone to decay pathogens (Warren and Sargent, 2011). Mechanical injury can be prevented by avoiding the usage of deep containers or buckets during fruit accumulation (Thompson et al., 2002a). Rough handling and transport damages fruit and exposure to high temperatures the fruit will become overheated if there is inadequate cooling or ventilation (Thompson et al., 2002b). Severe water loss and sun-scorch of the exposed fruit resulted from transport in an open truck, thus a closed and/or well-ventilated transport is frequently used to protect the fruit from the sun exposure (Thompson, 2002).

Carambola should be carefully placed during packing to avoid bruising or abrasion to the delicate ribs and thin skin. The use of paper wrap often waxed to reduce water loss or foam sleeve to reduce friction at the rib edges. The carton/cardboard can be made more protective by using cushioning pads of plastic or polyethylene to avoid compression, impact bruising and desiccation. A foam pad is usually placed on the top and the bottom of the fruit. Delicate handling during packing will ensure that commodity retains its market quality and that economic returns are maximized (Paull and Duarte, 2012).

2.2.2.2 Temperature and humidity control

Carambola fruit should be held around 5 to 7°C as soon as possible after harvest. At the warmer temperature and low humidity will hasten ripening and major physico-chemical changes. Golden Star and Arkin cultivars picked at the light green stage or color-break developed a normal yellow after 44 days of storage at 10°C. Meanwhile, the fruit was less yellow when kept at 5°C (Campbell et al., 1987; 1989). Storage of Malaysian carambola at 5°C significantly slowed down colour development than in 10, 15 and 20°C (Cecilia, 2008).

Ideally, the atmospheric humidity for carambola is in the range of 85 to 95% to reduce vapour pressure differential between the fruit and the surrounding atmosphere thus slowing down moisture loss and rib-edge desiccation (Paull and Duarte, 2012). Humidity below than 70% leads to severe ribs browning or dark discoloration. Fruit that is held below the ideal relative humidity (RH) can lose more than 5% of saleable weight (Cecilia, 2008). Golden Star and Arkin carambola exhibited serious weight loss when subjected to storage at 10°C (5.3-6.7% RH). However, lower water depletion was observed when stored at 5°C (4.2-4.4% RH).

Prior transferring from storage at 10 and 5°C to 20°C, Golden Star and Arkin experienced greater weight loss of 10% and 20% respectively (Campbell 1987; 1989). Likewise, Kenny and Hull (1986) reported that weight loss of Fwang Tung and Arkin cultivars was lower at 7.5°C than that under high temperature 10 or 21°C. Fruit attained water loss of 30-35% of their initial weight in 2 weeks when stored at 21°C. Carambola harvested at the light green stage or at colour break will resume developing colour until full yellow attainment. The skin colour will change significantly if carambola is subjected to 15 or 20°C storage (Paull and Chen, 2014) and attained full orange (overripe) after 2 weeks (Yon and Jaafar, 1994).

Carambola at the colour break stage will have a postharvest life of 3 weeks at 16°C, while at a higher temperature (21°C) fruit will have an approximately shorter shelf life of 2 weeks. Exposure to 5°C or below may result in failure to ripen normally and chilling injury (Cecilia, 2008). Likewise, Ali et al. (2004b) reported that rate of softening in B10 carambola is suppressed at 5°C (85 to 95% RH) than that fruit held at 10, 15 and 20°C for 30 days. Furthermore, carambola kept at 5°C was firmer than at 10°C.

2.2.2.3 Chilling injury

Chilling injury (CI) is a physiological disorder that influences tropical and subtropical horticulture. It manifests when the susceptible fruit is held above freezing temperature, but below a threshold temperature (Wang, 1994a). The cultivar, maturity stage, duration and temperature of postharvest storage are the decisive factors that determine the intensity of CI symptoms (Paull and Chen, 2004). Wan and Lam (1984) reported that carambola is a chilling sensitive fruit and unripe fruit are more sensitive compared than ripe fruit.

The common symptoms of CI in carambola are unsightly rib-edge browning, shriveled along the edge of the ribs, brown patches on the surface, skin pitting, failure to ripen properly prior transfer to 20°C and defective fruit is susceptible to decay pathogens (Campbell et al., 1985; O'Hare, 1993; Cecilia, 2008). The magnitude of the skin pitting may exist as small (less than 1mm) or large (1-2 mm). The pit may transform into deep and dark brown skin depression. Such symptoms usually dormant during low temperature and later appear when fruit is transferred to a higher temperature (O'Hare, 1993).

It has been established that storage at 5°C is considered the low temperature threshold in carambola and may develop CI when held lower than 5°C. However, external factors such as growing area and maturity at harvest need to be taken into consideration (Warren and Sargent, 2011). The appearance of CI was observed in carambola kept at 0°C for 2 weeks or at 5°C for 6 weeks, followed by subsequent storage at 20°C for 2 days (Cecilia, 2008). Carambola that harvested as premature does not ripen to full colour and more sensitive towards cold storage as they more susceptible physiological injury (Campbell et al., 1987).

On the other hand, mature carambola is more resistant to exposure to cold storage with less apparent the CI symptoms. This is probably due to major physiological changes was far advanced and making the fruit more tolerance when placed at chilling temperatures (Wang, 1994a).

2.3 Polyamines

The history of polyamines (PAs) started when Anton van Leewenhoek created a primitive microscope in 1674. He found the existence of substances in the form of crystalline in human semen in 1678 (Bachrach, 2010; Valero and Serrano 2010). Later, Vauquelin

identified in 1791 that the crystals were phosphate derivatives of unknown new compounds and in 1878 the unknown compounds were reported as an organic base (Bachrach, 2010). The work of Rosenheim in 1924 completed the early phase of PAs studies when correct structures were elucidated and putrescine, spermine and spermidine were successfully synthesized (Mattoo and Handa, 2008; Bachrach, 2010).

PAs are micromolecules, polycations with more than one amine group (Figure 2.1). These organic substances are ubiquitous in all eukaryotic cells and have profound effects on distinct biological functions (Kakkar and Sawhney, 2002; Kaur-Sawhney et al., 2003; Liu et al., 2006). The endogenous content of PAs in the plant is greater than other plant hormones. Their concentrations may vary from μM to more than mM (Kakkar and Sawhney, 2002; Imai et al., 2004; Bassard et al., 2010). Diamine putrescine (PUT, 1, 4-diaminobutane) is a major PAs in plants and a precursor for triamine spermidine (SPD, *N*-3-aminopropyl-1, 4-diaminobutane), and spermine (SPM, [bis (*N*-3-aminopropyl)-1, 4-diaminobutane]) (Kumar et al., 1997; Gill and Tuteja, 2010; Handa and Mattoo, 2010; Calzadilla et al., 2014).

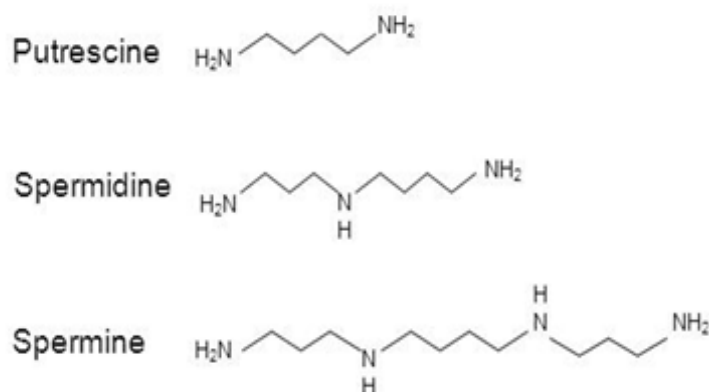


Figure 2.1: Putrescine, spermidine and spermine chemical structures in plants (Pang et al., 2007).

In plant tissues, PAs hold important regulatory functions during development and physiological processes such as cell division, elongation and differentiation, seed germination, flowering, fruit development and stress tolerance (Pang et al., 2007; Nambeesan et al., 2008). This wide variety of biological functions has direct implications on various aspects of the postharvest physiology of fruit and vegetable crops including quality, storage life, senescence, environmental stresses and diseases (Pang et al., 2007; Nambeesan et al., 2008; Gill and Tuteja, 2010).

Early studies on plant systems showed that PAs bind strongly to negatively charged membranes, nucleic acids and other macromolecules including a number of enzymes or proteins whose activities are highly influenced by PAs biological activity (Kakkar and Sawhney 2002; Carbonell and Blazquez, 2009; Bassard et al., 2010). PAs capability of binding is attributed to their positively charged nature at normal cellular pH (Carbonell and Blazquez, 2009; Bassard et al., 2010). These ionic interactions have been implicated in stabilizing membranes, regulating ion channels, scavenging oxygen radicals and affecting gene expression (Kaur-Sawhney et al., 2003; Gill and Tuteja, 2010; Handa and Mattoo, 2010; Calzadilla et al., 2014).

Significant information about PAs physiology and regulation during fruit development, ripening and senescence has emerged from analyzing endogenous PA levels, biosynthesis and metabolism (Nambeesan et al., 2008; Valero and Serrano, 2010). In recent years, a better understanding of PAs has also begun to emerge through experimental procedures aimed at manipulating endogenous PAs level in fruit through the exogenous supply of PAs under field conditions and after harvest and molecular aspects.

Evidence gathered in transgenic plants with modified PA metabolism and isolation of mutants with altered PAs biosynthetic deficient and inhibitor genes had opened PAs possible roles in cellular, physiological and biochemical processes including plant adaptive tolerances (Kumar et al., 2007; Liu et al., 2006; Bassard et al., 2010).

2.4 Polyamines regulation in plant tissues

2.4.1 Polyamines biosynthesis

The biosynthesis of the common polyamines (PAs) such as putrescine (PUT), spermidine (SPD) and spermine (SPM) is very tightly controlled due to their pivotal role in eukaryotic cells (Kusano et al., 2008; Valero and Serrano, 2010).

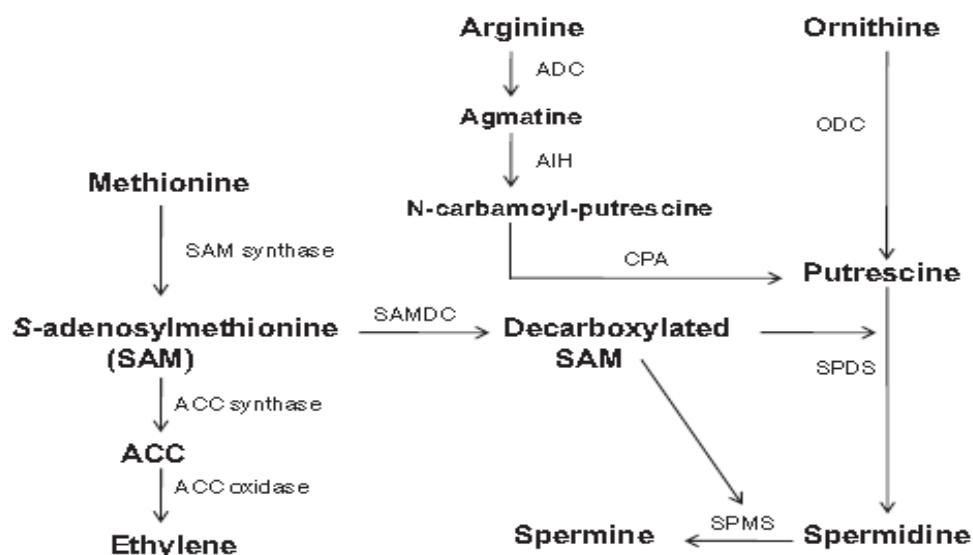


Figure 2.2: Biosynthetic pathway of PAs in plants. ADC: arginine decarboxylase; AIH: agmatine iminohydrolase; ODC: ornithine decarboxylase; SAMDC: SAM decarboxylase; CPA: N-carbamoylputrescine aminohydrolase; SPDS: spermidine synthase; SPMS: spermine synthase; SAMDC: S-adenosylmethionine decarboxylase; ACC synthase: 1-amino-cyclopropene-1-carboxylic acid synthase; ACC oxidase: 1-amino-cyclopropene-1-carboxylic acid oxidase (Wimalasekera et al., 2011).

As shown in Figure 2.2, PUT is synthesized from two alternative pathways through either decarboxylation of arginine or ornithine amino acid. Both pathways were catalyzed by arginine decarboxylase [EC 4.1.1.19, ADC] and the ornithine decarboxylase [EC 4.1.1.17, ODC] respectively, which is subsequently converted to PUT. In plants, arginine is converted to agmatine through arginine decarboxylase-catalyzed reaction. Agmatine is converted into N-carbamoyl-putrescine by two successive steps by agmatine iminohydrolase [EC 3.5.3.12, AIH], and later N-carbamoyl-putrescine aminohydrolase [EC 3.5.1.53, CPA] converts N-carbamoyl-putrescine to PUT (Mayer and Micheal, 2003; Bassard et al., 2010; Valero and Serrano, 2010; Wimalasekera et al., 2011).

Once PUT is initiated through ADC and/or ODC, the first PAs become the precursor of higher molecular weight PAs triamine SPD and tetramine SPM. These enzymatic reactions are sequentially catalyzed by SPD synthase [EC 2.5.1.16, SPDS] and SPM synthase [EC 2.5.1.22, SPMS] respectively. Conversion to SPD and SPM involved the sequential addition of aminopropyl moieties derived from decarboxylated S-adenosylmethionine (dcSAM) which is synthesized from the decarboxylation of SAM by SAM decarboxylase [EC 4.1.1.50, SAMDC]. The availability of dcSAM limits the formation of SPD and SPM, demonstrating that SAMDC plays a key role in the regulation of PAs homeostasis (Mehta et al., 2002; Valero et al., 2002; Kaur-Sawhney et al., 2003; Pang et al., 2007; Valero and Serrano, 2010; Wimalasekera et al., 2011).

Ripening hormone ethylene is synthesized in two step reactions from amino acid methionine via S-adenosyl-methionine (SAM) by SAM synthetase. Subsequently, SAM is converted to 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase. The final

step requires oxygen release from ACC oxidase action to produce ethylene. ACC oxidase is fondly known as ethylene forming enzyme (EFE) (Lin et al., 2009).

2.4.2 Polyamines localization

In higher plant polyamines (PAs) biosynthetic enzyme such as ODC has been mainly identified in the cytoplasmic and nuclear localization (Slocum and Flores, 1991). ADC predominantly detected to be localized in the thylakoid membrane of chloroplast of photosynthetic tissues and to the nucleus in non-photosynthetic tissues (Borrell et al., 1995). Meanwhile, SPDS were predominantly localized to the cytoplasm and chloroplast and PAO enzymes in the cell wall fraction (Kumar et al., 1997; Kaur-Sawhney et al., 2003).

In the plant, PUT is originated from two alternative pathways; synthesized directly from ODC or indirectly from ADC. Ornithine-dependent PUT production has been reported to be absent in *Arabidopsis thaliana* thus PUT is formed exclusively via ADC synthesis route (Bagni et al., 2001; Alcazar et al., 2010). In contrast, the PUT formation in mammals and fungi are synthesized solely via decarboxylation of ODC (Bagni et al., 2001; Valero and Serrano, 2010).

Synthesis of PUT via ADC and/or ODC proposed differential physiological roles and resulting in the specific regulation for ADC and ODC in plant compartment systems (Walden et al., 1997; Bouchereau et al., 1999). Formation of the diamine from different routes suggested that ADC and ODC undergo different evolutionary of origins (Alcazar et al., 2010).

2.4.3 Polyamines catabolism

The pool of cellular free polyamines (PAs) is controlled by oxidative deamination synthesized by copper containing diamine oxidases [EC 1.4.3.22, DAO]. Additionally, PAs catabolic pathway also requires the involvement of flavin containing polyamine oxidases [EC 1.5.3.11, PAO] (Kusano et al., 2007).

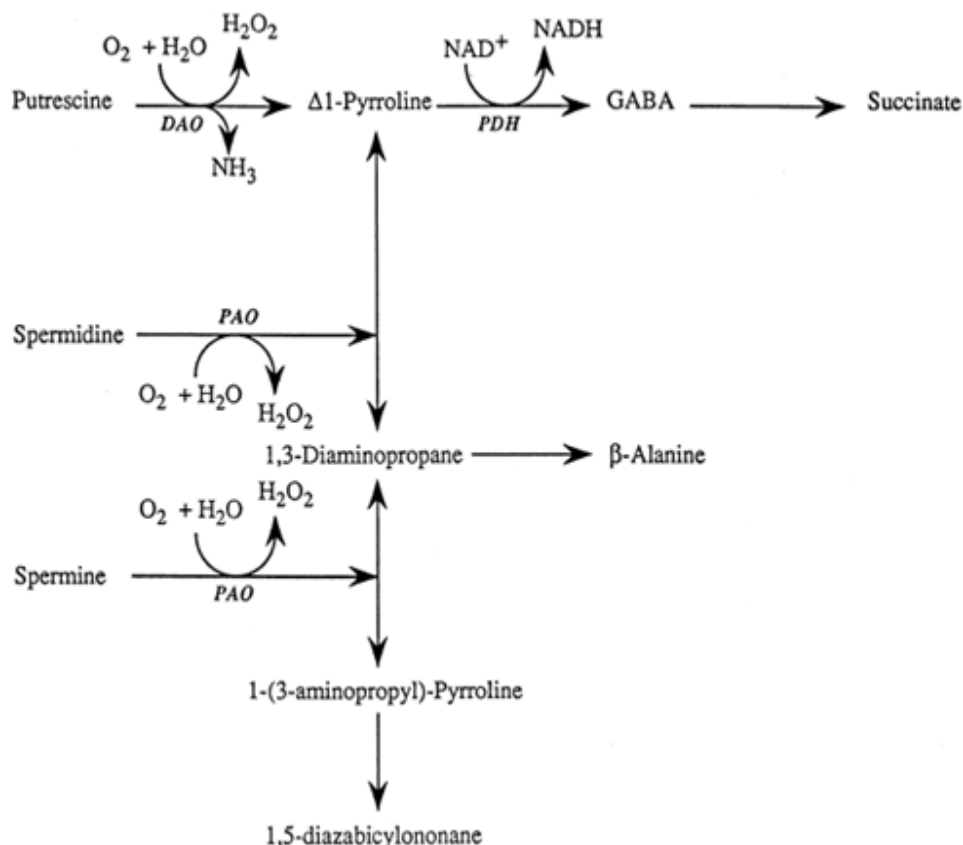


Figure 2.3: Polyamine catabolism (degradation) mediated by amine oxidases in plants. DAO: diamine oxidase; GABA: 7-aminobutyric acid; PAO: polyamine oxidase, PDH: pyrroline dehydrogenase (Bouchereau et al., 1999).

As shown in Figure 2.3, DAO catabolized PUT producing 1-pyrroline, in addition to ammonia (NH_3) and H_2O_2 as by products. 1-pyrroline is catalyzed into γ -aminobutyric acid (GABA) which is further metabolized to succinate and entered the Krebs cycle (Bouchereau et al., 1999; Seiler, 2004). PAO which has a high affinity for SPD and SPM

generate 1, 3-diaminopropane (DAP) and H_2O_2 along with 1-pyrroline and 1, 5-diabicyclononane respectively and DAP subsequently produces β -alanine (Bouchereau et al., 1999; Kusano et al., 2007).

This pathway allowed physiological back conversion of the secondary amine group (SPD and SPM) to PUT and the reactions depend on SPD/SPM acetyl transferase (SSAT) activity respectively. Reformation of PUT from by acetylation of SPM/SPD is controlled by SSAT and the oxidation by PAO in the peroxisome. Through back conversion pathway, SPM is utilized to SPD in the cytoplasm by oxidizing enzymes SPM oxidases (Seiler, 2004; Cona et al., 2006).

H_2O_2 released as byproducts in PAs catabolism are essential components for cell wall lignification and stiffening (Nambeesan et al., 2008) and may act as a defensive mechanism in the plant (Walters, 2003). Generation of H_2O_2 through increased PAO expression triggers production of antioxidant enzymes, namely POD, SOD, CAT allows redox homeostasis and minimizing oxidative damage (Angelini et al., 2008).

2.4.4 Conjugated and bound polyamines

In most plant systems, PAs frequently exist in forms of free, bound and conjugated. PAs can conjugate to micromolecules mainly cinnamic and phenolic acid or bounded to other macromolecules such as DNA, RNA, proteins and phospholipids. PAs are covalently conjugated to hydrocinnamic acid thru amide linkage using carboxyl groups from esters of coenzyme A (CoA) catalyzed by transferases (Bouchereau et al., 1999; Martin-Tanguy, 2001). The actual role of conjugated PAs is thought to serve as storage forms and when required, PAs be released into free forms during the growth phase (Valero et al., 2002). PAs

that conjugated with hydroxycinnamic acid may have important roles in long distance translocation of PAs and floral induction (Nambeesan et al., 2008).

Cationic PAs that are bound to proteins such as transglutaminases was reported to involve in flower development and compatibility in reproduction (Kakkar and Sawhney, 2002; Wimalasekera et al., 2011). In addition, conjugated PAs are also related to flower gender and fertility. Female flowers have more content in hydroxycinnamic amides than male and sterile organs contained less PAs than the fertile line (Liu et al., 2006).

2.5 Polyamines in human diet and health

Bioactive amines are substances with one or more amine group. They are low molecular weight organic bases synthesized by all living organisms normal metabolism. Bioactive amines can be traditionally classified in several criteria's (i) amine groups present in the molecule (ii) chemical structure (iii) physiological functions in the body or biosynthesis. The latter is the most widely accepted. Based on the biosynthetic pathway, the bioactive amines are divided into biogenic amines and neutral (polyamines) (Santiago-Silva et al., 2011; Rodriguez et al., 2014).

Biogenic amines are synthesized from direct enzymatic decarboxylation of their precursor amino acids. The common biogenic amines found in food are histamine (histidine), tyramine (tyrosine), tryptamine (tryptophan), serotonin (tryptophan), cadaverine (lysine) and phenylethylamine (phenylalanine) (Santiago-Silva et al., 2011). Putrescine (PUT) is classified in both groups (biogenic and PAs) and regarded as a true biogenic amine as the diamine is synthesized directly from precursor amino acid ornithine and also a precursor for SPD and SPM (Kalac, 2014; Rodriguez et al., 2014).

The PAs such as SPD and SPM are required for normal physiological functions such as growth, renewal and metabolism. Histamine, tryptamine and serotonin are reported to act as protective agents, meanwhile other biogenic amines can be used as an indicator for inappropriate and unhygienic storage and handling conditions (Kalac, 2014). Mammalian including human cells contained large amounts of PAs due to their participation in cell growth and proliferation (Hunter and Burritt, 2012). PAs have synthesized within human cells via three routes (i) intracellular synthesis (*de novo*) (ii) PAs as metabolites by gut microbiota (iii) dietary PAs (Hunter and Burritt, 2012; Kalac, 2014).

Dietary intakes such as fruit and vegetables can contain proper amounts of PAs and concentration varies depending on food types, ranging from nM to μ M per gram (Moinard et al., 2005). However, PAs concentration and composition can be affected by handling, processing and storage conditions (Eliassen et al., 2002). In contrast, Kalac et al. (2014) reported that PAs are physiologically stable molecules upon heat and acidic or alkaline environments. Red meat and poultry tend to be high in SPM but low in SPD while plant-derived foods are enriched in PUT and SPD (Moinard et al., 2005; Kalac and Krausova, 2005) and fresh fish contain low SPM levels (Kalac, 2014).

Cheese particularly is rich in PAs due to high microbial activity during the fermentation process (Eliassen et al., 2002). PAs are notably low in cereals such as wheat, maize and white rice except in wheat germ and rice bran (Nishimura et al., 2006; Cipolla et al., 2007; Binh et al., 2010). Legumes namely soybeans and fermented soybean products, particularly enriched in SPD and SPM excluding lentil and bean (Nishibori et al., 2007). Considerably low PAs have detected in potato including sweet potato and yam (Nishimura et al., 2006; Nishibori et al., 2007). Green pepper and spinach are high in PUT and SPD

respectively, meanwhile green peas and in some edible mushrooms are high in both PAs content (Nishimura et al., 2006; Kalac et al., 2005; Cipolla et al., 2007).

A study conducted by Larque et al. (2007) reported that oranges and pears are the richest fruit sources of PAs particularly PUT and SPD. Another study from Santiago-Silva et al. (2011) reported the prevalent amount of SPD are present in some Asian tropical fruits namely mango and passion fruit, whereas the significant SPM content present in guava and PUT in papaya. Accordingly, PAs content tends to decrease as fruit approaching the ripening stage and loss rapidly upon harvesting and length of time taken to reach consumers (Valero and Serrano, 2010).

Due to the importance of PAs in numerous human physiological processes such as growth, renewal and metabolism, PAs participation can be both favorable and injurious to human health. In line with this, of PAs regulation has been tightly controlled inside the cells due to an increased requirement for human growth and cell viability. PAs content in dietary intake has been cautioned because of their adverse effects on human health (Valero and Serrano, 2010; Kalac, 2014). Given the ubiquity of PAs within the body, young children possess higher PAs level compared to adults for growth and healthy body (Bardocz et al., 1995; Larque et al., 2007).

Studies from Cohen et al. (1976) and Soda et al. (2009) postulated that the erythrocytes and leukocytes contain the most PAs and to some extent the PAs level decreases as aging progress. Thus, there is a consensus among study that PAs levels in tissues and blood may improve longevity in human cells and resistance towards stress. The increment of oxidative stress is often correlated with aging process (Hunter and Burritt, 2012). Additionally, PAs is thought to maintain the human gut health, profound roles as anti-inflammation, wound

healing and post-operational recovery (Hunter and Burritt, 2012). On the contrary, PAs accelerate tumor growth, but not triggering cancer. Naturally, patients with tumor should be restricted from a diet with high PAs content (Santiago-Silva et al., 2011; Hunter and Burritt, 2012; Kalac, 2014).

2.6 Interaction between ethylene and polyamines in plant system

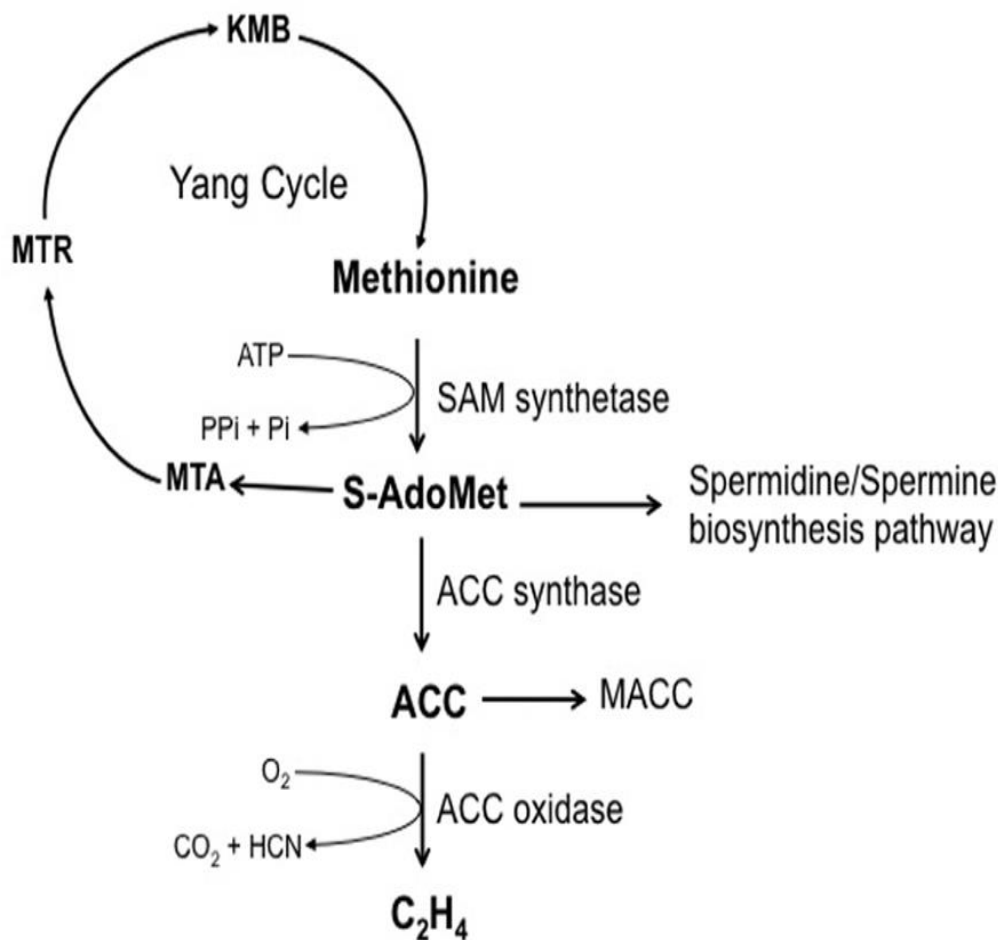


Figure 2.4: Ethylene biosynthetic pathway and regulation. S-AdoMet: S-adenosyl methionine; ACC: 1-aminocyclopropane-1-carboxylate; MACC: Malonyl-ACC; MTA: methylthiodenosine; MTR: methylthioribose; KMB: 2-keto-4-methylthiobutyrate; C₂H₄: ethylene (Arc et al., 2013)

Methionine (Met) is the precursor amino acid for ethylene biosynthesis (Saltveit, 1999; Lin et al. 2009). As shown in Figure 2.4, S-AdoMet is synthesized from Met by SAM synthetase at the expense of one ATP molecule. Met is recycled from methylthiodenosine (MTA), a byproduct of ethylene biosynthesis that permitting for the continued flux of Met (Yang) cycle involving various intermediate such as MTR (methylthioribose) and KMB (2-keto-4-methylthiobutyrate) (Arc et al., 2013).

The conversion of S-AdoMet into 1-aminocyclopropane-1-carboxylate (ACC) is catalyzed by ACC synthase and often regarded as a rate-limiting step for ethylene synthesis (Kepczynski and Kepczynska, 1997; Zhang and Wen, 2010). The production of malonyl-ACC (MACC) deprives ACC pool and consequently decreases ethylene emission (Lin et al., 2009; Arc et al., 2013). Using ACC as main substrate, ACC oxidase synthesizes ethylene and generates carbon dioxide and cyanide as by products (Lin et al., 2009; Arc et al., 2013).

Ethylene has the simple chemical structure of two carbons with a double bond (alkene) and considered unique because of gaseous nature yet involved in numerous plant development and physiology processes (Saltveit, 1999; Lin et al. 2009). Hence, ethylene has been termed as a ripening hormone. Ethylene is active and can stimulate ripening at a concentration of 0.1 part-per-million (ppm, $\mu\text{l l}^{-1}$) to as low as part-per-billion (ppb, nl l^{-1}) (Saltveit, 1999).

It is well documented that ethylene and polyamines (SPD and SPM) share SAM as starting point in their biosynthesis pathway. In ethylene pathway, SAM evolves into ethylene through ACC and the other SAM molecule is decarboxylated into SAMDC which serves as a donor of the aminopropyl groups to SPD and SPM. The competition between

these two plant growth modulators on SAM pools become decisive factors to inhibit ethylene biosynthesis or to suppress the accumulation of PAs (Mattoo and Handa, 2008; Valero and Serrano, 2010). PAs level was high during cell division and elongation at early fruit development and reduced thereafter. Declining in PAs concentration was detected at the final stage of fruit development and gives way to induction of signal for ripening (Kakkar and Sawhney 2002; Mattoo and Handa, 2008).

This relationship indicated a nexus interaction exists between two plant hormones that apparently possess antagonistic biosynthetic pathways (Mattoo and Handa, 2008).

2.7 Polyamines in fruit set, growth and development

Extensive literature depicts involvement of PAs and their conjugates during flowering, the early phase of fruit growth and development (Liu et al., 2006). There is a suggestion that ODC pathway may be involved in floral development, whereas ADC pathway appears to have a role in vegetative phase (Kaur-Sawhney et al., 2002). Evidence gathered indicates that PAs has been described to be necessary signaling molecules for floral initiation and development (Dios et al., 2006; Bassard et al., 2010).

Application of PAs biosynthetic enzyme inhibitors such as difluoromethylarginine (DFMA) and difluoromethylornithine (DFMO) causes strong inhibition of flowering, the inhibitory effect was reversed when PAs was supplied exogenously or inhibitors were absent. Utilization of PAs induces flowering under non-inductive conditions and mutant plant with deficient PAs exhibited delay and abnormal flowering patterns to the wild types (Liu et al., 2006).

Flower opening in damson plum is concomitant with rapid increases in free PA titers in the sepals, petals and ovarian development (Dios et al., 2006). Accordingly, high free SPD

and SPM titers are most often associated with floral organ development, viability and fruit set. This observation has been established in the grapevine (Aziz et al., 2001), pear (Franco-Mora et al., 2005) and apricot (Albuquerque et al., 2006). Moreover, PAs accumulation is also correlated to pollen germination, pollen tube growth, flower gender and fertility (Liu et al., 2006).

Normally, free PAs concentration changes during fruit set, growth, development and ripening. Experimental evidence of fruit species indicates that PAs are present at high levels during the early growth phase of cell division activity. PAs levels decrease thereafter at a later phase of fruit development and have been accepted as an initiation of the ripening process (Valero et al., 2002). The patterns of PAs changes have been widely studied in both climacteric and non-climacteric fruit as occurred in pepper (Serrano et al., 1995), tomato (Martinez-Madrid et al., 1996), strawberry (Ponappa and Miller, 1996), plum (Zuzunaga et al., 2001), peach (Bregoli et al., 2002), nectarine (Torrighiani et al., 2004), pomegranate (Mirdehghan et al., 2007a), plum (Khan et al., 2008), and table grape (Champa et al., 2014).

However, a few exceptions exist, such as a non-climacteric long keeping Alcobaca tomato displayed an increase in PUT level (Dibble et al., 1988), muskmelon demonstrated high PUT titers (Lester, 2000) and Golden Japan plum; a suppressed climacteric phenotype showed high both PUT and SPD content during ripening (Zuzunaga et al., 2001). This observation proves a discrepancy in the developmental trends of PA levels across different plant systems.

2.8 Polyamines in fruit ripening and senescence

Fruit ripening is the crucial final development stage that activates major shift in biochemical, metabolic and physiological pathways. Fruit attains desirable transformation

in colour, aroma, sensory (texture and flavour) and nutritional qualities (Giovannoni et al., 2004). Fruit is categorized into climacteric and non-climacteric nature by the increase on ethylene-associated respiratory (Srivastava and Handa, 2005). Climacteric fruit such as mango, banana and papaya exhibited a peak in respiration rate, concomitant with an ethylene emission burst at the onset of ripening. On the other hand, non-climacteric fruit such as carambola, pineapple and dragon fruit lack of ethylene production, indicating that the hormone gaseous is less needed for ripening (Lin et al., 2009).

As mentioned in sub-chapter 2.6, ethylene and PAs competing for the utilization of the SAM pool during fruit ripening (Valero et al., 2002). The interplay between PAs and ethylene during ripening has been studied between slow and fast ripening tomato (Saftner and Baldi, 1990; Martinez-Madrid et al., 1996). Liberty (slow), Pik Red and Rutgers (fast) tomato varieties exhibited a declines pattern of total PAs during growth. PAs endogenous level was low in Pik Red and Rutgers tomatoes, varieties with short life; the PAs level was high in Liberty, a variety with prolonged life.

Pik Red and Rutgers tomatoes produced up to 16 and 38% of ethylene respectively during ripening. Meanwhile, Liberty tomato possessed three to six more PAs than Pik Red and Rutgers. Increased PAs level in Liberty tomato indicated that the plant growth regulators accountable for low ethylene emission and longer shelf life (Saftner and Baldi, 1990). A similar observation was reported in Japanese pear cultivar Nakai as the cultivar displayed a higher level of PAs accompanied with long keeping quality. There was also a negative correlation between PA endogenous levels and the rate ethylene emission (Mora et al., 2005).

Transgenic tomato overexpressing SAMDC gene showed an increased in SPD, SPM level during ripening and experienced longer vine life. Moreover, mutant tomatoes also exhibited a two to threefold increase in lycopene and enhanced fruit juice quality. Apart from that, the study also revealed that these tomatoes emitted higher ethylene levels as compared to the parental line (Mehta et al., 2002). An inhibitor of ethylene receptor, 1-methylcyclopropene (1-MCP) delayed tomato ripening course and inhibited the PUT accumulation by downregulating PAs biosynthetic enzymes at the transcriptional level (Tassoni et al., 2006). Therefore, both outcomes suggested that PAs and ethylene can function in harmony instead of mutual inhibition and the competition between both of them is not expected since SAM are not limiting rate (Mehta et al., 2002).

PAs have been associated to be involved in plant senescence signaling cascades. In most plant systems, leaf aging and fruit ripening and senescence is associated with reduce in PAs levels. Exogenous treatment of PAs application often slows down or suppressed the progression of senescence (Sood and Nagar, 2003). The hypodermal-mesocarp tissue of Honey Dew muskmelon incubated with exogenous SPD and SPM had more chlorophyll, reduced membrane peroxidation, low lipoxygenase (LOX) and phospholipase-D (PL-D) production and higher H^+ -ATPase level (Lester, 2000).

PAs biosynthesis potent inhibitors namely difluoromethylarginine (DFMA) and methylglyoxal-bis-guanyldrazone (MGBG) hastened senescence of leaf discs of two different species of rose. On the other hand, free PAs primarily SPM suppressed the senescence by inhibiting of peroxidase and cellulase enzyme activity (Sood and Nagar, 2003). Cut carnation flowers which treated with 10 mM SPD displayed a delay in

senescence. It has been observed that the free and soluble SPD accumulated in treated petal flowers leads to stabilization and reduced degradation of DNA (Tassoni et al., 2006).

Creasing or albedo breakdown is a form of early senescence in the sweet orange cultivar. This undesired modification is mainly due to high pectin methylesterase and water-soluble pectin activity resulted in degradation of pectin linkages and the breakdown of albedo tissue connections. PUT spray application inhibited ethylene evolution, increased free PAs level in the flavedo and albedo tissue and caused substantial reduction of creasing incidence in sweet oranges (Hussain and Singh, 2015).

2.9 Pre- and postharvest polyamines treatment on fruit ripening and shelf life

This sub-section provides an overview of the effects of PAs treatment on fruit quality parameters during growing season and postharvest. A number of studies have proven that PAs as a preharvest treatment in the field caused a substantial reduction in ethylene production, reduce fruit drop, improve fruit quality attributes and slow down ripening progression PAs treatment from μM to mM concentrations has been investigated in a wide range of fruit such as field grown apricot (Paksasorn et al., 1995), peach (Bregoli et al., 2002; 2006; Ziosi et al., 2006; Torrigiani et al., 2012), mango (Malik et al., 2003; Malik and Singh, 2006; Ali et al., 2017), plum (Khan et al., 2007; 2008), nectarine (Torrighiani et al., 2004; Bregoli et al., 2006) and sweet orange (Hussain and Singh, 2015).

In peach, foliar spray of 10 mM of PUT, SPD (0.1, 1.0 and 5 mM) or 2 mM of SPM markedly suppressed ethylene evolution, delayed firmness loss and slowed down soluble solids concentration (SSC) accumulation (Bregoli et al., 2002; 2006). PUT treatments (0.5, 1.0 and 2.0 mM) were effective in reducing mango fruit rot index (Malik et al., 2003) meanwhile PUT (0.45 mM) increased chlorophyll accumulation and induced antioxidant

enzymes activity thus preventing fruit abscission. Additionally, fruit acidity levels and solids content ratio were also higher compared to the control (Ali et al., 2017). Mango peel colour development was retarded in the PAs-treated tree (0.01, 0.1 and 1.0 mM) and total carotenoid and ascorbic acid content were enhanced as compared to untreated fruit tree (Malik and Singh, 2006).

Field treatment of PUT (0.1, 1.0 and 2.0 mM) on plum significantly prevented firmness loss by inhibiting softening enzyme activity, exhibited a higher acidity level and suppressed ethylene associated respiratory by retarding ACS and ACO enzyme activity (Khan et al., 2007; 2008). In nectarine and peach, application of PUT and SPD counterattacked upregulation of ethylene (ACS and ACO) and SAMDC biosynthetic genes proposing that exogenous PAs modified ripening by interfering ethylene biosynthesis (Torrigiani et al., 2004; 2012; Ziosi et al., 2006). The effect of PUT by spraying at different concentrations (500 to 1000 μ M) during fruit set strongly reduced creasing index in sweet orange by inducing the accumulation of endogenous free PAs in the albedo and flavedo tissues (Hussain and Singh, 2015).

Accordingly, PAs as a postharvest treatment by vacuum, pressure infiltration or immersion has been proven to extend shelf life and improve fruit quality profiles such as delay in weight loss and peel colour changes, increase or retain flesh firmness, inhibit or reduce ethylene emission and respiration rate, suppress diminution titrable acidity and maintain higher soluble solids. Previously, the induction of exogenous PAs treatment on postharvest beneficial effects has been widely studied in both climacteric and non-climacteric horticultural crops, such as in apple (Wang et al., 1993), lemon (Valero et al., 1998a; Safizadeh et al., 2013), zucchini squash (Martinez-Tellez et al., 2002), plum

(Martinez-Serrano et al., 2003; Khan et al., 2007; 2008), kiwifruit (Petkou et al., 2004), strawberry (Khosroshahi et al., 2007), mango (Jawadha et al., 2012) and apricot (Khosroshahi and Esm-Ashari, 2017).

Application of PAs particularly PUT and SPD has been shown to boost the concentration of bioactive compounds with antioxidant activity during postharvest storage. Mirdehghan et al. (2007c) and Barman et al. (2011) revealed that PAs application has shown to enhance the accumulation of functional compounds in pomegranate. The concentrations of ascorbic acid, total phenolic and anthocyanins content was retained at a higher level as compared to untreated pomegranate. Similar findings were also reported in plum (Khan et al., 2008), table grape (Shiri et al., 2013; Champa et al., 2014), apricot (Davarynejad et al., 2013) mango (Malik and Singh, 2005; Razzaq et al., 2014) and orange (Habibi and Asghar, 2017).

Irrespective of the method of treatment, PAs have been regarded as regulatory molecules in plant responses to cope with unpredictable stresses, including low temperature (Gill and Tuteja, 2010). PUT and SPD (1.0 mM) by infiltration or immersion significantly alleviated chilling injury (CI), reduced skin browning and delayed the increment of electrolyte leakage in pomegranate throughout 60 days at cold storage (Mirdehghan et al., 2007c). Table grape treated with PUT and SPD (0.5, 1.0 and 1.5 mM) prior 75 days at cold storage exhibited lower decay incidence, reduced rachis browning and maintained a higher palatability rating than untreated fruit (Champa et al., 2014).

In addition, higher antioxidant enzyme activity such as catalase (CAT), peroxidase (POX), superoxide dismutase (SOD) and polyphenol oxidase (PPO) were also observed in PAs-treated apricot (Koushesh-Saba et al., 2012), pepper (Liu et al., 2013) and mango

(Razzaq et al., 2014). An elevated antioxidant enzyme was closely correlated with the ability of fruit to withstand chilling -induced oxidative injury (Imahori et al., 2008). Accordingly, reduced concentrations of lipid peroxidation products, malondialdehyde (MDA) were also recorded in PAs-treated pepper (Liu et al., 2013) and zucchini (Palma et al., 2015). Taken together, it appears that PAs treatment before cold storage could account for greater resistance to CI incidence (Kramer et al., 1991).

Mechanical damage or physical injury can hasten fruit deterioration by negatively affecting organoleptic properties and fruit tissue breakdown (Martinez-Romero et al., 2004). It appears that endogenous PAs content has been shown to increase in fruit tissue during mechanical damage. Application of compression force caused an increase in endogenous SPD levels in peach (Martinez-Romero et al., 2000) and plum (Perez-Vicente et al., 2002). Similarly, physically injured mandarin cv. Fortune exhibited an increase in PUT levels (Valero et al., 1998c) and an increment of both SPD and SPM level in lemon (Martinez-Romero et al., 1999).

From the previous literatures, PAs have been pictured as an antisenescence agent due to its antagonistic effect to fruit ripening and senescence during pre and postharvest conditions. Furthermore, it is well established that PAs efficacy is dependent on a number of conditions such as type, concentration and time of application.

2.10 Polyamines and abiotic stress adaptation

Plants are regularly exposed to numerous environmental stresses, despite being considered sensitive organisms. Environmental or abiotic stresses caused by unpredictable drastic environmental changes such as climate change, are seriously affecting crop productivity worldwide. Therefore, designing crops with traits conferring to abiotic stresses is a huge

challenge in merging biotechnology and agriculture. This is where PAs would hold the key player as stress messengers in plant responses to adverse stress (Liu et al, 2007; Gill and Tuteja, 2010; Hussain et al., 2011).

It is well documented that PAs have been implicated in regulating various abiotic stresses such as temperature extremes, drought, mineral deficiency and salinity (Wimalasekera et al. 2011; Hussain et al., 2011). PAs treatment has been noted as an effective alternative for enhancing crop productivity through increased crop tolerance under unfavorable conditions such as osmotic stress, heavy metals, water logging and flooding tolerance and (Gill and Tuteja, 2010).

It has been reported that the exogenous supply of PAs have been successful to increase resistance and equipped plants with tolerance towards extremely low and high temperatures (Nayyar et al., 2004; 2005; Murkowski 2001), drought (Zeid et al., 2006), hyperosmosis (Liu et al., 2004), waterlogging (Arbona et al., 2008), flooding-induced oxidative stress (Yiu et al., 2009), salinity (Chattopadhyay et al., 2002; Zapata et al., 2004; Verma et al., 2005; Ndayiragije et al., 2006; Liu et al., 2007) and heavy metal toxicity (Wang et al., 2007).

Activation of arginine decarboxylase (ADC2), induction of transcript level of ADC and regulation of amine oxidase activities was responsible for the induced PAs level in plants thus providing tolerance concerning salinity (Pang et al., 2007; Wimalasekera et al. 2011). Upregulated ADC2 expression by ABA (abscisic acid) modulation increase PAs level thus provide adaptation to drought tolerance by controlling stomatal movement (Alcázar et al., 2006; 2010; Caldazilla et al., 2014).

Plants under heat stress exhibited higher levels of free, bound and presence of uncommon PAs. It had been accepted that tolerant cultivar had the capacity to increase, maintain its total PAs pool by using the uncommon PAs as storage (Pang et al., 2007) and a higher level of PAO and ADC activities (Wimalasekera et al. 2011). It was suggested that upon heat stress PAs might directly induce HSPs (heat shock protein) synthesis or indirectly by altering the cell membrane properties (Königshofer and Lechner, 2002).

Previous studies reported that an increased in PUT levels in several plant species was detected after exposure to low temperatures (Bouchereau et al., 1999; Martinez-Romero et al., 2003). Under cold exposure free PUT levels increased coincident with an increment of ADC. Furthermore, up-regulation of ADC1, ADC2 and SAMDC2 is also shown to be a contributor in response to cold treatment (Wimalasekera et al. 2011). Some studies also postulated that a possible interaction between PA and ABA exists and suggesting a synergistic role between PAs and various hormones in modulating low temperature responses (Cuevas et al., 2008; Caldazilla et al., 2014).

Under the conditions of potassium (K^+) and magnesium (Mg^{2+}) deficiency have shown to cause in alteration of endogenous PAs metabolism (Wimalasekera et al. 2011). In Arabidopsis, accumulation of PUT especially conjugated and wall-bound forms were observed with concomitant increased of ADC level (Pang et al., 2007). PAs have been linked to providing tolerance towards heavy metal toxicity. Elevated PUT levels were shown when plant subjected to lead (Cd^{2+}), copper (Cu^{2+}) and mercury (Hg^{2+}). Adaptation towards heavy metal stress is highly influenced by inducing ADC and ODC activities and reduced DAO activity (Groppa et al., 2003; Pang et al., 2007).

Actual PAs action as signaling molecules during environmental stress is depending on stress type and the plant species. PUT, SPD and SPM contribute unique roles during abiotic stress exposure. In addition, modification of PUT: SPD and SPM ratios seem to be pivotal for plant adaptation to different environmental stress and markedly vary with plant species (Groppa and Benavides, 2008; Bassard et al., 2010).

2.11 Polyamines and biotic stress adaptation

PAs play crucial roles in wounding response and DAO activity was increased in plant cell walls (Scalet et al., 1991). Arabidopsis plant showed an increased in both ADC2 expressions and free PUT levels concomitant with a decline in the free SPM content during wounding (Perez-Amador et al., 2002). A similar observation was reported in wounded oilseed rape when the plant produced high levels of free PUT by decreases PUT catabolism rate and enhancing ADC activity (Cowley and Walters, 2005). Later, another study indicated that ODC1 gene transcript accumulated tremendously in wounded capsicum (Yoo et al., 2004).

An early study showed that PAs metabolism in the plant varies from an increase to decrease according to the type of microorganisms such as fungal pathogens, viruses or bacterial infection (Pang et al., 2007). The implication of PAs in plant disease resistance mechanisms significantly varies with plant tissue, species and infection systems (Wimalasekera et al. 2011). Marina et al. (2008) indicated a clear rule by indicating the concentration of total PAs and ratios between individual PAs greatly differs with every plant.

Prior to fungal and viral infection, an increase in PAs content and biosynthesis was observed where rapid levels of ADC, ODC and SAMDC activities were involved (Pang et

al., 2007). In infected plants, ODC was tightly controlled while SPD or SPM were enhanced accumulated in the apoplast (Marina et al., 2008; Bassard et al., 2010). SPD and/or SPM in apoplast was believed to be involved in regulating specific signal namely Ca^{2+} influx, pectic signal and H_2O_2 production through the SPM-signalling pathway (Messiaen and Van Cutsem, 1999; Cona et al., 2006; Kusano et al., 2007; Bassard et al., 2010).

SPD and SPM behaved as inhibitors for pectin dimerization by performing pectins linkages that inhibit pectin oligomerization and preventing the formation of endogenous elicitors (Messiaen and Van Cutsem, 1999). Oxidation of SPD/ SPM by PAO produced H_2O_2 may serve as a signal mediator and this response was common for a defensive mechanism in plants (Bassard et al., 2010). Also, H_2O_2 generated through PAs catabolism by PAO is necessary components during the oxidative burst for cellular defense responses (Angelini et al., 2008; Wimalasekera et al. 2011; Hussain et al., 2011).

Conversely, PUT stabilizes pectic fragments and prevents plant cell death (PCD) through H_2O_2 generation from PAO (Yoda et al., 2009; Bassard et al., 2010). The interplay of PUT, SPD:SPM concentrations, ratios between individual PAs, pectins complexes and inwards Ca^{2+} exert different phenomenon in disease resistance and carefully control balance of the homeostasis of PUT and SPD:SPM seems to be the fate between cell defense and PCD (Kusano et al., 2008; Bassard et al., 2010).

2.12 Molecular basis of polyamines role and effect

2.12.1 Polyamines effect at cellular level

PAs role has been implicated in a wide range of cellular processes such as cell proliferation, division, differentiation, growth and death, homeostasis, gene expression, regulation of

enzyme activities, protein synthesis and DNA replication (Kaur-Sawhney et al., 2003; Pang et al., 2007; Nambeesan et al., 2008; Gill and Tuteja, 2010; Handa and Mattoo, 2010; Calzadilla et al., 2014). Participation of PAs in fruit growth, ripening, shelf life, senescence and stress resistance attributed by their positively charged that allow PAs binding to negatively charged macromolecules (Kakkar and Sawhney, 2002; Carbonell and Blazquez, 2009; Bassard et al., 2010).

PAs maintain calcium cellular homeostasis and facilitate overall gene expression (Srivastava et al., 2007; Miller-Fleming et al., 2015), modulate specific metabolite (Mattoo et al., 2006) and protein profiles (Mattoo et al., 2007). However, the paucity of knowledge regarding the mode of action of PAs and their biosynthetic enzymes at cellular level remains one of the obstacles in understanding their biological role in plants (Nambeesan et al., 2008).

2.12.2 Polyamines interaction with macromolecules

In brief, PAs molecules with intracellular cations properties bind to DNA and RNA with intracellular polyanions for charge neutralization by targeting anionic phosphate of DNA (Panagiotidis et al., 1995; Kumar and Rajam, 2004). PAs interact and stabilize the helix binding of each DNA strand and remodeling chromatin structures (Kakkar and Sawhney, 2002; Childs et al., 2003; Keniry, 2003; Miller-Fleming et al., 2015). Apart from polycationic nature of PAs, other interactions such as hydrogen bonding, hydrophobic interactions and site-specificity have also been invoked in the binding of PAs to DNA (Panagiotidis et al., 1995).

PAs interaction also has profound effects on DNA molecule structures by affecting stabilization and transition conformation from B-DNA to A-DNA and induced B-Z

transition (Bryson and Greenall, 2000; Handa and Mattoo, 2010). These PAs-induced DNA conformational changes influence enzyme activities involved in DNA metabolism and modify binding activities of sequence DNA-specific binding proteins (Rajam et al., 1998; Kumar and Rajam, 2004). Furthermore, PAs can bind to RNA and modulate their stability and conformation through specific binding, and alter the regulation of synthesis and nucleic function (Kumar and Rajam, 2004; Miller-Fleming et al., 2015). PAs control elongation of mRNA (Yoshida et al., 2002) and phosphorylation of transcript proteins for translation (Landau et al., 2010).

In bacteria *Escherichia coli*, 89.7% of SPD and 47.9% of PUT exist as PA-RNA complexes that closely related to structural changes in RNA, thus add to its proposed role in translation (Igarashi and Kashiwagi, 2010). A role of PAs in regulating translation has been studied in modified *Escherichia coli* and eukaryotes and the term of ‘PA modulon’ was surfaced. It explained the importance of PAs presence in increasing the transcription rate by promoting the initiation of genes translation (Yoshida et al., 2004; Nishimura et al., 2009).

2.12.3 Polyamines influence on membrane functions

PAs prevent photosynthesis cessation and proteins and chlorophyll progressive degradation, lipid peroxidation and ion leakage rate reduction, cell wall and organelle structures stabilization. PAs inhibit the intensive breakdown of cellular compositional and processes that are associated with senescence (Lester, 2000; Pandey et al., 2000; Kumar and Rajam, 2004; Nambeesan et al., 2008). Apart from protecting membrane integrity, PAs also able to stabilize protoplasts or spheroplasts, mitochondria, lysosomes and subcellular elements such as plasma membranes and microsomes (Schuber, 1989).

PAs delay senescence effect on plant cells by interacting and stabilizing the proton-secreting system in oat protoplasts, thylakoid membranes and prevented chlorophyll degradation in senescing barley and oat leaves (Popovic et al., 1989; Schuber, 1989; Besford et al., 1993). The previous report showed that SPD bind to barley leaf discs prevented RNase activity, degradation of chlorophyll and protein of thylakoid membranes, thus inhibited the senescence process (Legocka and Zajchert, 1999). Supplied PAs in oat leaves reduced levels of malondialdehyde thus preventing lipid peroxidation activities (Borrell et al., 1997). Conjugated PAs interact with transglutaminases (TGase) and regulate senescence by affecting conformation and function of specific proteins (Griffin et al., 2002; Serafini-Fracassini et al., 2002; 2009).

2.12.4 Polyamines and their involvement in genes expression

PAs regulate translation by affecting protein synthesis through 'PA modulon'. The analysis of PA modulon described mechanisms that can regulate expression of certain genes. In modified *Escherichia coli* proved that 58 out of 309 genes were upregulated by PAs and these genes were responsible for a cellular process such as central intermediary metabolism, energy metabolism, iron and zinc transport mechanisms. On the other hand, PAs modulon influence translation by downregulating genes that associated with amino acid metabolism, cofactor biosynthesis, prosthetic groups and carriers (Yoshida et al., 2004; Igarashi and Kashiwagi, 2006).

Analysis of metabolite profiling using NMR spectroscopy from transgenic tomato overexpressed with SAMDC gene revealed the presence of higher SPD/SPM level during ripening. Overexpression of SAMDC gene in transgenic tomato lead to accumulation of metabolites used in protein biosynthesis such as glutamine, asparagine, choline, glutamic

acid, citrate, malate and fumarate compared to the wild type. On the other hand, metabolites such as valine, threonine, monosaccharides glucose and disaccharides sucrose exhibited the reverse pattern. Similar levels of leucine, phenylalanine, GABA and monosaccharides fructose were observed in both transgenic and wild type tomato (Mattoo et al., 2006; Mattoo and Handa, 2008).

The accumulation of these metabolites suggested that carbon metabolism and nitrogen sensing/signaling pathways are stimulated in transgenic fruit with low PUT but high SPD/SPM levels (Mattoo et al., 2006; Mattoo and Handa, 2008). Transcriptome analyses from over 1,000 transgenic tomato cDNAs indicated 25% genes were upregulated by which representing a wide range of anabolic pathways (Srivastava et al., 2007). Several phenotypes were improved in transgenic tomatoes such as higher ethylene emission and better juice attributes. These outcomes propose that significant changes in SPD/SPM levels not only can affect the transcriptional and translational level, but also functional biosynthesis pathways such as isoprenoid, carotenoids and flavonoid (Srivastava et al., 2007).

A similar observationn was reported in transgenic tomato with overexpressing the spermidine synthase gene (Md-SPDS1). Functional compounds like carotenoids were enhanced with lycopene being the highest (1.3 to 2.2- fold) accompanied with a marked shift in ethylene emission (1.2 to 1.6-fold) (Neily et al., 2011). The results revealed higher PAs accumulation in transgenic tomato caused a significant enhancement of lycopene content as well as downregulating genes involved in the lycopene degradation (Neily et al., 2011).

In *Arabidopsis thaliana* with overexpressed ADC, genes associated stress signaling pathway such as transcription factors involved in salt, cold and dehydration were suppressed while genes involved in gibberellin biosynthesis and pathogen response were upregulated (Alcazar et al., 2005). Further support for interactions between PAs regulation and gene expression is upregulation of transcription factor genes responsible for chilling tolerance in modified *Arabidopsis thaliana* overexpressed spermidine synthase gene (SPDSYN). In addition, other genes such as cytochrome P450, calmodulin-related proteins, protein kinases and peroxidase are also enhanced in transgenic *Arabidopsis* plant (Kasukabe et al., 2004).

2.13 Polyamine-ethylene nexus: an alternative strategy for fruit ripening modulation

The current postharvest biotechnology is exclusively emphasizing two main areas: delay of fruit ripening and suppression in fruit softening through downregulation of ethylene metabolism and alteration of cell membrane properties (Hamilton et al., 1990; Oeller et al., 1991; Lashbrook et al. 1998; Brummell et al., 1999; Wang et al., 2005; Xie et al., 2014). Notably, ethylene manipulation has been directed towards antisense technology of ACC synthase and ACC oxidase. Whereas, cell membrane metabolism alteration have been concentrated on suppressing PG and PE activities (Gray et al. 1992; Stearns and Glick, 2003; Bennett and Labavitch, 2008).

Tomato was the first plant model for transgenic approach with manipulated ethylene biosynthesis pathway (Hamilton et al., 1990; Oeller et al., 1991; Ayub et al., 1996). Antisense orientation of ACO gene led an effective shut-off of the rate of ethylene biosynthesis in fruits by 97%. Similarly, the expression of an antisense ACS gene resulted

in an inhibition by 99.5% of ethylene emission in fruits (Brummel and Harpster, 2001). These two pieces of evidence opened the way to other successful attempts at manipulation of ethylene production to achieve extended storage life, such as melon (Ayub et al., 1996; Senior, 1998). These ‘low ethylene’ transgenic plants developed an increase resistance to over ripening either attached to and off the vine.

This antisense technology approaches, however, equipped with several drawbacks and limitations. Despite the timing of the onset of ripening was unaffected, the technology faces shortcomings that transgenic melon fruits are short in shelf life following ethylene induced ripening as the wild types (Pandey et al., 2015). Later, transgenic ‘low ethylene’ plants produced fruit with longer shelf life, but resulted to be affected in the normal development of color, taste and aroma (Brummel and Harpster, 2001; Xiong et al., 2005; Pandey et al., 2015). Such fruits would have moderate ripening attributes; reddening of the fruit was reduced due to a clear reduction of carotenoid pigments, especially lycopene and mRNA encoding for phytoene synthase (Picton et al., 1993; Ayub et al., 1996; Senior, 1998).

The biotechnology of inhibited cell wall metabolism by targeting the dominant degradation enzymes i.e. PG and PE have been proposed as a superior alternative to that of the former as it hardly interferes with the ripening course. The antisense technology has shown that it is possible to ‘turn off’ the expression of existing genes in transgenic plants and this has effectively extended vine life of fruit, mechanical damage and reduced incidence of disease (Senior, 1998). Disadvantages of this approach that softening regulation is redundant by multigene families and the performance of enzymes are interrelated and interdependent (Gupta et al., 2013; Pandey et al., 2015).

However, there was a huge obstacle in accomplishing the objective of single gene manipulations since the existence of enzyme multiple isoforms. This occurrence can complement the suppressed isoform making antisense technology impossible (Xiong et al., 2005; Madhulatha et al., 2014). Thus, a newer insight has been introduced through developing an approach the suppression of more than one gene (Gupta et al., 2013).

From the perusal of literatures, major PAs biosynthesis genes, namely SAMDC, ODC, ADC, SPDSYN have been identified, isolated and cloned (Mehta et al., 2002; Kasukabe et al., 2004; Alcazar et al., 2005; Mattoo et al., 2006; Srivastava et al., 2007; Mattoo and Handa, 2008; Neily et al., 2011). Stable integration and expression of these genes have given strategy targeting biosynthetic pathway regulation and metabolic engineering flexibility (Madhulatha et al., 2014; Pandey et al., 2015).

PAs are implicated to have a more direct effect on the metabolic shift at the SAM flux. Being an antagonist to ripening and senescence, these molecules can divert the PA's metabolism resulted in the inhibition of ethylene biosynthetic enzymes directly. Therefore, the strategy of manipulation of PAs titers especially during ripening armed with specific promoter would result in downregulation of ethylene synthesis. This allowed ethylene actions to be contrasted which in turn suppress the fruit ripening and softening. In this regard, the strategy exerts benefit of permitting natural ripening to take place in a normal way without affecting the quality and nutritional attributes (Mehta et al., 2002; Srivastava et al., 2007).

Competitive interaction of PAs with that ethylene can be utilized to modify genes involved in cell wall degradation since PAs could also perform as a cellular signal in cross talk with ethylene pathway. Therefore, for delayed softening, perception and action

employing overexpression of PAs gene is a better option than chimeric genes designed to knockout softening enzyme mRNA since antisense inhibition suffers from unpredictable and variable results (Theologis, 1992; Hackett et al., 2000). Additionally, PAs are known to impart tolerance and protection against abiotic stress, such as chilling conditions (Gill and Tuteja, 2010; Hussain et al., 2011). Thus, PAs would negate negative effects physiological damages in cases of fruits and vegetables kept at low temperature during transportation and storage.

With a better understanding of the PAs role in modulating fruit ripening and senescence, it is possible that biotechnological strategies such as exploiting genes encoding for PAs along with that of the ethylene or cell wall degrading enzymes might offer greater importance. Indeed, the manipulations of polyamine-ethylene concerted action can be considered as a better alternative device for postharvest biotechnology after considering transgenes effect on the trait of interest and implications and their metabolic modulations (Kumar and Rajam, 2004).

CHAPTER THREE

GENERAL MATERIALS AND METHODS

3.1 Fruit materials

Carambola cultivar B10 at Index II (Appendix A) were purchased from packing houses (DRS Sdn Bhd) located in Kajang, Selangor state of Malaysia. Fruit of medium and uniform sized, the absence of mechanical defects and diseases were selected for this study. Fruit was delivered to the laboratory immediately after harvest, rinsed with distilled water for cleaning purpose and air dried at ambient temperature ($22\pm1^{\circ}\text{C}$) before PAs treatment.

3.2 Methods

3.2.1 Fruit preparation and treatment

Unless otherwise stated, all of the chemicals and reagents used in this study were analytical grades with the highest purity available and all solutions were prepared with distilled or deionized water. Polyamines (diamine putrescine, triamine spermidine and tetraamine spermine) and Tween 20® were purchased from Sigma-Aldrich Co., USA. Three different PAs concentration was selected for this study: 0.1, 1.0 and 2.0 mM (Chapter 4). For putrescine (PUT) application, the fruit was immersed in aqueous solutions of PUT in 10 liter storage box for 5 min with chosen concentrations. A similar routine was applied for spermidine (SPD) and spermine (SPM) treatment. Surfactant 0.2% Tween 20® (polyoxy ethylenesorbitan monolaurate polyethylene glycol) was added into PAs aqueous to enhance infiltration of the solutions. Control fruit was dipped in distilled water with 0.2% Tween-20 for 5 min.

Following the PAs treatment, the fruit was allowed to air-dry overnight, placed in cardboard boxes and stored at 3°C (± 0.5) with a relative humidity of 85-90%. The data were collected at the day of harvest (day 0), 7 day intervals for 4 weeks at cold storage followed by 3 day intervals for 6 days upon transferring to ambient ($22 \pm 1^\circ\text{C}$) for shelf life analysis. For tissue sampling, the whole fruit excluded the seeds was cut into small cubes ($\sim 1\text{cm}^3$), frozen in liquid nitrogen and kept at -80°C until needed for further analysis.

3.3 Statistical analysis

Experiments were arranged in completely randomized design (CRD) with three replications. The effects of treatments, storage period and their interactions on different parameters were subjected to analysis of variance (ANOVA). Mean separations were performed by Tukey test and differences at $p < 0.05$ were considered significant using statistical analysis Genstat (Version 16, VSN International Ltd, UK). Pearson correlation among selected parameters studied was calculated. The significance level was $*p < 0.05$ and $**p < 0.01$.

CHAPTER FOUR

PRELIMINARY STUDIES: SELECTION OF STORAGE TEMPERATURE AND POLYAMINES CONCENTRATION

4.1 Introduction

Most horticultural fruit of tropical and subtropical origin is susceptible to physiological injury when subjected to a temperature drop below the threshold temperature but above their freezing temperature (Wang, 1994a). Susceptibility to chilling injury (CI) in carambola is modulated by a number of factors, of which the most decisive components, including genotype, cultivar, maturity stage at the time of harvest and growing area (Ali et al., 2004b; Cecilia, 2008; Warren and Sargent, 2011). The threshold temperature for carambola is 5°C and storage between 5-10°C effectively counteracts respiration rate, moisture loss and maintaining fruit firmness (Ali et al., 2004b; Paull and Chen, 2004). However, there is a lack of information about the development of CI symptoms in carambola following cold exposures. Hence, a preliminary study was carried out to determine the chilling-inducing temperature in B10 carambola and to observe commonly occurring visual symptoms that translate into postharvest losses.

Numerous studies have been focused postharvest treatments designed to impede ripening course, alleviate CI and the symptoms resulting from this injury. Treatments that have shown promise include pre-treatments with plant growth regulators such as polyamines (PAs). However, exogenous application of PAs could be considered as chemical treatments and may cause harmful effects on horticultural fresh produce because of their potential toxicity (Valero et al., 2002). Beneficial effects of PAs treatment in tropical and subtropical fruit have been observed at fairly low concentrations in the range

of 0.1 to 10 mM (Valero and Serrano, 2010). However, the optimal PAs concentration may vary between fruit and among cultivar itself. Hence, another preliminary study was conducted to select PAs (PUT, SPD and SPM) concentration that not only preserves the keeping quality of carambola but also without phytotoxic effects on the treated fruit.

4.2 Materials and methods

4.2.1 Selection of storage temperature

Carambola cultivar B10 (Index II) was purchased from the local supplier and immediately transported to the laboratory (as described in sub-chapter 3.1). Fruit were rinsed with tap water and air-dried, divided randomly into four lots and placed in cardboard boxes and stored at four different temperatures: room temperature (RT) for until full yellow attainment, cold storage at 10°C, 5°C and 3°C for 28 days and transferred to RT on the following day for shelf life study. The experiment was arranged randomly with three replicates each consisting of 10 fruit (30 fruit per lots). Visual observations on colour changes, chilling injury symptoms and decay development were observed and recorded every day throughout cold storage and shelf life.

4.2.2 Polyamines concentration

Carambola cv. B10 were purchased and undergo a similar preparation as described in sub-section 4.2.1. The fruit was divided randomly into seven lots and dipped in aqueous solutions of polyamines (PUT, SPD and SPM) at different concentrations: 0.1, 1.0, 1.5, 2.0, 2.5 and 3.0 mM respectively and 0.2% Tween 20 as a surfactant for 5 min. Prior to dipping fruit were air-dried, placed in cardboard boxes and stored at chosen cold temperature. The fruit was kept for seven days at 22±1°C and visual inspections of phytotoxic effects were observed and recorded. The experiment was carried out randomly

with three replicates each consisting of 10 fruit (30 fruit per lots).

4.3 Results and discussion

4.3.1 Selection of storage temperature

Carambola at Index II was selected due to higher resistance to mechanical damage and postharvest decay which allowed shelf life analysis to be carried out. During storage at ambient ($22\pm 1^\circ\text{C}$), carambola with mature green maturity required 12 days to reach full yellow skin attainment and some of fruit achieved overripe stage (Figure 4.1). Overripe fruit showed the appearance of rib-edge browning and was prone to decay after 15 days.

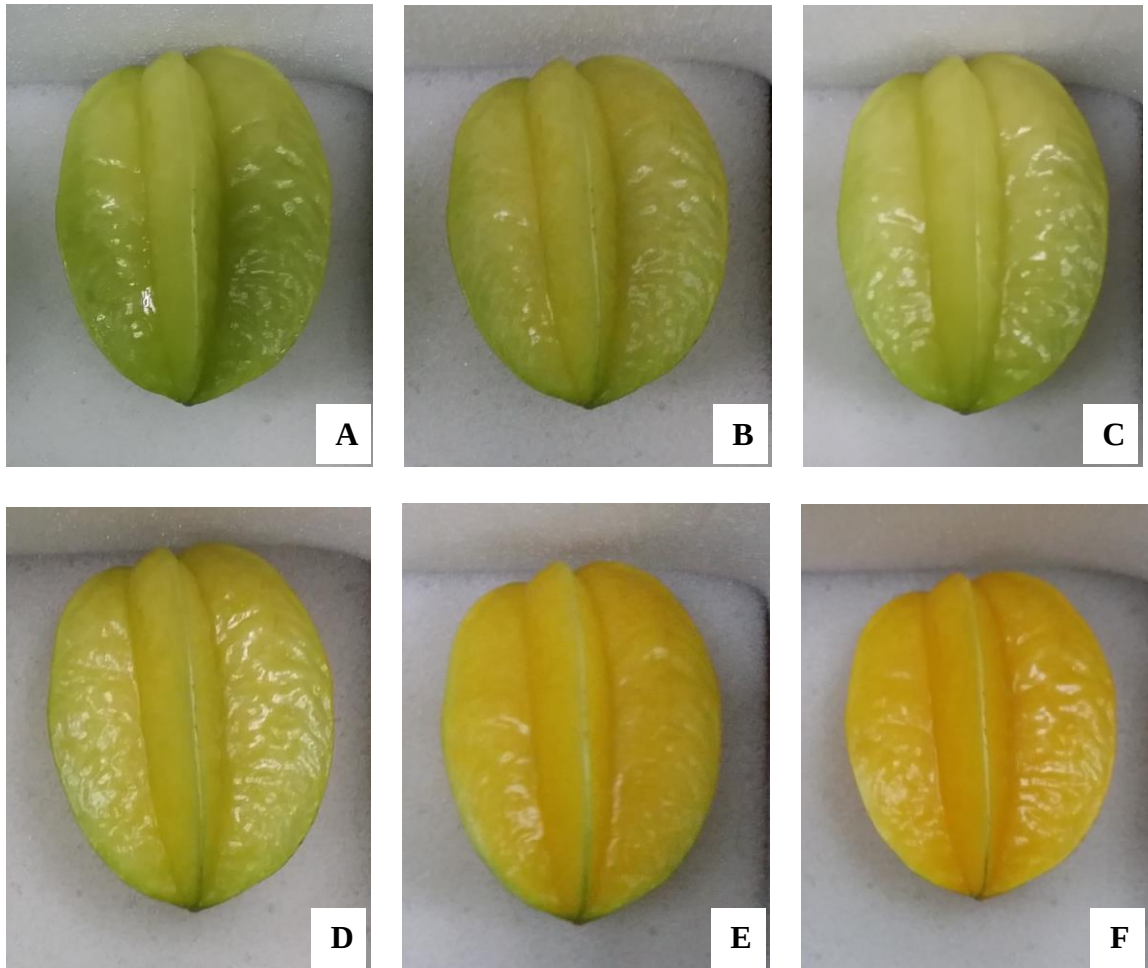


Figure 4.1: Colour changes in B10 carambola stored for 15 days at $22\pm 1^\circ\text{C}$. (A) At harvest (B) Day 3 (C) Day 6 (D) Day 9 (E) Day 12 (F) Day 15

At 10°C storage, no CI symptoms were observed and fruit maintained green colour throughout cold storage. The fruit was visually acceptable for presentation throughout 28 days at 10°C. However, carambola changes colour faster when fruit was exposed to higher temperatures (22±1°C). On day 1 at RT, fruit displayed several areas of sunken brownish discoloration and increases in number and size as storage advances. On day 3 at RT, development of slight decay in the sunken areas of the skin was observed and almost 50% of the fruit surface is affected by decay afterwards (day 6) (Figure 4.2). At 10°C, fruit colour changes from mature green to yellow, indicating a normal ripening pattern, yet the riper the fruit the more severe the decay. The outcome of this study revealed that CI symptoms are not common to occur at 10°C by which considered as the non-chilling temperature in carambola (Warren and Sargent, 2011).



Figure 4.2: Decay development in B10 carambola after stored at 10°C for 28 days followed by an additional 6 days at 22±1°C for shelf life. (A) Day 1 at RT (B) Day 3 at RT (C) Day 6 at RT

Carambola maintained visually acceptable for presentation and consumption during storage at 5°C throughout 28 days. By contrast, fruit deteriorated very quickly when fruit was transferred and held at 22±1°C and within 3-6 days fruit developed defects such as sunken brownish depression on the ribs and browning of the ribs edges. In addition, some

fruit displayed symptoms such as surface pitting, rib-edge browning and uneven ripening (Figure 4.3).



Figure 4.3: CI-like symptoms of the surface lesion and (A) skin pitting (B) browning of the ribs (C) uneven ripening in B10 carambola during shelf life at $22\pm1^{\circ}\text{C}$ for 6 days after storage at 5°C for 28 days.

Carambola exposed to 3°C maintained satisfactory visual quality for 21 days. Some fruit displayed minor defects on the peel such as round sunken lesion and subtle rib-edge browning becoming evident on day 28 of storage. Deterioration of fruit was hastened when transferred to $22\pm1^{\circ}\text{C}$. Postharvest quality of the fruit was reduced significantly by the appearance of CI such as severe surface pitting, shriveled and darkened ribs, dark skin depressions and failure to ripen (Figure 4.4). Hence, storage at 3°C was considered as chilling injury-inducing temperatures for B10 carambola and selected for further postharvest quality analysis.

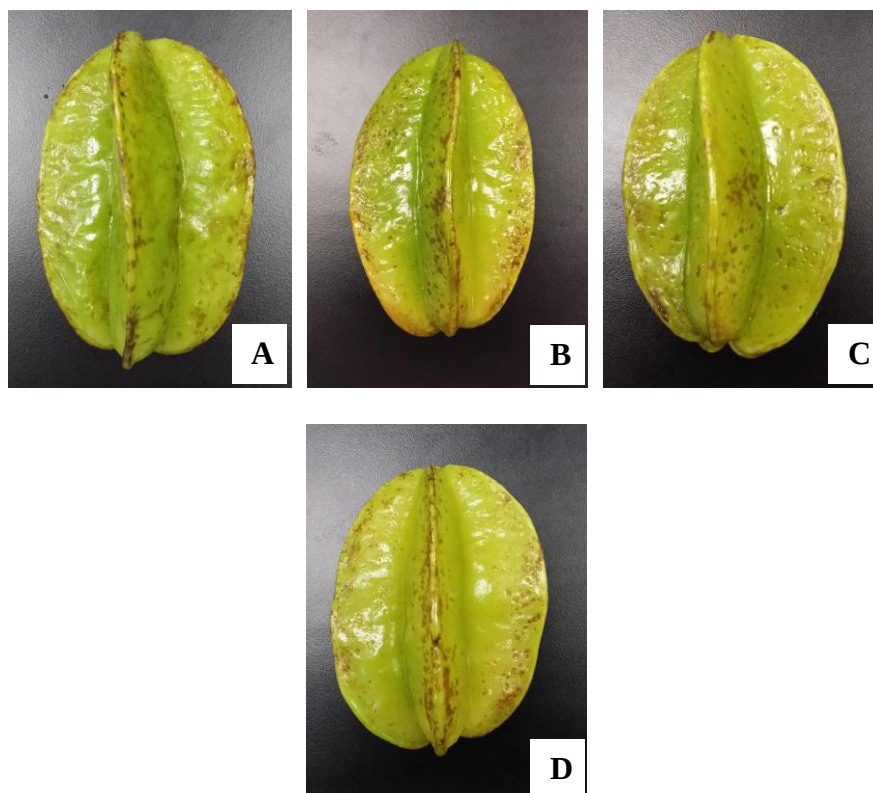


Figure 4.4: CI symptoms (A) surface pitting (B) severe surface pitting and browning of the ribs (C) severe surface pitting and skin depressions (D) failure to ripen and skin pitting in B10 carambola during shelf life at $22\pm1^{\circ}\text{C}$ for 6 days after storage at 3°C for 28 days

4.3.2 Selection of polyamines concentration

Fruit treated with various concentrations of PUT, SPD and SPM (0.1, 1.0, 1.5, 2.0, 2.5 and 3.0 mM respectively) retained acceptable presentation throughout 28 days at 3°C storage. However, carambola treated with 3.0 mM of PAs (PUT, SPD and SPM) experienced phytotoxic injury. The symptom showed up as dark brown spots on skin after several days (day 3-6) at $22\pm1^{\circ}\text{C}$ and the injury occurred all at once (Figure 4.5). Therefore, PAs concentrations at 0.1, 1.0 and 2.0 mM were selected for further postharvest quality analysis.

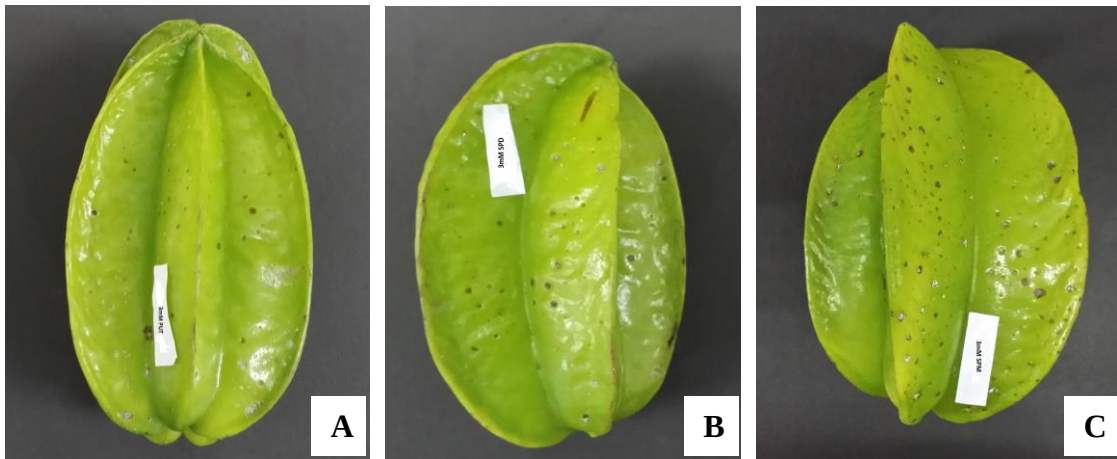


Figure 4.5: Phytotoxicity (dark brown spots on skin) on B10 carambola from PAs treatment (A) 3 mM PUT (B) 3 mM SPD (C) 3 mM SPM after several days at $22\pm1^{\circ}\text{C}$.

4.4 Conclusion

Postharvest storage at 3°C was chosen as the chilling-inducing temperature in carambola and the experiment for cold storage ended after 28 days due to appearance of chilling defects. In addition, PAs concentration of 0.1, 1.0 and 2.0 Mm possesses no phytotoxicity effects on the fruit.

CHAPTER FIVE

EFFECT OF POLYAMINES APPLICATION ON POSTHARVEST QUALITY AND SHELF LIFE OF CARAMBOLA

5.1 Introduction

Carambola (*Averrhoa carambola* L.) is categorized as slow-ripening fruit with relatively long storage life (Ali et al., 2004b). However, this unique tropical fruit is highly susceptible to postharvest losses due to the perishable nature of the fruit. Therefore, the cold storage is widely used to delay the acceleration of physical and biochemical that lead to major changes in ripening and decay development (Maul et al. 2011; Tietel et al. 2011). However, carambola held for prolonged cold storage elicits a set of physiological disorders manifested as chilling injury (CI) (Warren and Sargent, 2011). As for chilling-injured carambola, the most generated symptoms are surface pitting, shrivelled and darkened rib-edge, failure or uneven ripening, dark skin depressions and prone to decay (Chapter 4).

Several postharvest attempts have been carried out to preserve the shelf life during cold storage and minimize the incidence of CI in carambola, including modified atmosphere packaging (MAP) (Ali et al., 2004b), heat treatment (Hallman, 1991; Miller and McDonald, 1997) and coatings (Miller et al., 1993; Nurul Hanani et al., 2012). However, all of above treatments encountered issues or problems that took shape in loss of fruit quality, off flavour development and undesirable texture attributes.

Characterized by polycationic properties and capability to form ionic interactions and stabilize numerous biological macromolecules, PAs have been pictured as antisenescence agents (Carbonell and Blazquez, 2009; Shiri et al., 2013). Free forms of PAs have a profound effect on ethylene emission, reduce respiration, increase firmness, suppress

color changes, reduce mechanical damage and diminish chilling symptoms (Valero and Serrano, 2010).

To the best of our knowledge, there is no information available on the role of PAs as a postharvest application in carambola during cold storage and shelf life. In the present work, selected concentrations of PAs (0.1, 1.0 and 2.0 mM) was applied to mature green carambola by immersion for 5 min at room temperature ($22\pm1^{\circ}\text{C}$) and stored at 3°C . Physicochemical quality and chilling injury symptoms in carambola were evaluated during 4 weeks of cold storage and further shelf life at ambient for 6 days. Thus, the objective of this chapter was to examine the effects of PAs treatment on the postharvest quality and to unveil the PAs treatment effectiveness in alleviating chilling injury in carambola.

5.2 Materials and methods

Carambola was subjected to the treatments described in sub-section 3.2.1 and used for assessing the physicochemical quality prior PAs treatment during cold storage and shelf life at ambient. Three replicates each consisting of five fruit ($n=15$) were used to assess physicochemical quality. Meanwhile, chilling injury (CI) evaluation was carried out in three replicates, each consisting of ten fruit ($n=30$). Experiments were repeated to confirm the reproducibility of results and data were pooled across two independent experiments.

5.2.1 Weight loss

Fruit was weighted using a digital balance (Model GF-6100, A&D Co. Ltd., Japan) at day 0 (at harvest), 7, 14, 21 and 28 during cold storage and upon transferring to ambient for 6 days (day 3 and 6) to determine the weight loss due to respiration and transpiration during

storage. Weight losses were calculated as a percentage relative to the initial carambola weight.

5.2.2 Colour

Colour was determined using the Hunter lab colour difference meter (MiniScan XE Plus, Model: 45/0-5, USA). The colourimeter was first calibrated with standard black tile and white tile with values of $X=79.0$, $Y=83.9$, $Z=87.9$. The values of L^* , a^* , b^* were recorded and hue angle (h°) $\arctan(b^*/a^*)$ and chroma (C^*) $[(a^*)^2 + (b^*)^2]^{1/2}$ were calculated. The readings were measured on three specified locations along one of the fruit ribs.

5.2.3 Firmness

Fruit firmness was determined using an Instron universal testing machine (Instron 2519-104, Norwood, MA) equipped with an 8 mm plunger tip. The fruit was subjected to a puncture test at a constant speed of 20 mm^{-1} . The reading was expressed in N as the mean of three measurements recorded on each fruit on three specified locations along one of the fruit ribs.

5.2.4 Soluble solid contents (SSC)

SSC was determined using a Palett digital Refractometer (Model: PR-32 α ; Atago Co, Ltd. Japan) and calibrated against sucrose. The fruit juice was obtained by homogenizing 10 g of fruit pulp in a kitchen blender (Tefal, USA) with 40 ml of distilled water. The mixture was filtered through a cheese cloth and fruit juice was expressed in percentage.

5.2.5 Titratable acidity (TA)

Titrate acidity was determined by adding 2-3 drops of 0.1% (v/v) phenolphthalein solution to 5 ml of fruit juice and titrates against 0.1 N NaOH until the pH reached 8.1. The obtained results were expressed as the percentage of oxalic acids in carambola.

5.2.6 Gaseous exchange analysis

5.2.6.1 Respiration rate

Carbon dioxide (CO₂) and ethylene production were assessed by placing fruits in a plastic container which was in turn tightly seal with a lid. 1 ml of the head-space gas was withdrawn after 1 hour of incubation and CO₂ was quantified on a gas chromatograph (500 Clarus, Perkin Elmer, USA) fitted with a thermal conductivity detector (TCD) and a stainless-steel Porapak R column 80/100 mesh (Agilent, USA). The injector, oven and detector temperatures were 150, 60 and 200°C respectively. Helium was used as the carrier gas at a flow rate of 138 ml m⁻¹. 1 ml of CO₂ gas (1.0%) (Scotty gases, Beltifonte, PA, USA) was used as the external standard for calibration. The amount of CO₂ production was expressed in ml kg⁻¹ h⁻¹.

5.2.6.2 Ethylene production

To determine the ethylene production, 1 ml of gas samples were taken from the headspace and injected into a gas chromatograph equipped with a Carbonplot column (CAT 115-3133, Agilent, USA) and a flame ionization detector (FID). The injector, oven and detector temperatures were 200, 120 and 250°C respectively with nitrogen as the carrier gas at a flow rate of 10 psi. 1 ml of ethylene gas (10 ppm) (Scotty gases, Beltifonte, PA, USA) was used as an external gas standard was injected for calibration. The amount of ethylene was expressed in µl kg⁻¹ h⁻¹.

5.2.7 Chilling injury (CI) evaluation

CI was evaluated on the 7 days interval for 4 weeks during cold periods and followed by 3 days interval for 6 days upon transferring the fruit to ambient (22±1°C). The severity of symptoms was evaluated visually in a three-stage scale: 0=no CI symptoms; 1= CI symptoms covering <25% of the fruit surface; 2= CI symptoms covering <50% but >25% of surface; 3= CI symptoms covering >50% of the surface. The CI index was calculated using the following formula:

$$\text{CI Index} = \frac{\sum [(\text{CI scale}) \times (\text{number of fruit at the CI scale})]}{(\text{Total number of fruit})}$$

5.2.8 Sensory evaluation

Sensory evaluations of carambola on the final day of shelf life were rated by 20 untrained member panels. The sensory qualities were carried out using a hedonic scale of 1-9 (Appendix B). Scores of 5 and above were considered to be acceptable for commercial purpose. Control fruit and three PAs treatment (2.0PUT, 1.0SPD and 0.1SPM) were selected based on postharvest qualities assessment. 20 fruit for each treatment were used for this study.

5.3 Results

5.3.1 Weight loss

Weight loss was significantly affected by PAs treatment since control fruit experienced higher losses of weight throughout storage at 3°C and shelf life for 6 days at ambient (Figure 5.1). In addition, there were significant differences ($p < 0.001$) in weight loss found among PAs treatment and in all cases, weight loss increased ($p < 0.001$) significantly with storage time. Control fruit lost 10% of their initial weight at the end of 28 days at cold storage, while reverse trend was observed in PAs-treated fruit. Cumulative weight loss was reduced in PUT-treated fruit (7.2%) followed by SPM (8.3%) and SPD (9.4%).

The maximum physiological weight loss occurred at the end of experiment with cumulative weight loss was 20% in control fruit. While, the percentage was lower in SPD-treated fruit (17%), SPM (14%) and in PUT was even less (13%). With regard to different doses, highest PUT concentration (2.0 mM) demonstrated higher efficacy than lower concentrations (1.0 and 0.1 mM).

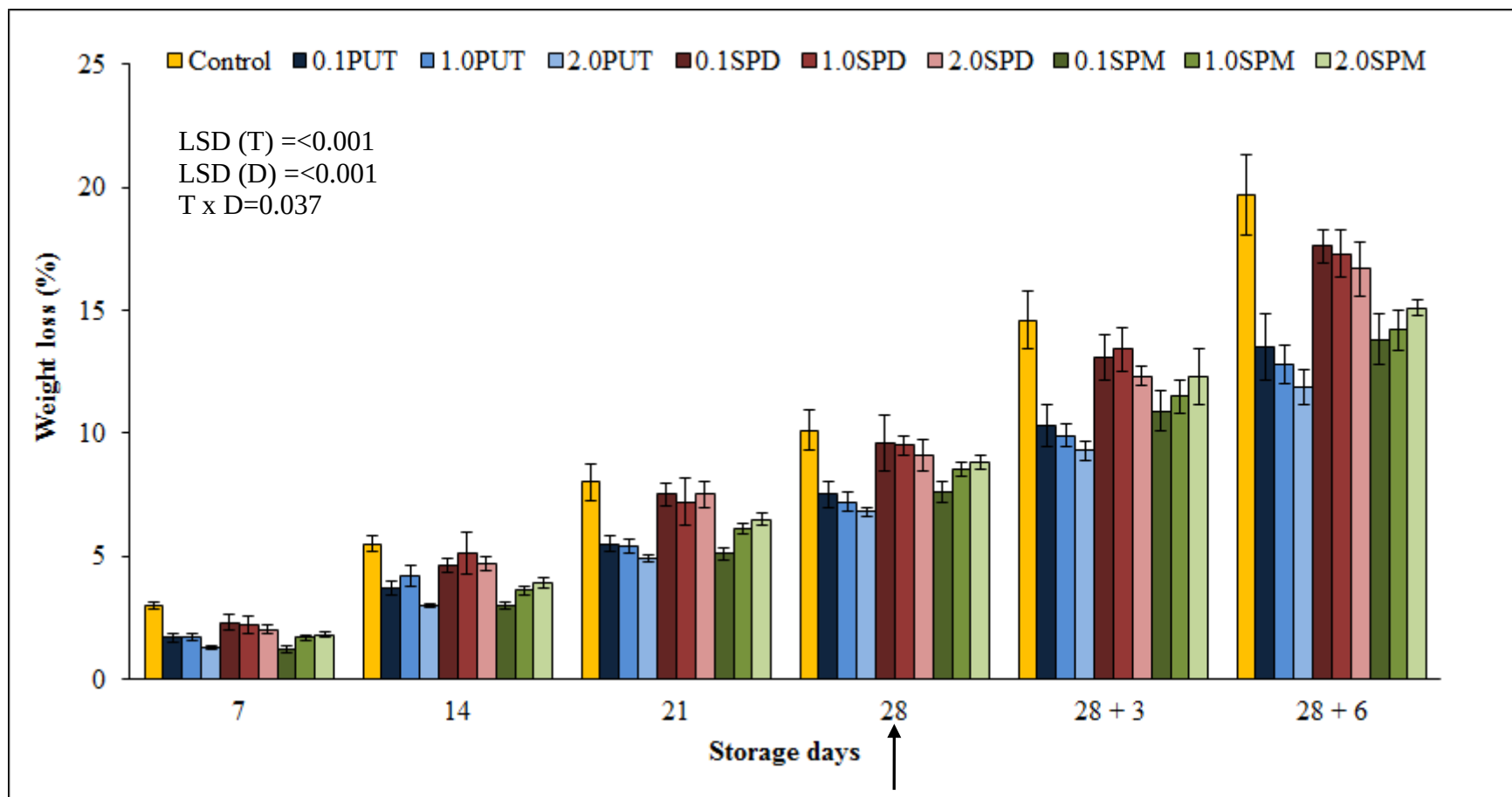


Figure 5.1: Effect of PUT, SPD and SPM treatment on weight loss (%) of B10 carambola during cold storage (3°C) for 28d followed by 6d at 22±1°C (shelf life). Vertical bars represent ±SE of pooled mean from two independent experiments. One experiment: n=15. An arrow (↑) indicates fruit was transferred from cold storage to ambient conditions for shelf life study. T=treatments, D=days, treatments x days = 0.037

5.3.2 Colour

At the optimum harvest stage, carambola is characterized by a dark green skin colour, with CIE lab parameters of 22.56 ± 1.2 (L^*), 16.24 ± 0.33 (C^*) and 118.07 ± 0.53 (h°). Throughout the experiment, lightness (L^*) and saturation(C^*) exhibited an increasing trend up to 28 days at 3°C and the values increased progressively after transferred to ambient for 6 days regardless the treatment. However, a significant delayed in lightness (Table 4.1) and saturation (Table 4.2) enhancement were observed in PA-treated fruit. Amongst concentrations used 2.0PUT, 1.0SPD and 1.0SPM being the most effective in suppressing the increment of lightness (L^*) and saturation(C^*).

Meanwhile, hue angle (h°) dropped progressively throughout storage irrespective of treatment. A reduced in hue angle was observed in control during 3°C storage and sharp decline occurred after fruit transferred to ambient (Table 4.3). Significant reduction in hue angle was slowed down in PAs-treated fruit particularly in 2.0PUT, 1.0SPD and 1.0SPM throughout cold storage. However, the hue angle declined progressively during shelf life at ambient even for fruit treated with the highest concentration of PUT.

Table 5.1: Effect of PUT, SPD and SPM treatment on luminosity (L^*) of B10 carambola during chilling storage (3°C) for 28d followed by 6d at 22±1°C (shelf life). Data are ±SE of pooled mean from two independent experiments. One experiment: n=15. Means in a column with the same letter are not significantly different ($p<0.05$) between treatments according to LSD. An arrow (↓) indicates fruit was transferred from cold storage to ambient conditions for shelf life study.

L^*	Conc. (mM)	Storage period (days)						
		At harvest	7	14	21	28	28 + 3	28 + 6
Control	0	22.56±1.2	30.00±1.32 a	32.06±0.67 a	32.19±1.48 a	33.24±0.62 a	37.01±0.98 a	39.82±1.16 a
PUT	0.1		26.63±1 ab	27.91±0.75 b	29.33±0.47 abc	30.78±0.31 ab	31.92±0.46 b	34.52±0.67 bcde
	1.0		26.50±0.7 ab	27.85±1.01 b	28.94±0.58 abc	30.75±0.87 ab	31.49±0.64 b	34.09±0.41 cde
	2.0		24.41±1.67 b	25.39±1.27 b	25.48±0.86 c	27.60±1.04 b	30.61±1.55 b	32.31±0.87 e
SPD	0.1		28.08±0.58 ab	28.97±0.54 ab	30.55±1.47 ab	31.36±2.41 ab	34.15±0.94 ab	38.34±1.41 ab
	1.0		25.66±0.56 b	26.65±0.5 b	28.04±0.38 bc	29.4±0.6 ab	31.09±0.66 b	32.28±0.6 e
	2.0		26.95±0.41 ab	28.19±1.39 ab	29.48±0.46 abc	31.11±0.66 ab	32.13±0.67 b	35.61±0.71 bcde
SPM	0.1		27.45±0.39 ab	28.88±0.24 ab	30.12±0.57 ab	31.20±0.9 ab	33.95±1.13 ab	36.88±0.58 abcd
	1.0		26.39±0.93 ab	27.8±0.74 b	28.36±0.54 abc	30.28±0.49 ab	31.43±0.71 b	32.95±0.96 de
	2.0		28.39±0.09 ab	29.09±0.51 ab	31.04±1.02 ab	32.70±0.38 a	36.90±0.59 a	37.88±0.65 abc
LSD ($p<0.05$)			0.006	<0.001	<0.001	0.02	<0.001	<0.001

Table 5.2: Effect of PUT, SPD and SPM treatment on saturation (C^*) of B10 carambola during chilling storage (3°C) for 28d followed by 6d at $22\pm 1^{\circ}\text{C}$ (shelf life). Data are $\pm\text{SE}$ of pooled mean from two independent experiments. One experiment: $n=15$. Means in a column with the same letter are not significantly different ($p<0.05$) between treatments according to LSD. An arrow ($\downarrow$) indicates fruit was transferred from cold storage to ambient conditions for shelf life study.

C^*	Conc. (mM)	Storage period (days)						
		At harvest	7	14	21	28	28 + 3	28 + 6
Control	0	16.24 $\pm$ 0.33	21.55 $\pm$ 0.5 a	21.56 $\pm$ 0.8 a	22.66 $\pm$ 0.66 a	26.06 $\pm$ 1.62 a	26.48 $\pm$ 0.9 a	29.35 $\pm$ 0.82 a
PUT	0.1		20.17 $\pm$ 0.58 ab	20.76 $\pm$ 0.44 ab	21.05 $\pm$ 0.44 abc	21.87 $\pm$ 0.44 b	22.82 $\pm$ 0.67 abc	24.28 $\pm$ 1.5 abc
	1.0		21.03 $\pm$ 0.58 a	20.34 $\pm$ 0.55 ab	20.94 $\pm$ 0.38 abc	21.52 $\pm$ 1.06 b	22.54 $\pm$ 0.73 abc	23.93 $\pm$ 1.92 bc
	2.0		18.37 $\pm$ 0.52 b	18.97 $\pm$ 0.63 b	19.63 $\pm$ 0.35 c	20.48 $\pm$ 0.47 b	20.75 $\pm$ 1.15 c	21.77 $\pm$ 0.8 c
SPD	0.1		21.4 $\pm$ 0.64 a	21.51 $\pm$ 0.55 ab	21.97 $\pm$ 0.25 ab	22.72 $\pm$ 1.31 ab	24.11 $\pm$ 0.59 abc	26.29 $\pm$ 1.17 abc
	1.0		18.48 $\pm$ 0.36 b	19.50 $\pm$ 0.3 ab	20.16 $\pm$ 0.26 bc	20.59 $\pm$ 0.6 b	21.56 $\pm$ 0.65 bc	22.84 $\pm$ 0.31 bc
	2.0		20.23 $\pm$ 0.26 ab	20.93 $\pm$ 0.74 ab	21.62 $\pm$ 0.54 abc	21.99 $\pm$ 0.76 ab	23.38 $\pm$ 1.06 abc	24.37 $\pm$ 0.76 abc
SPM	0.1		20.90 $\pm$ 0.32 a	21.27 $\pm$ 0.51 ab	21.80 $\pm$ 0.7 abc	22.33 $\pm$ 0.65 ab	23.72 $\pm$ 0.89 abc	25.21 $\pm$ 0.84 abc
	1.0		19.31 $\pm$ 0.32 ab	19.76 $\pm$ 0.45 ab	20.45 $\pm$ 0.41 abc	20.81 $\pm$ 0.47 b	22.01 $\pm$ 0.69bc	23.13 $\pm$ 1.1 bc
	2.0		21.54 $\pm$ 0.74 a	21.56 $\pm$ 0.28 a	22.16 $\pm$ 0.59 ab	24.06 $\pm$ 0.42 ab	25.57 $\pm$ 1.16 ab	27.27 $\pm$ 0.9 ab
LSD ($p<0.05$)			<0.001	0.008	<0.001	<0.001	<0.001	<0.001

Table 5.3: Effect of PUT, SPD and SPM treatment on hue angle (h°) of B10 carambola during chilling storage (3°C) for 28d followed by 6d at 22±1°C (shelf life). Data are ±SE of pooled mean from two independent experiments. One experiment: n=15. Means in a column with the same letter are not significantly different (p<0.05) between treatments according to LSD. An arrow (↓) indicates fruit was transferred from cold storage to ambient conditions for shelf life study.

h°	Conc. (mM)	Storage period (days)						
		At harvest	7	14	21	28	28 + 3	28 + 6
Control	0	118.07±0.53	112.86±0.66 c	110.52±0.89 d	108.59±0.83 c	95.48±1.99 e	85.82±2.14 d	78.25±3.11 c
PUT	0.1		115.45±0.67 ab	114.57±0.29 abc	114.46±0.45 a	112.94±0.43 ab	96.79±0.83 bc	85.59±1.04 abc
	1.0		115.52±0.38 ab	115.23±0.39 abc	114.54±0.88 a	113.16±0.47 ab	111.16±0.71 a	88.55±0.84 ab
	2.0		116.74±0.23 a	116.16±0.87 a	115.81±0.5 a	115.65±0.38 a	102.30±0.81 b	90.49±2.01 a
SPD	0.1		114.77±0.6 abc	113.39±0.55 bc	111.21±0.51 bc	105.96±2.15 cd	89.75±1.16 d	81.20±1.69 bc
	1.0		116.21±0.34 ab	115.93±0.8 ab	115.75±0.32 a	115.40±0.58 a	102.26±0.61 b	88.69±2.22 ab
	2.0		115.34±0.43 abc	114.47±0.35 abc	114.28±0.59 a	111.52±0.94 ab	91.67±1.72 c	84.00±1.54 abc
SPM	0.1		115.13±0.5 abc	114.30±0.39 abc	113.71±0.67 ab	109.52±0.57 bc	89.87±1.8 d	82.81±1.22abc
	1.0		115.94±0.84 ab	115.30±0.33 ab	114.66±0.41 a	114.51±0.46 ab	101.62±1.05 b	88.58±2.43 ab
	2.0		114.04±0.51 bc	112.61±0.49 abc	114.24±0.54 a	102.81±1.02 d	86.58±1.53 d	78.50±2.28 c
LSD (p<0.05)			<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

5.3.3 Firmness

Carambola firmness declined during storage both in PAs-treated and control fruit (Figure 5.2). However, the firmness loss was significantly ($p < 0.001$) reduced by PA treatment and in all cases, loss of firmness increased ($p < 0.001$) with storage time. The rate of reduction was different among three PAs and between concentrations examined. Fruit dipped in the highest concentration of PUT (2.0 mM) maintained a significantly higher firmness than with lower concentrations (0.1 and 1.0 mM), but a dissimilar pattern was showed by SPD and SPM. Lower doses of SPD, SPM at 1.0 and 0.1 mM respectively maintained comparatively higher firmness than 2.0 mM.

Carambola treated with 2.0PUT exhibited 2.4-fold higher firmness followed by 1.0SPD (2.2-fold) and 0.1SPM (2.0-fold) as compared to control at the end of the experiment (Figure 5.2). However, there was no significant difference observed between 2.0PUT, 1.0SPD and 0.1SPM. Nevertheless, fruit treated with SPD and SPM displayed a rapid firmness reduction in comparison to fruit treated with PUT at the end of the experiment.

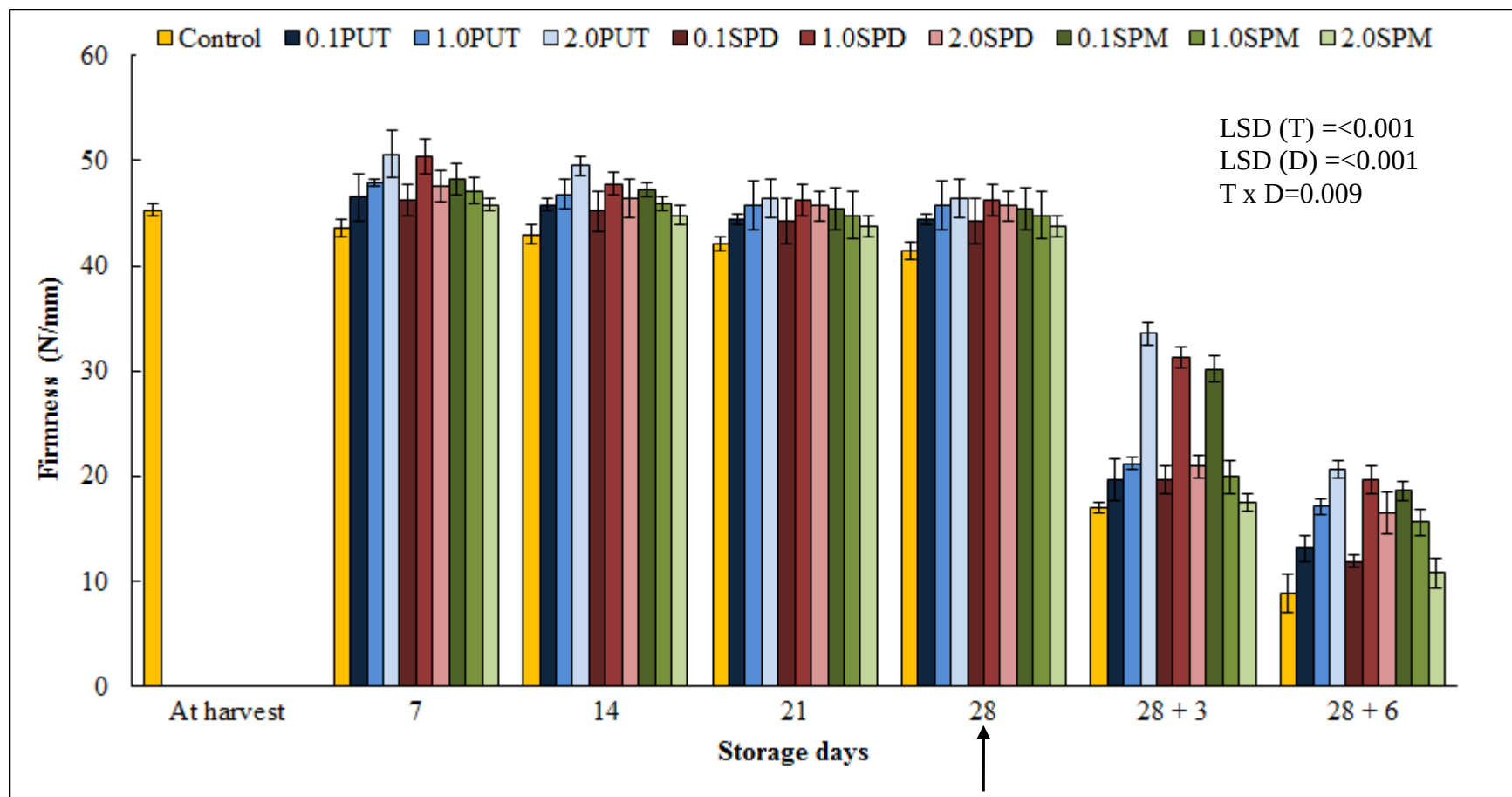


Figure 5.2: Effect of PUT, SPD and SPM treatment on firmness (N) of B10 carambola during cold storage (3°C) for 28d followed by 6d at 22±1°C (shelf life). Vertical bars represent ±SE of pooled mean from two independent experiments. One experiment: n=15. An arrow (↑) indicates fruit was transferred from cold storage to ambient conditions for shelf life study. T=treatments, D=days, treatments x days = 0.009

5.3.4 Soluble solid contents (SSC)

SSC was significantly affected by PAs treatment. SSC exhibited a gradual increase trend throughout the storage, with control fruit showed a greater increase in contrast to fruit treated with PAs. Irrespective treatments, SSC remained constant for 21 days during cold storage and soon rose tremendously from day 28 onwards and during ambient condition (Figure 5.3). The linear increase of SSC in control fruit was 1.7 to 2.2-fold while in all SPM-treated fruit was 1.5 to 2.2-fold than at harvest. Parallel to SPM, SPD with all three doses also showed increasing rate in SSC (1.6 to 2.2-fold) during the same period and the increase was comparable to SPM.

On the other hand, these changes were significantly lower in PUT-treated fruit. At this point, SSC value was 1.5 to 2.1-fold higher than at harvest with 2.0PUT being the most effective (Figure 5.3). In addition, there were significance differences ($p < 0.001$) in SSC found among PAs treatment and in all cases, SSC increased ($p < 0.001$) with storage time. At the end of the experiment, the effect exerted by PAs did not show SSC reduction and remained higher irrespective the treatments.

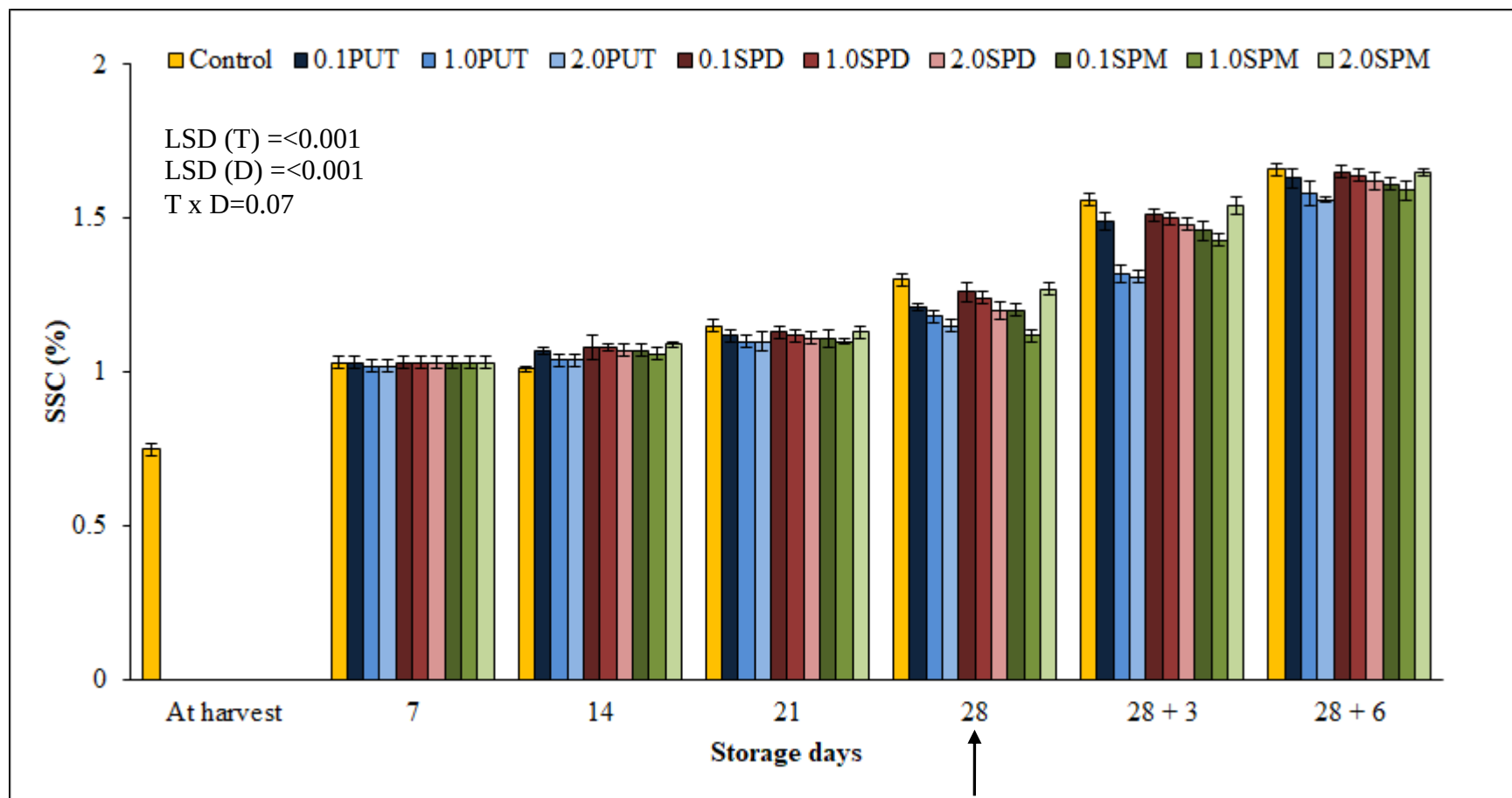


Figure 5.3: Effect of PUT, SPD and SPM treatment on soluble solids content (%) of B10 carambola during chilling storage (3°C) for 28d followed by 6d at 22±1°C (shelf life).). Vertical bars represent ±SE of pooled mean from two independent experiments. One experiment: n=15. An arrow (↓) indicates fruit was transferred from cold storage to ambient conditions for shelf life study. T=treatments, D=days, treatments x days = NS (p>0.05); NS = not significant

5.3.5 Titrable acidity (TA)

Regardless the treatments, TA exhibited reduction on day 7 and onwards than at harvest. Control fruit demonstrated the greatest reduction in TA throughout the storage at 3°C and became prominent during shelf life (Figure 5.4). However, a slight increase was detected on day 28. Among the three PAs, PUT retained a higher level of TA in comparison to SPD and SPM, and among the concentrations, 2.0PUT, 1.0PUT and 1.0SPM maintained higher TA in comparison to other doses (Figure 5.4). Moreover, PUT concentrations at 2.0 and 1.0 mM were equally effective in inhibiting the rate of TA reduction although there was no significance difference between two concentrations. PAs significantly delayed the diminution of TA ($p < 0.001$) over the control up throughout cold storage and TAs levels were declined ($p < 0.001$) with advancement pf storage time irrespective the treatment.

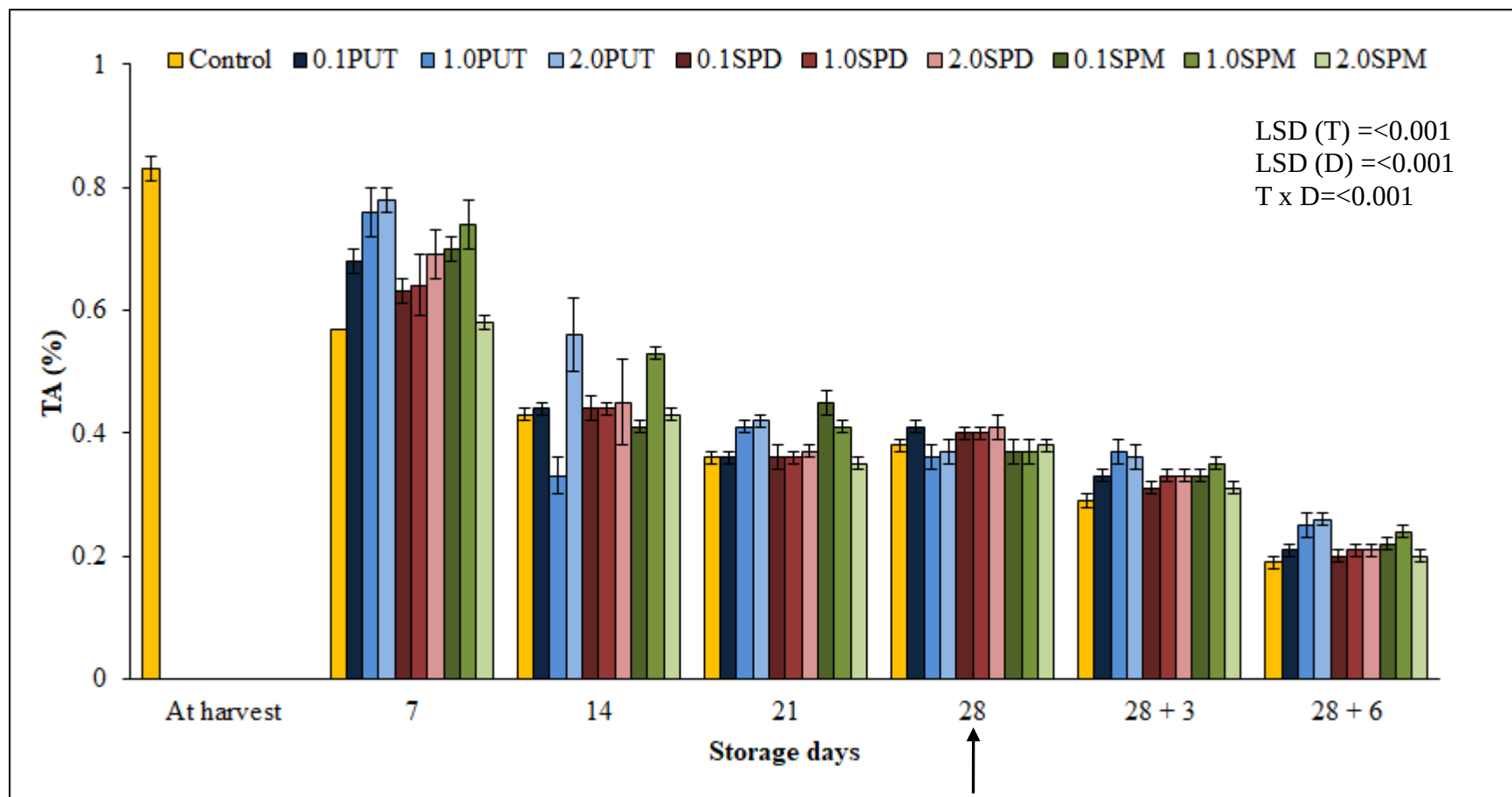


Table 5.4: Effect of PUT, SPD and SPM treatment on titrable acidity (%) of B10 carambola during chilling storage (3°C) for 28d followed by 6d at 22±1°C (shelf life). Vertical bars represent ±SE of pooled mean from two independent experiments. One experiment: n=15. An arrow (↓) indicates fruit was transferred from cold storage to ambient conditions for shelf life study. T=treatments, D=days, treatments x days = <0.001

5.3.6 Respiration rate

The rate of CO₂ production in carambola ranged from 0.54 to 45 ml kg⁻¹ hr⁻¹ and was significantly lower in PAs-treated fruit (Figure 5.5). The CO₂ level was detectable as soon as after harvest time point. Irrespective the treatment, similar patterns in respiration rate were observed throughout the cold storage. A considerable increase in the respiration rate was observed on day 7 and followed by a gradual decrease afterwards until day 21 at 3°C. An advanced increase of CO₂ production was observed on the final day at cold storage and respiration level rose upon transferring to ambient. The CO₂ level in control fruit showed 38.3 fold higher rate of CO₂ production than at harvest (0.54 to 20.7 ml kg⁻¹ hr⁻¹) and later increased greatly with 84 fold higher at the end of the experiment (0.54 to 45.4 ml kg⁻¹ hr⁻¹) (Figure 5.5).

On the contrary, fruit treated with PAs significantly ($p<0.001$) reduced the respiration rate with PUT application presented the lowest respiration rate and highest dose of PUT (2.0 mM) being the most effective. After 28 days at 3°C, 2.0PUT markedly suppressed respiration rate by 25 fold higher than at harvest (0.54 to 13.4 ml kg⁻¹ hr⁻¹) and delayed the increment of the CO₂ level by 56 fold higher during shelf life at ambient (0.54 to 30.1 ml kg⁻¹ hr⁻¹). In addition, lower doses of PUT and SPM (1.0 mM) were also found to be effective in suppressing the level of CO₂ production in carambola although there was no significant difference between the two doses throughout storage.

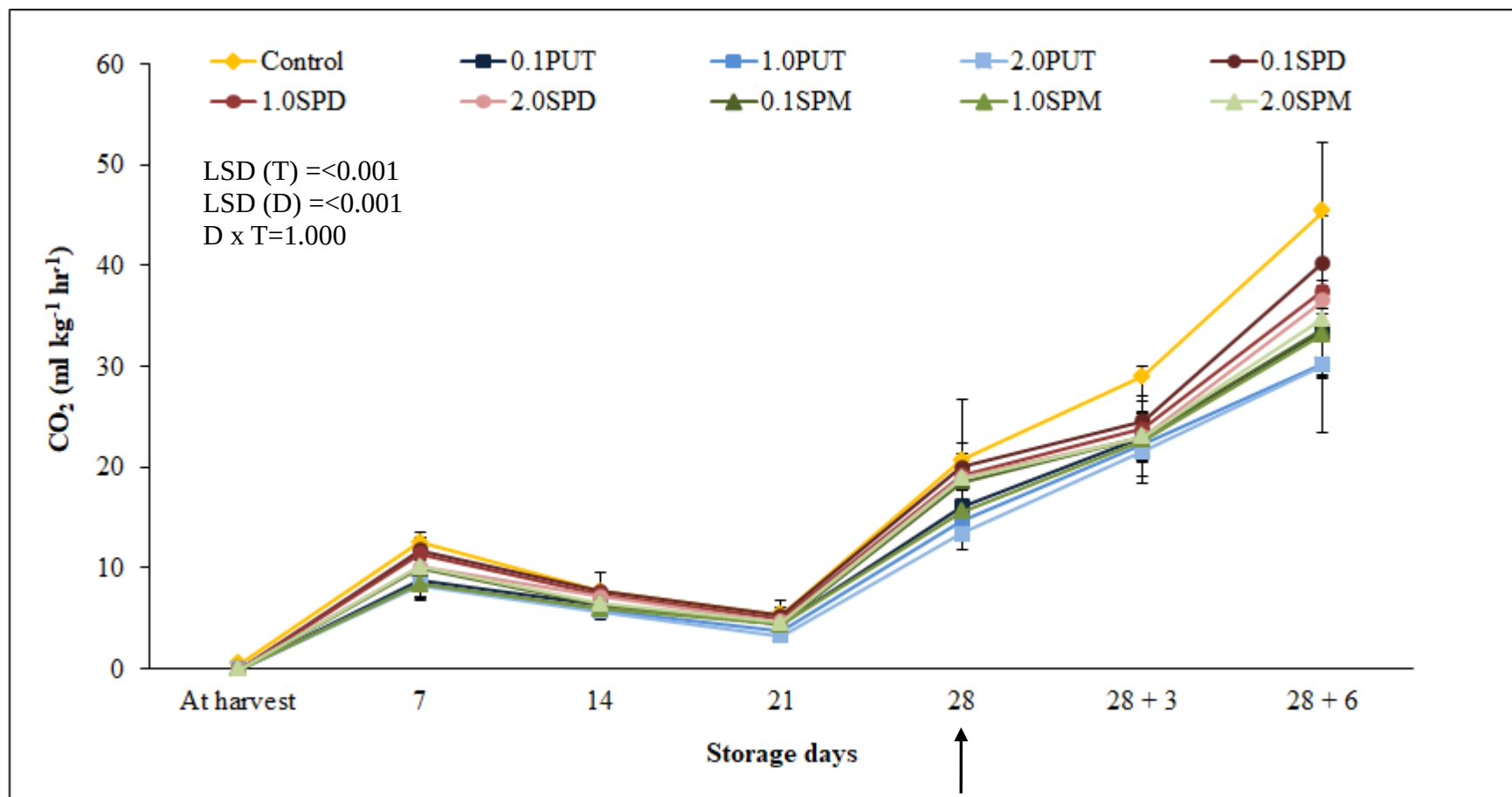


Figure 5.5: Effect of PUT, SPD and SPM treatment on the respiration rate of B10 carambola during cold storage (3°C) for 28d followed by 6d at 22±1°C (shelf life). Vertical bars represent ±SE of pooled mean from two independent experiments. One experiment: n=15. An arrow (↑) indicates fruit was transferred from cold storage to ambient conditions for shelf life study. T=treatments, D=days, treatments x days = NS (p>0.05); NS = not significant

5.3.7 Ethylene production

Ethylene production in control fruit was noticeable and showed an increasing pattern until the end of storage with values about $0.07 \mu\text{l kg}^{-1} \text{hr}^{-1}$ to $2.92 \mu\text{l kg}^{-1} \text{hr}^{-1}$ (Figure 5.6). On the other hand, all PAs-treated fruit began to emit ethylene at day 7 and at much lower rate than control. This increase was more relevant on day 21 at 3°C storage and a considerable increase in the ethylene production was observed when fruit was transferred to ambient. Postharvest application of PAs demonstrated a delay in the ethylene production with highest concentrations of PUT, SPM (2.0 mM) and 1.0PUT were the most effective. Throughout cold storage, ethylene emission in 2.0PUT, 2.0SPM and 1.0PUT were strongly reduced reaching levels of 0.47, 0.5 and $0.52 \mu\text{l kg}^{-1} \text{hr}^{-1}$ respectively as compared to control fruit ($0.78 \mu\text{l kg}^{-1} \text{hr}^{-1}$) (Figure 5.6).

The increment of ethylene production in 2.0PUT, 2.0SPM and 1.0PUT were higher with values of 2.32, 2.37 and $2.39 \mu\text{l kg}^{-1} \text{hr}^{-1}$ respectively on the final day at ambient. However, there was no significance difference between three doses. The ethylene evolution in carambola was produced in a small amount ($<3 \mu\text{l kg}^{-1} \text{hr}^{-1}$) following the non-climacteric ripening pattern. A sharp peak of ethylene on the final day of shelf life may have been a consequence of the presence of microbial activity due to over ripening.

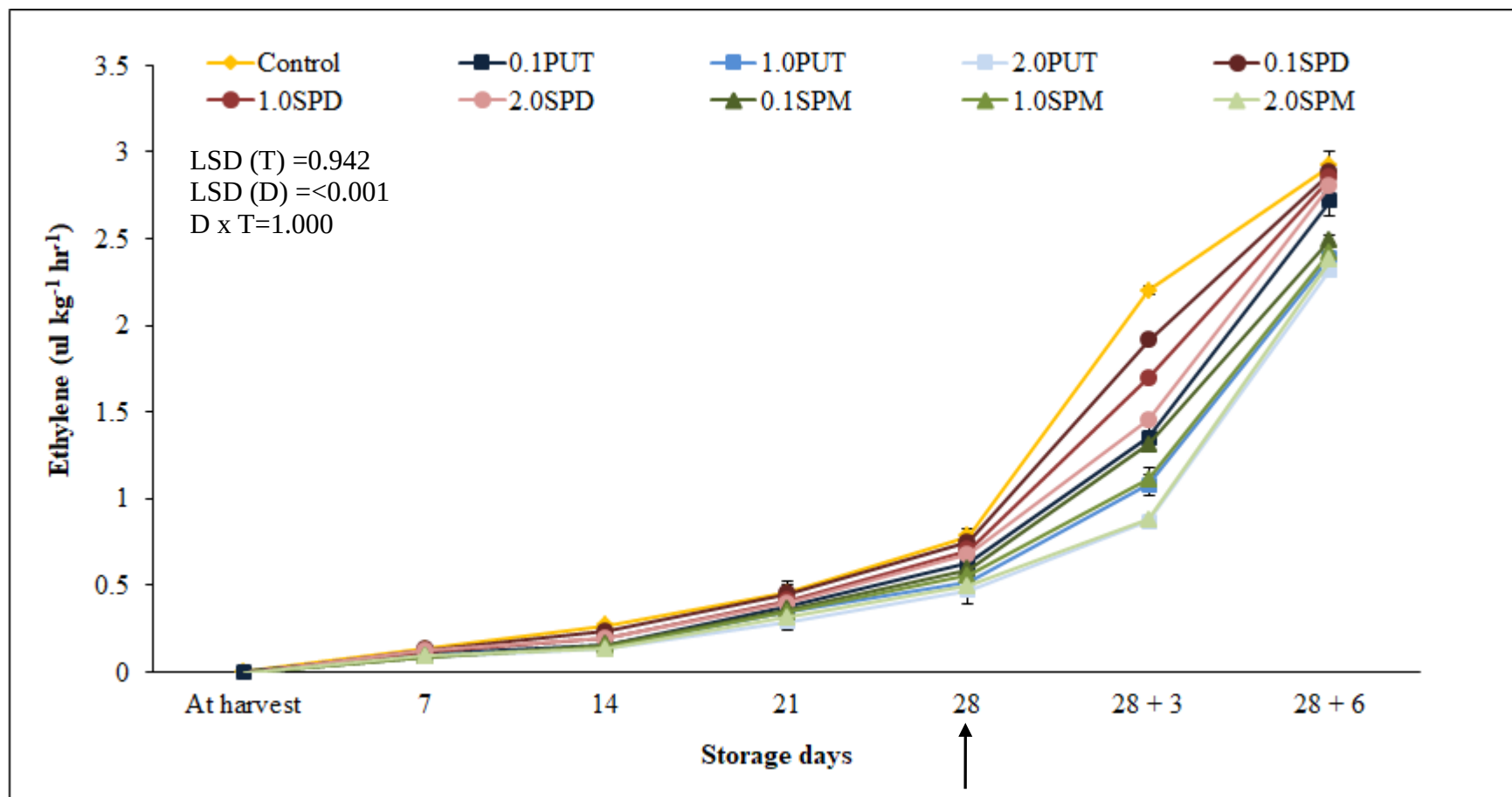


Figure 5.6: Effect of PUT, SPD and SPM treatment on ethylene production of B10 carambola during cold storage (3°C) for 28d followed by 6d at 22±1°C (shelf life). Vertical bars represent ±SE of pooled mean from two independent experiments. One experiment: n=15. An arrow (↑) indicates fruit was transferred from cold storage to ambient conditions for shelf life study. T=treatments, D=days, treatments x days = NS (p>0.05); NS = not significant

5.3.8 Chilling injury (CI) evaluation

Overall, PAs treated carambola stored at 3°C maintained acceptable visual quality throughout cold storage. All fruit appeared normal and no visible symptoms of CI were observed in control and PAs-treated fruit up until 14 days of storage. However in control fruit, the minor defects such as round sunken lesion and subtle rib-edge browning manifested on day 21 and onwards during cold storage. On the other hand, CI symptoms in PAs-treated fruit becoming evident on day 28 except fruit dipped in 2.0PUT and 1.0SPD. Carambola kept at 3°C for 28 days demonstrated severe surface pitting and peel browning and differences in CI severity became more apparent upon transferring to ambient. As storage progressed, the symptoms severity increased significantly ($p < 0.001$) (Figure 5.7).

However, the CI indexes were significantly affected ($p < 0.001$) by PAs treatment, suggesting that CI symptoms onset and development were inhibited or diminished by PAs. Among the three PAs studied, PUT displayed significantly the lowest level of CI index with 2.0PUT the most effective; CI value was 1.73 than that in control which surpassed 2.5 at the end of shelf life (Figure 5.7). Similarly to firmness loss, fruit which received the 1.0SPD and 0.1SPM doses exhibited lower CI index than the control on day 28 and during shelf life though there was no significance difference between two doses. At the end of the experiment, the CI indexes of 1.0SPD and 0.1SPM fruit were 2.32 and 2.4 respectively (Figure 5.7).

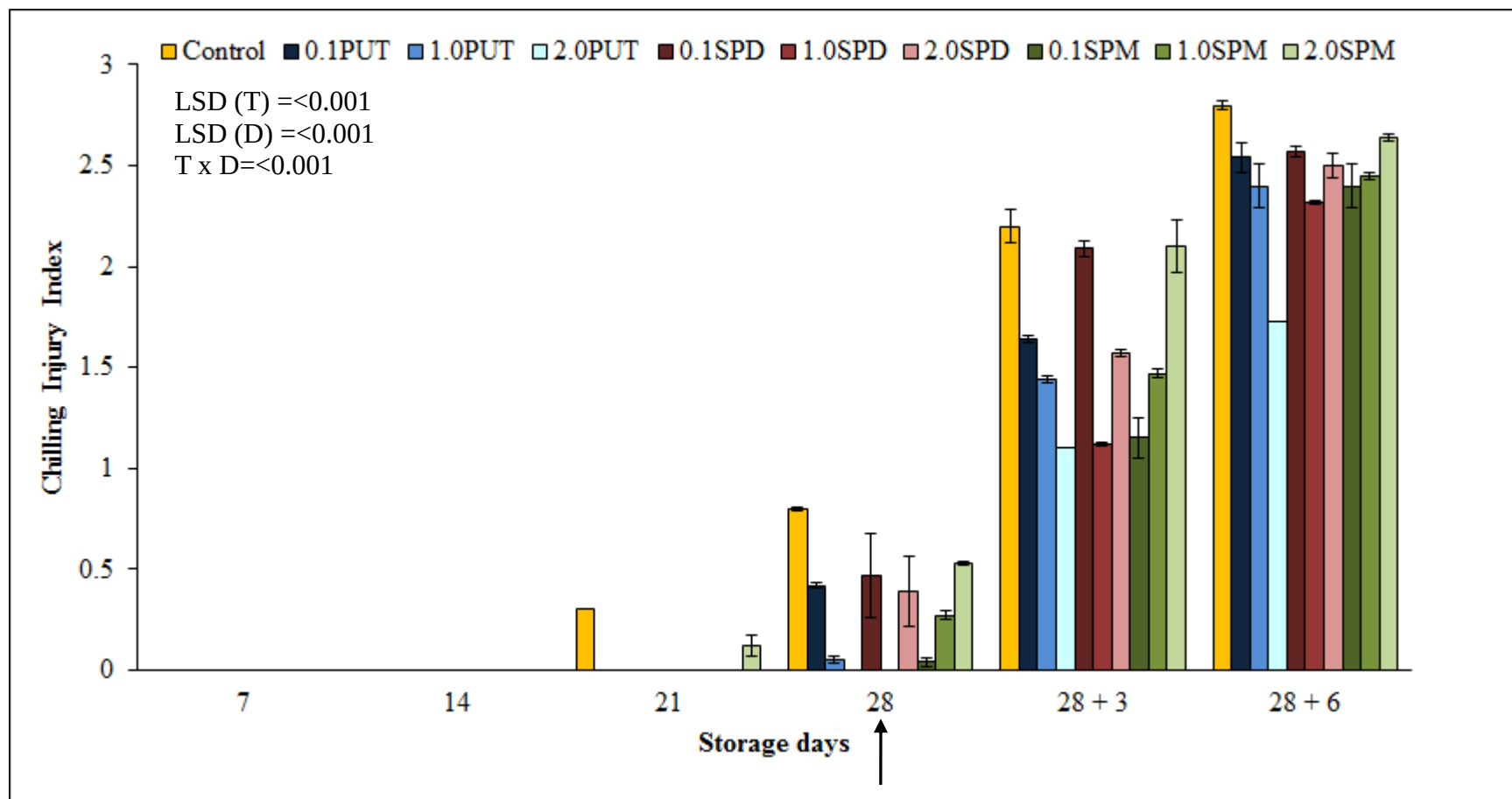


Figure 5.7: Effect of PUT, SPD and SPM treatment on the chilling injury of B10 carambola during cold storage (3°C) for 28d followed by 6d at 22±1°C (shelf life). Vertical bars represent ±SE of pooled mean from two independent experiments. One experiment: n=30. An arrow (↑) indicates fruit was transferred from cold storage to ambient conditions for shelf life study. T=treatments, D=days, treatments x days = <0.001

5.3.9 Sensory evaluation

Table 5.4: Effect of 2.0PUT, 1.0SPD and 0.1SPM treatment on sensorial parameters of B10 carambola during chilling storage (3°C) for 28d followed by 6d at 22±1°C (shelf life).

Treatment	Appearance	Pulp colour	Smell	Sweetness	Texture	Overall acceptability
Control	4.92 ^a	4.94 ^a	4.53 ^a	5.43 ^c	5.26 ^c	5.18 ^c
2.0PUT	6.23 ^a	5.92 ^a	5.21 ^a	6.41 ^a	6.05 ^a	6.15 ^a
1.0SPD	5.91 ^a	5.35 ^a	4.75 ^a	6.09 ^b	5.73 ^{ab}	5.83 ^a
0.1SPM	5.43 ^a	5.33 ^a	4.64 ^a	5.64 ^{bc}	5.52 ^{bc}	5.65 ^b

A 9 point hedonic scale where 1=extremely dislike 3=dislike 5=acceptable 7=good 9=excellent. Means in a column with the same letter are not significantly different ($p<0.05$) between treatments according to LSD.

There were significant differences ($p<0.05$) between PAs-treated and control fruit in term of sweetness, texture and overall acceptability. However, there was no significant difference in appearance, pulp colour and smell. Carambola treated by PUT at 2.0 mM received highest appearance ratings by the taste panellists and control fruit attained lowest scores due to the occurrence of the physiological injury on peel prior to cold storage. Control fruit also obtained lowest ratings for pulp colour and smell from sensory test mainly because of over ripening and off-odour development at the of end storage.

Fruit treated by 2.0PUT gained higher scores for sweetness and texture test in contrast to 1.0SPD, 0.1SPM and control, as demonstrated previously in soluble solids content and firmness analysis respectively. The sensory result revealed that 2.0PUT and 1.0SPD were assigned superior overall acceptability scores by the taste panels after prolonged cold storage and 6 days at ambient.

5.4 Discussion

5.4.1 Effect of PAs treatment on physicochemical profiles of carambola

Fruit ripening is genetically programmed and tightly regulated process that leads to the major physical and biochemical changes. Ripening allows fruit to attain desirable transformation in colour, aroma, sensory (texture and flavour) and nutritional qualities (Giovannoni et al., 2004). This particular process is heavily influenced by plant hormones; ethylene is accepted as the main cause but at least other hormones such as abscisic acid (ABA) and gibberellins are equally responsible (Giovannoni, 2001). In non-climacteric fruit, polyamines (PAs) and ABA might play a much more crucial role because of the lesser importance of ethylene fruit development and ripening (Martinez-Madrid et al., 1996) and fruit lack the ethylene autocatalytic response (Kader, 2002).

During prolonged cold storage and shelf life of control carambola fruit, the decline in firmness and titrable acidity (TA), a substantial increase in respiration rate, water loss, colour development and soluble solids content (SSC) were observed. In the present study, the acceleration of physicochemical changes which was associated with ripening was significantly suppressed by PAs application. PAs as postharvest treatment has been reported to suppress ripening and prolong shelf life in a wide range of horticultural products, such as apple (Wang et al., 1993), lemon (Valero et al., 1998a; Safizadeh et al., 2013), peach (Bregoli et al., 2002; 2006), apricot (Martinez-Romero et al., 2002; Khosroshahi and Esm-Ashari, 2017), plum (Perez-Vicente et al., 2002; Serrano et al., 2003; Khan et al., 2007; 2008), zucchini (Martinez-Tellez et al., 2002; Palma et al., 2015), kiwifruit (Petkou et al., 2004), mango (Malik and Singh, 2005; Jawadha et al., 2012; Razzaq et al., 2014), pomegranates (Mirdehghan et al., 2007a; Ramezani and Rahemi, 2010), strawberry (Khosroshahi et al., 2007) and table grape (Champa et al., 2014).

Even being detached from the parent tree, the fruit continues to respire and transpire through the skin and fruit undergoes water exchange caused by vapour pressure differentials. Cellular metabolism during ripening process resulted in losses in the substrate and water vapour thus accelerating weight loss (Woods, 1990; Shiri et al., 2013). Consequently, weight loss was significantly reduced in PAs-treated fruit with PUT treatment being the most effective. PAs might affect weight loss in fruit during storage by maintaining biophysical properties such as delaying the removal of waxy skin layers and retaining epicuticular thickness since water vapour mainly lost through the aerial part and fruit skin (Martinez-Romero et al., 2002; Shiri et al. 2013). Moreover, PAs due to their antisenescence abilities (Valero et al., 2002) can reduce cellular metabolism by slowing down carbohydrates breakdown and decrease respiration rate through inhibition of ethylene biosynthesis leading to significant reduction in weight loss of carambola.

Carambola peel colour attainment was suppressed by PAs treatment throughout cold storage and shelf life at ambient when lower values for L^* , C^* and hue were recorded as compared to control fruit. Amongst supplied PAs, PUT appeared to be the most effective in retarding chlorophyll degradation thus causing a delay in ripening (Valero and Serrano, 2010). Previously, PAs treatment has been proven to ameliorate chlorophyll breakdown in wheat leaves by a reduction in peroxidase activity (Ma-Jun et al., 1996) or by inhibition of hydrolytic activities acting on chloroplast thylakoid membrane in muskmelon (Lester, 2000).

Most studies reported that PAs as postharvest treatment caused no major changes in soluble solid contents (SSC) evolution during storage (Serrano et al., 2003; Mirdehghan et al., 2007a; Khan et al., 2008). In contrast, PAs application significantly suppressed the

SSC increase throughout cold storage and shelf life in carambola. Similarly, titrable acidity (TA) in PAs-treated fruit was maintained at higher concentrations and the TA level diminution was significantly delayed than in control fruit. The reasons for PAs modifying soluble solutions and titrable acids remains unclear however most reports have postulated that the PAs reduce respiration rate thus delay the ripening and senescence process. This inhibition leads to slow process of breakdown of carbohydrates to sugars (Malik and Singh, 2006) as well as suppress the consumption of organic acids metabolism (Torrighiani et al., 2004).

Texture or firmness is an important quality that affects consumer's acceptability of fresh fruit. The firmness of control fruit was gradually declined throughout storage at 3°C and a rapid softening activity occurred thereafter during shelf life at ambient. PAs application markedly suppressed softening in carambola during postharvest storage by retaining higher fruit firmness in contrast to the control. Among the three PA studied, PUT was the most effective in maintaining tissue firmness and the effect was greater with the highest PUT concentration (2.0 mM). Effect of PAs on fruit firmness could be due to PAs affinity to bind and cross-link to pectin substances associated with the cell wall, resulting in stabilization and rigidification of membranes and cell walls (Champa et al., 2014).

PAs also have been proposed to regulate softening mechanisms by inhibiting cell wall degrading enzymes activity such as pectin esterase, pectin methylesterase, exo- and endoPG therefore, reducing the softening rate during storage (Champa et al., 2014; Razzaq et al., 2014). PAs also involved directly in the modification of ethylene biosynthesis probably by delaying the action of ACS and ACO activities (Khan et al., 2007). It has been suggested that PAs selectivity to the cell wall and membranes could be

attributed to their positive charges number availability that is $SPM^{4+} > SPD^{3+} > PUT^{2+}$ (Valero et al., 2002). Our experimental data showed that firmness in carambola was significantly improved by PUT treatment in a dose-dependent manner that is $2.0 > 1.0 > 0.1$ mM. The efficacy of PUT was greater than SPD and SPM, probably due to the fact that carambola epicarp is sensitive and permeable for PUT intake (Bregoli et al., 2006; Torrigiani et al., 2008). Neither SPD nor SPM at higher concentrations was effectively prevented firmness loss. This differential effect of PAs could be contributed to PAs binding site saturation at cell wall pectins or possibly due to PAs intake limitation in the mesocarp at the epicarp membrane level (Torrighiani et al., 2004). On the other hand, the exact biological function of PUT alone on fruit postharvest improvement has yet to be fully elucidated.

5.4.2 Effect of PAs treatment on respiration rate and ethylene emission in carambola

Respiration takes place in the presence of oxygen substrate this process converts stored sugar or starch to energy thus hastening ripening and senescence (Kader, 2002). Even though carambola exhibited a relatively lower or little ethylene (Paull and Chen, 2014), the postharvest quality diminishes quickly and impairs its long term storability. It has been known that respiration rate is generally proportional to the development of deterioration and senescence of fruit (Yang et al., 2014). Ethylene action has been proved as responsible for the induction of respiration rate in fruit ripening (Reid, 2002; Razzaq et al., 2014). Hence, for longer shelf life and better quality preservation respiration and ethylene activity levels must be kept at a minimum level.

Our result revealed that PAs application substantially delayed and/or reduced respiration rate and ethylene emission in carambola. Briefly, the PAs-treated fruit had a

suppressed pattern of respiration rate and ethylene production in contrast to control fruit throughout cold storage. Later, tremendous increases in carbon dioxide and ethylene were observed after transferred at ambient storage however, the rise in both respiration and ethylene production rate were significantly lower in PAs-treated fruit. The sudden high emission of ethylene and respiration peak prior ripening at ambient could be due to fruit recovered its normal metabolism and chemical reactions went faster after returned to a higher temperature. The influence of cold temperature is known to stimulate moderate stress and provoke momentary stop of metabolism reactions in a wide range of perishable crops (Kader, 2002).

PUT demonstrated the greater potential of suppressing carbon dioxide and ethylene peak compared to SPM and SPD during postharvest storage, which also caused a delay in the decay of the harvested fruit (Torrighiani et al., 2004). The slower respiration values in PAs-treated fruit could be explained from reducing rates of chemical metabolisms such as hydrolysis of sucrose into reducing sugars (glucose and fructose) and utilization of carbohydrates for metabolic activities (Champa et al., 2014). In addition, PAs may control sugar breakdown (Malik and Singh, 2006) and organic acid consumption (Torrighiani et al., 2004) during ripening hence leading to a reduction of weight loss in carambola which has been evidenced in SSC and TA quality traits pattern.

On the contrary, the reduction in ethylene emission may be attributed to the regulatory effect of PAs and ethylene synthesis to the fact that ethylene and PAs demonstrated competitive biosynthesis mechanisms for a common intermediate SAM and to inhibition of ACS and ACO biosynthesis, transcription and activity (Valero et al., 2002; Khan et al., 2008). In addition, PAs exert their free-radical scavenging ability by

removing and eliminating superoxide free radical which is a crucial part to convert ACO to ethylene resulting in the prevention of ethylene biosynthesis (Drolet et al., 1986).

Corresponding to PAs application, its efficacy was dependent on fruit type or cultivar and ethylene evolution at the climacteric peak as observed in Black Diamond and Black Star plum by Serrano et al. (2003). This study suggested that carambola with lowest ethylene production experienced high ethylene inhibition by 2.0PUT (80%) in agreement with other findings in kiwifruit, mango and plum (Petkou et al., 2004; Malik and Singh, 2005; Khan et al., 2007). Contrasting results were found in two apple cultivars since PUT treatment did not reduce or moderately inhibited ethylene production (Kramer et al., 1991; Wang et al., 1993). The inhibitory effects of PUT in ethylene production was inversely correlated to the maximum ethylene emission at the climacteric peak and the failures of PUT treatment to inhibit ethylene could be contributed to high level of ethylene emission (Valero et al., 2002; Serrano et al., 2003).

5.4.3 Effect of PAs treatment on chilling injury and sensory evaluations

Chilling injury (CI) symptoms in control fruit manifested as minor defects and later the chilling severities translated into decay development and subsequently postharvest losses. By contrast, all three PAs maintained acceptable visual quality throughout cold storage without the significant appearance of CI symptoms. However, this physiological disorder started to develop upon transferring to ambient temperature and the chilling incidences become more pronounced and serious with the advancement of storage time. Nonetheless, the occurrence of chilling damage was lesser than in the control.

PAs application has been suggested to stabilize and strengthen membrane bilayer surface by binding with anionic sites present on the membrane or interact with negatively

charged phospholipids components (Slocum et al., 1984). This mechanism prevents membrane deterioration and maintains membrane integrity upon chilling stress. Among the PAs used, fruit treated with 2.0 mM PUT provided the best protection against CI since fruit displayed the lowest reduction of chilling injury index. Accordingly, this result in agreement with previous studies that have been observed in other chilling-sensitive crops such as litchi (Jiang and Chen, 1995), pomegranate (Mirdehghan et al., 2007a; Ramezani and Rahemi, 2010), table grape (Champa et al., 2014) and zucchini (Palma et al., 2015).

Under chilling conditions, loss of membrane integrity is thought to be the primary event in defining the physiological basis of CI. It has been shown that physical changes occur in membrane lipids of the chilling sensitive plant at a critical temperature of the tissue which leads to a phase change in membrane lipid transitions from liquid-crystalline to a solid-gel state (Liu et al., 2013). Abrupt alteration in membrane lipids could induce direct increase on membrane permeability and leakage of ions.

Furthermore, the ratios of unsaturated and saturated fatty acids appear to determine how membrane cellular integrity was maintained (Gomez-Galindo et al., 2004). Increase proportion of unsaturated fatty acids has been suggested as a mechanism of acclimation to cold temperatures which could induce higher resistance under chilling conditions, and subsequently lower electrolyte leakage (Campos et al., 2003; Mirdehghan et al. 2007a).

In contrast, the application of PAs particularly PUT appears to reduce severity in CI symptoms, a diminished manifestation of skin pitting and postponed decay development in carambola. PAs treatment has been suggested to provide resistance to the cold condition by inducing cold acclimation resulted in membrane fluidity maintenance and

membrane deterioration retardation (Gomez-Galindo et al., 2004). Moreover, alleviation of CI in PAs-treated carambola could be associated with accumulation of endogenous PAs in response to low temperatures (Bouchereau et al., 1999). Exogenously applied PUT may increase the endogenous PAs level through inducing of PAs biosynthesis pathway. However, under chilling conditions endogenous PAs levels were metabolized as a substrate for the formation of other metabolites required to augment cold tolerance (Palma et al., 2015).

In other literatures, PUT has been proposed to induce the highest cold tolerance by regulating hormones activity which involved in cold tolerance such as γ -aminobutyric acid (GABA) or abscisic acid (ABA) (Alcazar et al. 2010; Shelp et al., 2012). GABA results from the indirect synthesis of PUT via enzyme DAO and 4-aminobutyraldehyde dehydrogenase (ABALDH) activities or pyrroline dehydrogenase (PDH). Succinate originating from this pathway has been proposed for its role in modulating tolerance towards chilling environments (Shelp et al., 2012).

The cross-talk between PAs and ABA in regulating abiotic stress has been well established. ABA accumulation in response to CI seems to be linked with its ability to regulate stomatal closure and restraining dehydration (Caldazilla et al., 2014). Under cold stress, PUT reduces chilling injury by regulating the levels of ABA by decreasing its content in plant tissue. Palma et al. (2015) suggested that PUT-treated fruit is capable of overcoming chilling stress by having lower ABA levels than control fruit.

The beneficial effect of PUT on reducing CI symptoms possibly could be one of the reasons for assigning highest scores for organoleptic evaluation. Postharvest treatment by PUT at 2.0 mM resulted in an improved sensory quality particularly in appearance,

sweetness, texture and overall quality. Similar results were reported in PUT-treated strawberry (Khosroshahi et al., 2007), mango (Jawandha et al., 2012) and table grape (Shiri et al., 2013; Champa et al., 2014).

5.5 Conclusion

In nutshell, PAs application by immersion suppressed the ripening-related changes by delaying firmness loss, reducing weight loss, maintained the L^* , C^* and hue values as well as TA and SSC. Moreover, the PAs application prolonged the shelf life in carambola by decreasing respiration rate and ethylene emission. Among PAs treatment, PUT at a concentration of 2.0 mM effectively alleviated fruit from CI and received highest organoleptic ratings. Thus, PAs may have positive effects in maintaining postharvest quality and extending shelf life in carambola stored at low temperature.

CHAPTER SIX

INFLUENCE OF POLYAMINES TREATMENT ON CELL WALL DEGRADING ENZYMES DURING COLD STORAGE AND RIPENING IN CARAMBOLA

6.1 Introduction

During fruit ripening, softening or texture modifications occurs making fruit more palatable and desirable for human consumption. Furthermore, textural changes are associated with the development of organoleptic attributes such as accumulation of sugars and pigments, and production of volatile aromatic compounds (Paniagua et al., 2014). However, ripening is accompanied with undesirable characteristics such as excessive softening, off flavours development and susceptible to diseases and decay (Goulao et al., 2008). It has been well established that fruit softening is a consequence of progressive compositional and structural alteration of the primary cell wall as fruit undergoes ripening (Giovannoni, 2001; Fonseca et al., 2005).

In general, the primary cell wall is composed of three co-extensive polysaccharides networks: cellulose, hemicellulose and pectin (Brummel et al., 2004; Nishiyama et al., 2007; Gilbert, 2010). Hemicellulose and pectin are increasingly solubilized and depolymerized during ripening and alterations in cell wall polymers involve the concerted effort range of cell wall degrading enzymes (Brummel, 2006; Goulao et al., 2008). A number of softening enzymes including polygalacturonase, pectin methylesterase and β -galactosidase have been implicated in the softening of many fruits (Brummel et al., 2004; Rugkong et al., 2010; 2011).

Involvement of PAs suppressing the fruit softening which are triggered by events of hydrolytic enzymes has been reported in apple (Kramer et al., 1991), zucchini

(Martinez-Tellez et al., 2002), plum (Khan et al., 2007), table grape (Champa et al., 2014) and mango (Razzaq et al., 2014). To note, no information has been done on the effect of postharvest treatment of PAs on softening enzymes activity in slow ripening fruit such as carambola. Relatively, B10 carambola takes about 12 days (Chapter 4) to ripen fully at ambient and mature fruit are not as firm, and do not soften as much as fast ripening fruit (Chin et al., 1999).

Activity of polygalacturonase (PG), pectin methylesterase (PME) and β -galactosidase (β -gal) were detected in carambola implying that these dominant degrading enzymes hold a significant role in regulating the softening course of the carambola (Lazan et al., 1995; Ali et al., 1995, 1998; Chin et al., 1999; Balasubramaniam et al., 2005). Therefore, this study aim was to evaluate the influence of PAs treatment on major softening enzymes activity notably PG, PME and β -galactosidase in B10 carambola during cold storage and shelf life.

6.2 Materials and methods

Carambola was subjected to the treatments described in sub-section 3.2.1 and freeze-dried fruit mesocarp kept at -80°C was used for evaluating activities of major softening enzymes prior PAs treatment during storage at 3°C and shelf life at ambient. For all PAs concentration including control fruit, three replicates each consisting of five fruit ($n=15$) were used to analyze PG, PME and β -galactosidase activity. All crude enzyme extraction and assay procedures were done on the ice at all time (4°C).

6.2.1 β -galactosidase (β -gal, EC 3.2.1.23)

Extraction of crude extract for β -gal and PME was carried out according to Ali et al. (1998). Approximately 10 g of freeze-dried fruit mesocarp was homogenized in 10 ml of 0.1 M sodium citrate (pH 4.6) containing 1 M sodium chloride, 13 mM EDTA, 10 mM β -mercaptoethanol and 1% (w/v) polyvinylpyrrolidone (PVP-40). The crude enzyme was left for 1 hour on ice with occasional stirring. The supernatant was recovered by centrifugation (Centrifuge 5810 R, Eppendorf, USA) at 12,000 rpm for 25 min (4°C).

Assay for β -gal activity was carried out according to Chin et al. (1999). β -gal was assayed in a reaction mixture containing 0.52 ml 0.1 M sodium citrate (pH 4.1), 0.4 ml 0.1% (w/v) bovine serum albumin and 0.4 ml 13 mM substrate *p*-nitrophenyl- β -D-galactopyranosidase (Sigma-Aldrich, USA) and 0.08 ml of crude extract incubated for 15 min at 37°C. The reaction was stopped by adding 2 ml 0.2 M sodium carbonate and the amount of *p*-nitrophenol (Merck, Germany) formed was determined from the absorption at 415 nm using a UV-VIS Spectrometer (Varioskan Flash Multimode Reader, Thermo Fisher Scientific, USA). A standard curve was developed using *p*-nitrophenol stock solution (Appendix C). Boiled enzyme was used as a control in all the assays and β -gal activity was expressed as nkatal g⁻¹ fresh weight.

6.2.2 Pectin methylesterase (PME, EC 3.1.1.11)

PME activity was determined according to Lazan et al. (1995). The activity was defined by titrating the release of carboxyl groups by the action of PME on the substrate with 0.1 M NaOH to pH 7.3 for 10 min. The assay mixture consisted of 0.5 ml of crude extract and 25 ml 1% pectin. Boiled enzyme was used as a control in all the assays. Boiled enzyme was used as a control in all the assays and PME activity was expressed as nequivalent

carboxyl group formed $\text{s}^{-1} \text{g}^{-1}$ fresh weight.

6.2.3 Polygalacturonase (PG, EC 3.2.1.67)

PG activity was determined by the dinitrosalicylic acid (DNS) based on the spectrophotometric determination of reducing groups released (Miller, 1951). Extraction of crude extract was as described in Fabi et al. (2009) with slight modifications. One gram of freeze-dried fruit mesocarp was homogenized with cold 100 mM sodium acetate buffer (pH4.6), consisting 1 M sodium chloride, 13 mM EDTA, 1% (w/v) PVP-40 and 10 mM β -mercaptoethanol. The supernatant was recovered by centrifugation at 12,000 rpm for 30 min (4°C) and was filtered through cheese cloth. The supernatant undergoes dialyzed procedure for overnight using a 12,000 Da to 14,000 Da size exclusion cellulosic membrane (Sigma-Aldrich, USA) in cold 100 mM sodium acetate buffer pH5 containing 100 mM sodium chloride.

PG assay was carried out with 0.02 ml of dialyzed extract was homogenized in 0.1 ml 50 mM sodium acetate buffer pH 5.5 containing 1 mg of polygalacturonase previously washed with 80% ethanol. The reaction was incubated in a water bath (Stuart, SWBD, Biocote, UK) at 30°C for 1 hour followed by addition of DNS reagent. The reaction was terminated by heating the reaction mixture in a boiling water bath for 5 min. The formation of reducing groups was estimated against monogalacturonic acid (Sigma-Aldrich, USA) as the standard for measuring the absorbance at 540 nm (Appendix C). Boiled enzyme served as a control in all the assays and PG activity was expressed as the amount of the enzyme that reduces 1 μmol of reducing groups per ml at 30°C .

6.3 Results

6.3.1 β -galactosidase activity (β -gal)

Control fruit demonstrated a steady increase in β -gal activity of about 4.8-fold higher at the point at harvest till final day at cold storage. The greatest β -gal activity was detected after 3 days storage at ambient with 6.9-fold higher than at harvest and thereafter the level slightly decreased (Figure 6.1). Clearly, a comparable pattern was observed in fruit receiving PAs treatment but the β -gal activity appeared to be suppressed. PUT, SPD and SPM treatments inhibited the increase of the enzyme activity significantly throughout the storage however, the mechanism of inhibition for each treatment were different depending on the concentrations applied. Prior to concentration, the increment of β -gal was delayed throughout storage periods with fruit subjected to the highest concentration of PUT and SPM (2.0 mM) showed the greatest suppression by maintaining lowest β -gal activity.

Fruit treated by 2.0PUT and 2.0SPM exhibited comparable β -gal activity at the end of cold storage about 4.3 and 4.2-fold higher respectively followed by a maximum enzyme activity occurred after 3 days storage at ambient with enzyme levels of 6.3 and 6.2-fold higher than at harvest respectively (Figure 6.1). Even though β -gal enzyme advancement was postponed, there was no significance difference ($p>0.05$) between two concentrations during the mentioned storage period. In all cases, a slight fall of β -gal activity was observed on the final day of shelf life and the reduction was not significant ($p>0.05$) for all treatments.

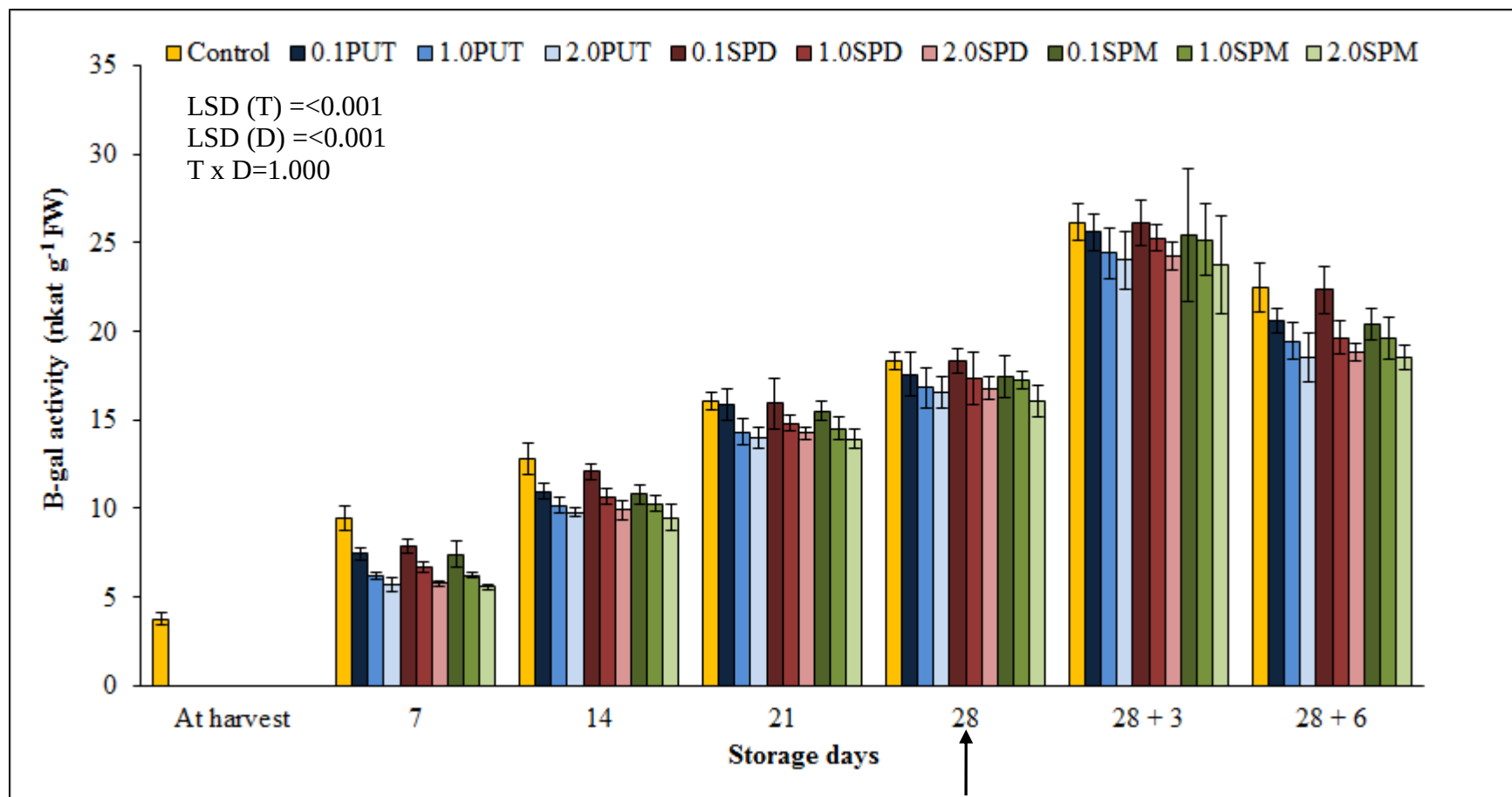


Figure 6.1: Effect of PUT, SPD and SPM treatment on the β -gal activity of B10 carambola during cold storage (3°C) for 28d followed by 6d at 22±1°C (shelf life). Vertical bars represent $\pm$ SE of pooled mean. n=15. An arrow ($\uparrow$) indicates fruit was transferred from cold storage to ambient conditions for shelf life study. T=treatments, D=days, treatments x days = NS ($p>0.05$); NS = not significant

6.3.2 Pectin methylesterase activity (PME)

PME activity in carambola was significantly ($p < 0.001$) affected by PAs application and storage time throughout the experiment. Overall, PME activity increased slowly up to 21 days and decreased thereafter on the final day at cold storage then exhibited a rise after transferred to ambient for 3 days. At end of cold storage, control fruit exhibited the highest PME activity about 8-fold higher than at harvest. The reverse pattern was observed in PAs-treated fruit with PME activity significantly lower as compared to control. PUT treatment was found to be more effective in delaying PME activity increment than SPD and SPM. At end of cold storage, fruit dipped PUT (2.0 and 1.0 mM) demonstrated equal PME activities about 7.0-fold higher than at harvest (Figure 6.2). However, the activity was lower than that in control fruit.

Irrespective treatments, PME activity demonstrated an increased after transferred to ambient for 3 days with control fruit exhibited the highest activity about 12-fold higher than at harvest. On the other hand, 2.0PUT and 1.0PUT showed comparable levels of PME about 11-fold higher than at harvest. Although, PUT application suppressed PME activity at this time point nevertheless, there was no significance difference ($p > 0.05$) between PUT-treated and control fruit throughout the experiment (Figure 6.2).

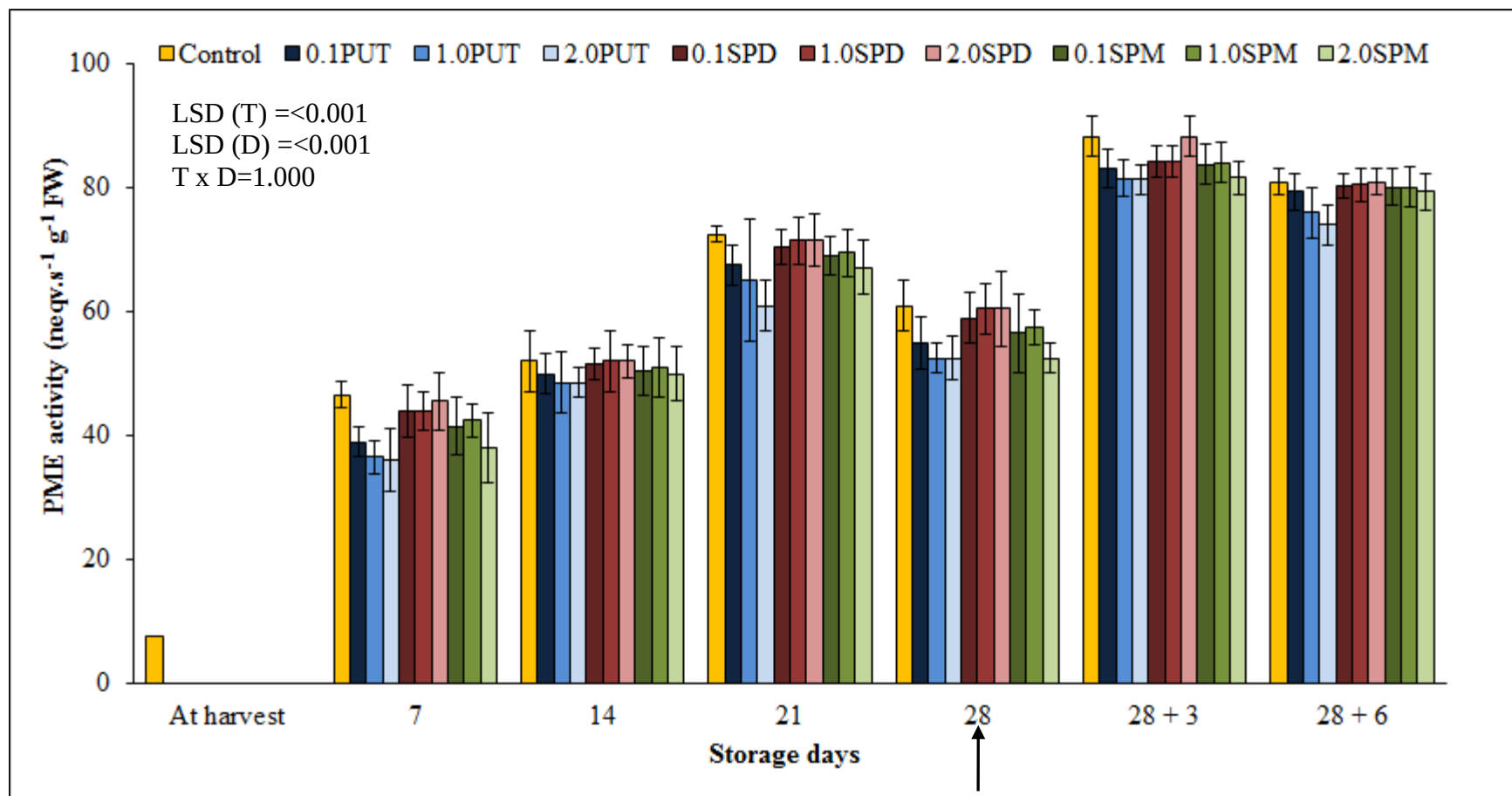


Figure 6.2: Effect of PUT, SPD and SPM treatment on PME activity of B10 carambola during cold storage (3°C) for 28d followed by 6d at 22±1°C (shelf life). Vertical bars represent ±SE of pooled mean. n=15. An arrow (↑) indicates fruit was transferred from cold storage to ambient conditions for shelf life study. T=treatments, D=days, treatments x days = NS (p>0.05); NS = not significant

6.3.3 Polygalacturonase activity (PG)

PG activity in control fruit increased gradually up to 21 days then showed a slight fall at the end of cold storage. The highest activity occurred after transferred to ambient for 3 days and declined thereafter on the final day of shelf life. At end of cold storage, control fruit demonstrated highest PG activity about 3.15-fold higher than at harvest, subsequently similar trend was observed during the next 6 days at ambient with the activity values of 3.86-fold higher than at harvest. Regardless PAs treatment, PG activity was reduced significantly ($p < 0.001$) throughout the experiment (Figure 6.3).

Fruit treated with the highest concentration of SPM and PUT (2.0 mM) were the most effective in reducing PG activities than that in control fruit. 2.0SPM and 2.0PUT significantly delayed the activity of PG throughout cold storage and later suppressed the increasing trend of PG activity which occurred during ripening at ambient. Throughout the cold period, the enzyme activity in 2.0PUT and 2.0SPM were 3.0 and 2.6-fold higher than at harvest respectively. Later, PG activity for both treatments reached higher values throughout ripening at ambient about 3.6 and 3.5-fold higher than at harvest (Figure 6.3). Even though, PAs application suppressed PG level during shelf life at ambient, there was no significant difference ($p > 0.05$) between 2.0PUT and 2.0SPM from the control fruit.

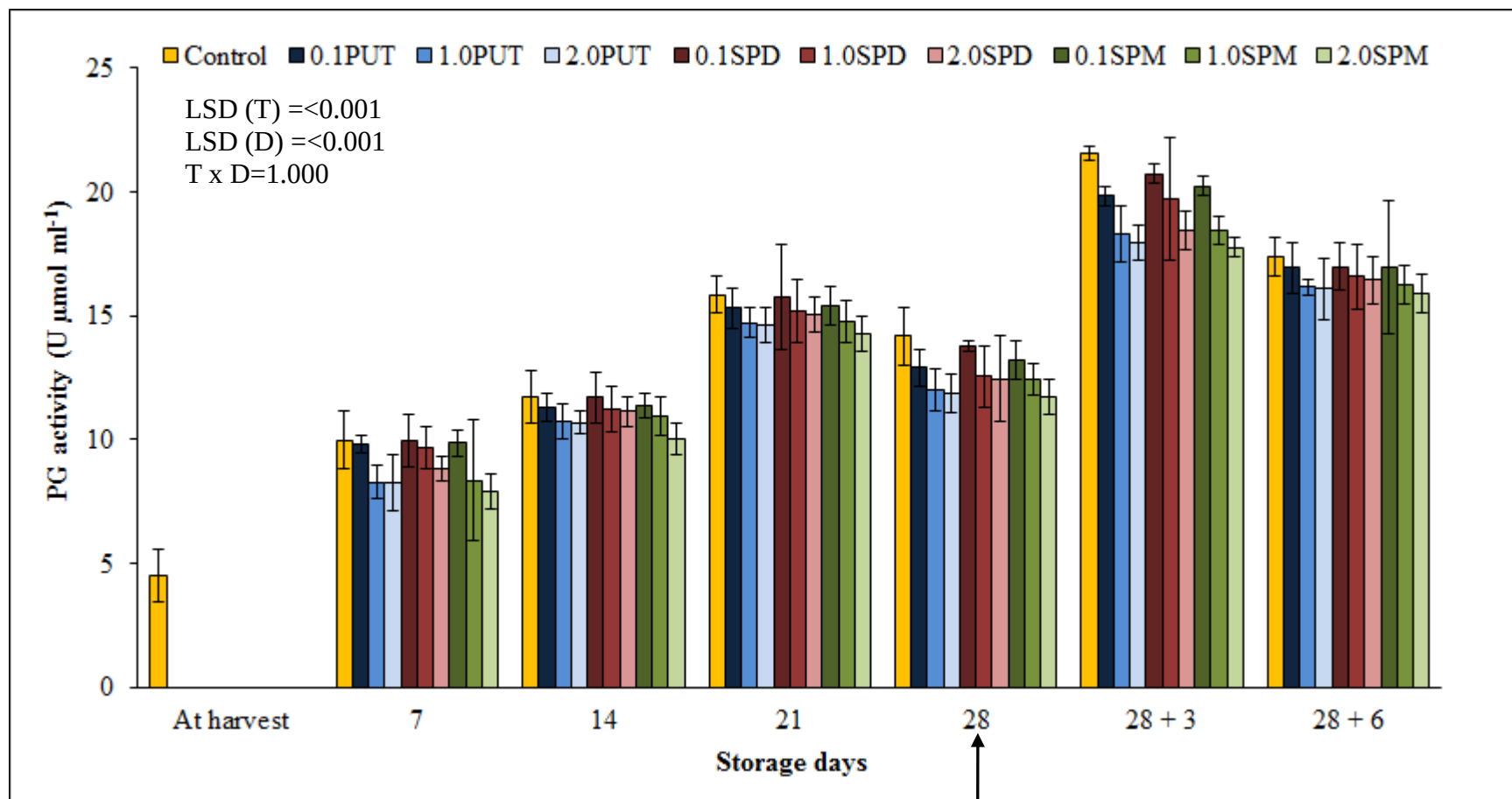


Figure 6.3: Effect of PUT, SPD and SPM treatment on PG activity of B10 carambola during cold storage (3°C) for 28d followed by 6d at $22\pm 1^{\circ}\text{C}$ (shelf life). Vertical bars represent $\pm\text{SE}$ of pooled mean. $n=15$. An arrow ($\uparrow$) indicates fruit was transferred from cold storage to ambient conditions for shelf life study. T=treatments, D=days, treatments x days = NS ($p>0.05$); NS = not significant

6.4 Discussion

6.4.1 Cell wall degrading enzymes involvement during fruit ripening

Polysaccharides are organic compounds that are present ubiquitously in nature and plant cell walls are primarily made of 90% polysaccharides (Barnavon et al., 2000). The plant cell wall consists mainly of cellulose, hemicellulose and pectin (Gilbert, 2010). These three polysaccharides make up rigid, tough and flexible polymer networks that provide both protection and structural strength (Minic and Jouanin, 2006; Nishiyama et al., 2007). During ripening, plant cell wall generally undergoes dismantling and disintegration via the interdependent and coordinated act of cell wall modifying enzymes (Brummel and Harpster, 2001; Minic and Jouanin, 2006; Payasi et al., 2009).

During early ripening stage, earliest modifications occur in pectins and a loss of neutral sugars mostly galactose and arabinose on the side groups attached to pectins (Regwell et al., 1997). Operationally, β -galactosidase (β -gal) hydrolyzes galactosyl residues on the side chains of pectin polymers (Vicente et al., 2005; Sekine et al., 2006; Martinez and Civello, 2008). β -gal is closely associated with pectin solubility by removing galactose residues during fruit ripening (Tateishi et al., 2001; Sozzi et al., 2002; Chavez Montes et al., 2008). However, although the galactose content in cell wall decreased continuously it is not easy to relate this loss to β -gal activity and to determine whether galactose loss can have direct implications on the softening of fruit. The β -gal activity could be involved in a more generalized ripening course (Barnavon et al., 2000).

Polygalacturonase (PG) and pectin methylesterase (PME) are two major enzymes that known to have concerted and synergistic actions in pectin degradation. PME hydrolyzes the methylester groups from galacturonyl residues and is thought to be responsible for the accessibility for degradation of demethylated polygalacturonan by PG (Rugkong et al.,

2011). Meanwhile, PG hydrolyzes pectin together with the side chain of polygalacturonic acid by releasing galacturonic acid (exo-PG) or oligogalacturonate (endo-PG) leading pectin disassembly (Wei et al., 2010). PG activity has been implicated as the major determinant for pectin depolymerisation and solubilisation and polyuronide degradation in ripening tomato and hence largely responsible for regulating papaya, carambola and tomato softening (Lazan et al., 1995; Ali et al., 2004a; Payasi et al., 2009).

Additionally, these particular hydrolases occur in a number of isoforms, apparently encoded by multigene families but not all of isoforms have the capacity to degrade neither neutral sugars nor the ability to modify fruit cell wall (Smith and Gross, 2000; Trainotti et al., 2001). For instance, in carambola at least three isoforms of β -gal were identified with β -gal I is the predominant form (Ali et al., 1998; Sekine et al., 2006). This enzyme isoform is thought to modify and solubilizes less tightly-bound pectins mostly at the early stage of ripening (Chin et al., 1999; Balasubramaniam et al., 2005; Sekine et al., 2006).

6.4.2 Influence of PAs treatment on cell wall degrading enzymes during cold storage and ripening in carambola.

In carambola, a range of glycosidases and pectinases that contribute to primary cell wall alteration and disassembly including β -gal, PME and PG were identified (Chin et al., 1999; Ali et al., 2004b). In general, activities of β -gal, PME and PG in control and PAs-treated carambola exhibited gradual upshift throughout cold storage. However, dissimilar patterns were observed in PG and PME activities when both enzymes demonstrated a distinct downshift at the final day of cold storage (day 28). This finding suggested that these two enzymes may not directly involved in affecting an overall softening rate that gives rise to firmness loss at cold temperature. Moreover, previous

studies showed that low temperature conditions affect normal cell wall metabolism by retarding ethylene biosynthesis and emission. However, the cell wall degrading enzymes level advanced progressively once fruit was transferred to ambient temperature indicating metabolism for ripening was recovered once fruit return to a higher temperature (Wang et al., 2006; Almeida and Huber, 2008; Rugkong et al., 2011).

On the other hand, the activity of β -gal increased throughout cold storage, although gradual but the increment of β -gal activity was significant and corresponding with a slow drop in firmness. Among the enzymes studied, the activity of β -gal increased in close parallel with firmness loss rate. Hence, it would be tempting to suggest that this enzyme may be important to cell wall disassembly during ripening in carambola. Moreover, remarkable and distinct reduction in firmness was observed on the final day of shelf life (Figure 6.2) proposing that it is unlikely for β -gal, PG and PME action to be the decisive factor for triggering softening course in carambola due to highly complex of cell wall architecture. A concerted and synergistic action of other degrading enzymes such as cellulases, glycosidases and glycanases would be required to alter and regulate textural changes during fruit ripening (Ali et al., 2004a; 2004b; Brummell et al., 2005).

PAs application by immersion was examined as a possible means to delay softening during postharvest storage. In carambola, the activity trends of β -gal, PME and PG were suppressed in PAs-treated fruit but essentially followed patterns of accumulation and increment that paralleled the trends in control fruit (Figure 6.1 - 6.3). Nevertheless, PAs treatment significantly suppressed the advancement of softening enzymes activity throughout the experiment. Among PAs used, PUT was found to be the most effective in affecting the performance of cell wall degrading enzymes and effect being greater as

concentrations increased from 0.1 to 2.0 mM. Reduction of cell wall degrading enzymes by PUT treatment has been observed in plum (Khan et al., 2008) and mango (Razzaq et al., 2014). Furthermore, immersion treatment by highest concentration SPM (2.0 mM) was also effective in delaying softening enzymes activity, while no significant effects were observed in fruit treated by SPD treatment throughout the experiment.

Galactose is one of major neutral sugar that present in carambola and as the fruit ripens the level of galactose sugar decrease caused substantial loss of wall materials (Ali et al., 1998; Chin et al., 1999; Nishiyama et al., 2007). In carambola, noticeable loss of galactose occurred during the early stage of ripening which resulted from hydrolysis of side-chain of loosely bound pectins (Chin et al., 1999). β -gal could hydrolyze galactosyl residues on the side chain of rhamnogalacturonans and the removal of neutral sugar weaken cohesive forces binding adjacent polysaccharides together and promote pectin network depolymerization (Smith et al., 1998). Taken together, PAs may delay degradation and solubility of pectins by blocking β -gal actions. PAs can bind to anionic membranes producing rapid rigidification and physically stabilize the cell wall composition (Martinez-Romero et al., 2002; Perez-Vicente et al., 2002; Valero and Serrano, 2010).

In carambola, degradation of tightly-bound Na_2CO_3 -soluble polyuronides polymers and substantial loss of polyuronides takes place relatively later during the ripening period. At this particular stage, more than 70% of tissue firmness declined by represented by 12-24 days of ripening phase. At this stage, there was a decrease in all pectin classes as indicated by the decrease in Na_2CO_3 -soluble polyuronides coincident with well advanced softening and textural changes (Chin et al., 1999). PG is thought to be responsible for

solubilisation of polyuronides from the pectin polymer (Brummell et al., 2004).

Treatment with PAs postponed fruit softening and attenuated PG and PME activity through binding to pectic acid and as a result, the pectic substances in the cell walls were strongly cross-linked and become resistance to decay (Kramer et al., 1991; Valero et al., 1998a). Since PAs are polycations, they may stabilize and maintain cell wall to a greater extent than do monocations, PAs may make cell wall polymers less accessible to PG and PME action and ultimately enhancing carambola firmness (Valero et al., 2002; Valero and Serrano, 2010).

Positive effects of PAs application on fruit firmness is well documented in a wide range of horticultural crop such as peach (Martinez-Romero et al., 2000), plum (Perez-Vicente et al., 2002; Serrano et al., 2003; Khan et al., 2007), kiwifruit (Petkou et al., 2004), strawberry (Khosroshahi et al., 2007), pomegranate (Mirdehghan et al., 2007a), table grape (Champa et al., 2014) and mango (Razzaq et al., 2014).

6.5 Conclusion

PAs postharvest application by immersion suppressed β -gal, PME and PG activity in carambola fruit throughout 3°C storage and shelf life. The treatment with PUT at 2.0 mM demonstrated higher efficacy than SPD and SPM on delaying cell wall degrading enzymes activity advancement particularly during ripening at ambient. β -gal activity patterns may be more closely associated with the dissolution and disintegration of cell wall integrity than PG and PME.

CHAPTER SEVEN

EFFECT OF POLYAMINES APPLICATION ON MAINTENANCE OF FUNCTIONAL PROPERTIES, REDUCTION OF LIPID PEROXIDATION AND ION LEAKAGE IN CARAMBOLA DURING COLD STORAGE AND RIPENING

7.1 Introduction

Reactive oxygen species (ROS) are formed as by-products of cellular aerobic respiration (Yahia et al., 2007). Accumulation of these reactive molecules is primarily as a consequence of endogenous enzymatic reaction through mitochondrial electron transport and cytochrome P450 system. A class of highly active pro-oxidants includes superoxide (O_2^-), hydrogen peroxide (H_2O_2), the hydroxyl radical ($OH^\bullet$) are generated from metabolism of mitochondrial respiration (Holmstrom and Finkel, 2014; Yang et al., 2014). Under normal condition, the production and scavenging of ROS are tightly and well-regulated and are necessary for signaling of various plant developmental processes however, under extreme environment such as chilling, wounding or infections can lead to excess formation of ROS thus resulting oxidative damage (Chen and Yang, 2012; Noctor et al., 2014).

Oxidative damage also has been addressed to explain the appearance of chilling damage and senescence in fruit (Hodges et al., 2004). Apart from CI development, increase concentrations of lipid peroxidation products (MDA content) and membrane permeability were also detected indicating reduces the functionality of membranes (Nukuntornprakit et al., 2015). Naturally, plants possess two interrelated lines of defence in order to combat the excess formation of ROS and to repair oxidative damage that involves enzymatic and non-enzymatic antioxidants defence system (Tan et al., 2012; Xu

et al., 2012). Accordingly, high activity of antioxidant has been associated with tolerance to low temperature and prevention of CI incidents (Sala, 1998; Huang et al., 2008; Imahori et al., 2008) and reduction of oxidative stress associated with a delay in senescence commencement (Hodges et al., 2004).

Tropical fruits are characterized by distinct nutraceutical properties that are attributed to the antioxidant activity of the fruits. Carambola is an excellent dietary source of the antioxidants such as phenolic compounds that include hydroxycinnamic acids and proanthocyanidins (Leong and Shui 2002), enriched with vitamin C, and minerals (Shui and Leong, 2006). In particular, carambola has higher total antioxidant activity (TAA) than many other tropical fruits such as guava, mangosteen, papaya and mango (Leong and Shui, 2002) thanks to their high phenolic concentrations (Lim et al., 2007).

Nowadays, frequent consumption fruits and vegetables have been closely correlated with reduced degenerative diseases illness such as cardiovascular, cancer, aging and much more (Yahia et al., 2007; Díaz-Mula et al., 2009; Alothman et al., 2010). For this reason, high dietary fruits intake is thought to be one of the main influences of a healthy regime, in addition to exercise, age and genetic factors (Yahia et al., 2007; Alothman et al., 2010).

Much research evidence indicates that PAs in their free forms may act according to their multi-faceted nature, which includes working as effective antiradicals, anionic and cationic-binding properties in radical scavenging and a membrane stabilizer (Bassard et al., 2010; Hussain et al., 2011; Wimalasekera et al., 2011). However, there is scarce knowledge about effects of PAs as a postharvest application on the functional properties with antioxidant activity in tropical fruits. This study was carried out to evaluate the impact of PAs treatment on the bioactive compounds with antioxidant activity in

carambola during cold storage and ripening at ambient.

We also hypothesized that increasing of MDA level and membrane ion leakage are associated with oxidative stress would be due to inadequate antioxidant response and membrane lipid composition is compromised. Ideally, a sufficient antioxidant level would reduce or even nullify an overload of the free radicals, leading to inhibition of membrane disintegration subsequently slowing down senescence process and thus, an extension of shelf life. PAs have been implicated in improving the retention membrane integrity (Valero et al., 2002) and we therefore also studied and the beneficial effect of PAs application in reducing lipid peroxidation level and ion leakage in carambola exposed to chilling temperature.

7.2 Materials and methods

Carambola was subjected to the PAs treatment as described in sub-section 3.2.1 and freeze-dried fruit mesocarp kept at -80°C was used for evaluating antioxidants activity, lipid peroxidation level and membrane leakage prior cold storage and shelf life at ambient. For all PAs concentration including control fruit, three replicates each consisting of five fruit (n=15) were used in this experiment.

7.2.1 Ascorbic acid (AA) content

The ascorbic acid content determination was carried out according to Ranganna (1977) using the titration method. DCPIP solution was prepared by dissolving 24 mg of sodium bicarbonate in 150 ml of hot distilled water followed by adding 50 mg of DCPIP dye (R&M Chemicals, UK). The solution was cooled and the volume was made up to 200 ml by adding distilled water and kept at cold temperature. 10 g of pulp tissue was homogenized

with 90 ml of 3% (w/v) metaphosphoric acid using a kitchen blender (Tefal, USA). The mixture was filtered through a cheese cloth and an aliquot of the volume of the mixture was making up to 100 ml using the 3% metaphosphoric acid. 10 ml of the juice was placed in a dry beaker and slowly added the DCPIP solution to the juice until the solution just starts to change colour to pink. The formation of the pink color solution is the end point and volume of DCPIP dye that was added to the solution was recorded. The ascorbic acid content was expressed as mg of ascorbic acid per 100 g of fruit using the formula below:

$$\text{AA content (mg/ 100 g)} = \frac{\text{Dye factor (mg/ml)} \times \text{titre value (ml)} \times \text{volume made up (ml)}}{\text{Weight of sample (g)} \times \text{aliquot taken for estimation (ml)}}$$

The dye factor was estimated by mixing 5 ml *L*-ascorbic acid solution (0.1 mg/ml) with 5 ml 3% metaphosphoric acid and titrated against DCPIP solution.

$$\text{Dye factor} = \frac{0.5 \text{ mg ascorbic acid}}{\text{Titer value (ml)}}$$

7.2.2 Total phenolic content (TPC)

The total phenolic content was determined using a method from Camacho-Cristobal et al. (2002). 5 g of pulp tissue was homogenized with 20 ml of cold methanol using a kitchen blender (Tefal, USA). The homogenate was centrifuged at 4000 rpm at 4°C for 15 min (Centrifuge 5810 R, Eppendorf, USA). The supernatant was added with 100 µL of freshly diluted 50% Folin-Ciocalteu reagent (R&M Chemicals, UK). After 3 min, the reaction was neutralized by adding 2 ml of 2% (w/v) sodium carbonate. After 30 min incubation at room temperature, the absorbance of the blue-colored solution was determined at 750 nm using UV-VIS Spectrometer (Varioskan Flash Multimode Reader, Thermo Fisher

Scientific, USA). A standard curve was prepared using the same procedure with gallic acid (0, 25, 50, 75 and 100mg/L) (Biobasic Inc) (Appendix D). The total phenolic content of the carambola samples was expressed in mg of gallic acid equivalent (GAE) per gram of fresh weight.

7.2.3 Total antioxidant activity

The antioxidant activity (TAA) was carried out by evaluating the free radical-scavenging effect on the DPPH radical. The determination was performed based on the method by Alothman et al. (2010) with slight modifications. 1 g of pulp tissue was homogenized with 10 ml of absolute methanol and centrifuged at 4000 rpm for 15 min (Centrifuge 5810 R, Eppendorf, USA). 10 µl of supernatant was mixed with 90 µl of distilled water and 3.9 ml of 0.0250g L⁻¹ DPPH (Sigma-Aldrich, USA) methanolic solution. The mixture was vortex-mixed and kept in the dark for 30 min. The absorbance was taken at 515 nm using a UV-VIS Spectrometer (Varioskan Flash Multimode Reader, Thermo Fisher Scientific, USA) against a blank (methanol without DPPH). Results are calculated according to the following equation:

$$\% \text{ inhibition of DPPH} = \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \times 100$$

Results are expressed as the percentage of inhibition of the DPPH radical where Abs control is the absorbance of the DPPH solution without the extracts.

7.2.4 Membrane ion leakage

To determine the rate of ion leakage, the method described by McCollum and McDonald (1991) was used with slight modification. 15 fruit were randomly selected and held at 23°C for 30 min before 1 cm diameter pericarp discs (1 cm diameter/2 mm thickness) were excised with a stainless steel cork borer. 45 discs were washed three times with deionized water and incubated in 25 ml of 0.4 M mannitol (Merck, Germany) in 50 ml plastic tubes under constant shaking. The solution conductivity was measured after 1 hour with a conductivity tester (ECScan-40, Thermoline, Australia). A preliminary experiment showed that the ion leakage rate was constant between 1 and 2 hours. The reaction was heated in boiling water for 10 min and total conductivity was measured after cooling down to room temperature. The rate of ion leakage was expressed as a percentage of the total conductivity in 1 hour.

7.2.5 Lipid peroxidation (MDA content)

MDA is the main product of lipid peroxidation. The extraction was carried out according to Hodges et al. (1999) with slight modifications. 1 g pulp tissue was homogenized with inert sand in 1:25 (gFW:ml) 80% ethanol, followed by centrifugation at 4000 rpm for 20 min (Centrifuge 5810 R, Eppendorf, USA). 1 ml aliquot of appropriately diluted sample was added to a plastic tube with a 1 ml of either (a) –TBA solution comprised of 20% (w/v) trichloroacetic acid (R&M Chemicals, UK) and 0.01% butylated hydroxytoluene (Sigma-Aldrich, USA) or (b) +TBA solution (Merck, Germany) containing the above solution plus 0.65% TBA. The reaction mixture was mixed vigorously and heated at 95°C in a water bath for 25 min, cooled on ice for 10 min and centrifuged at 4000 rpm for 10 min. Absorbances were measured using a UV-VIS Spectrometer (Varioskan Flash

Multimode Reader, Thermo Fisher Scientific, USA) at 440, 532 and 600 nm. MDA equivalents were calculated using the following formula:

$$1) [(Abs\ 532_{+TBA}) - (Abs\ 532_{-TBA}) - (Abs\ 532_{-TBA} - Abs\ 600_{-TBA})] = A$$

$$2) [(Abs\ 440_{+TBA} - Abs\ 600_{+TBA}) \cdot 0.0571] = B$$

$$3) \text{MDA equivalents (nmol.mL}^{-1}\text{)} = (A - B / 157\ 000) \cdot 10^6$$

7.3 Results

7.3.1 Ascorbic acid (AA) content

In general, AA content initially showed a gradual increasing pattern up to 21 days followed by slight declined by the end of 28 days of cold storage. A similar trend was observed during shelf life at ambient when a sharp rise was observed after 3 days at ambient and a fall during the next 3 days (Figure 7.1). AA content in carambola was significantly ($p<0.001$) affected by PAs application and length of storage. Among the PAs examined, lower doses of SPD (1.0 and 0.1 mM) and highest dose of SPM (2.0 mM) had the greatest increase in AA content throughout the experiment.

A significant ($p<0.05$) increase of AA content during 3 days of shelf life particularly in fruit treated with 1.0SPD and 2.0SPM when both treatments recorded similar increment about 12.8-fold higher than at harvest. Meanwhile, fruit treated by lowest doses of SPD (0.1 mM) also showed an increasing trend up in AA content about 12.5-fold higher and control fruit had the lowest increment of AA content about 10-fold higher than at harvest (Figure 7.1). However, a rapid reduction in AA content was observed in all treatments by the end of the experiment. The declined in AA content was prominent in control fruit in comparison to PAs-treated fruit. Similarly, carambola treated with 1.0 and 0.1 mM of SPD and 2.0 mM of SPM also experienced losses in AA content, though it was relatively lower than in control fruit (Figure 7.1).

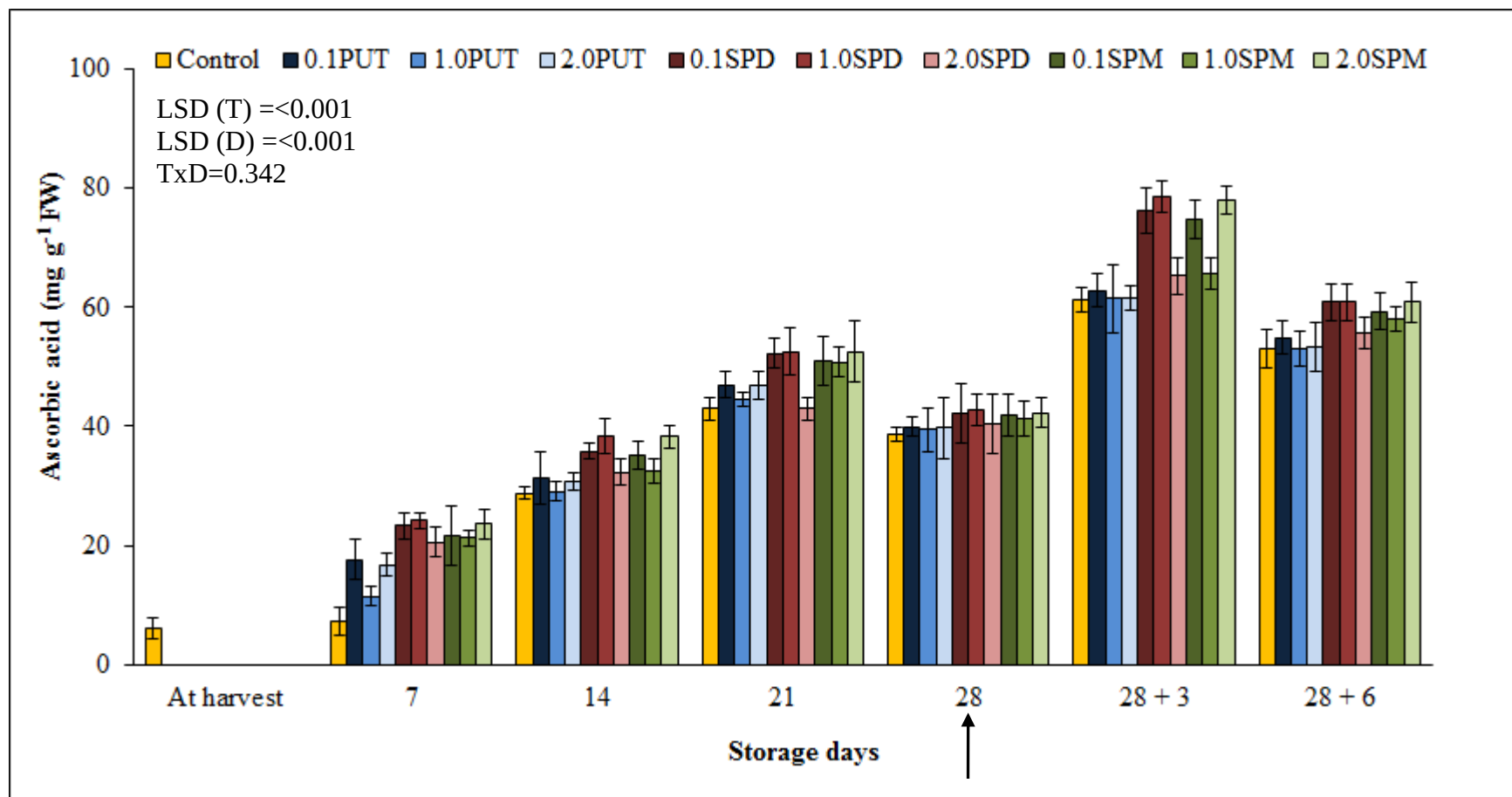


Figure 7.1: Effect of PUT, SPD and SPM treatment on the ascorbic acid level of B10 carambola during cold storage (3°C) for 28d followed by 6d at 22±1°C (shelf life). Vertical bars represent ±SE of pooled mean. n=15. An arrow (↑) indicates fruit was transferred from cold storage to ambient conditions for shelf life study. T=treatments, D=days, treatments x days = NS (p>0.05); NS = not significant

7.3.2 Total phenolic content (TPC)

Total phenolic content (TPC) was significantly ($p<0.001$) affected by PAs treatment and storage time. Initially, TPC increased gradually up to 7 days and thereafter exhibited a sharp rise coming to a peak at 14 days of cold storage, then demonstrated a sharp fall until the end of the experiment (Figure 7.2). Highest doses of SPM and SPD (2.0 mM) were equally effective boosting the TPC level than highest dose of PUT. During 14 days of cold storage, 2.0SPM showed greatest phenolic content about 14-fold higher followed by 2.0SPD and 1.0SPM with 12.5 and 10-fold higher than at harvest respectively (Figure 7.2). There was a significant ($p<0.001$) difference among the treatment during 14 days of cold storage.

After this point, TPC level declined drastically regardless the treatments and greatest reduction of TPC occurred at the end of the experiment. Control fruit displayed highest losses of the phenolic compound while fruit treated with 2.0SPM, 2.0SPD and 1.0SPM were equally effective in inhibiting the rate of TPC reduction. In addition, There was no significance difference ($p>0.05$) of all fruit with or without PAs treatments on day 21 onwards. SPM treatment affected TPC in dose dependent manner, where higher concentrations of phenolic compound were maintained by the highest doses of SPM (2.0 mM).

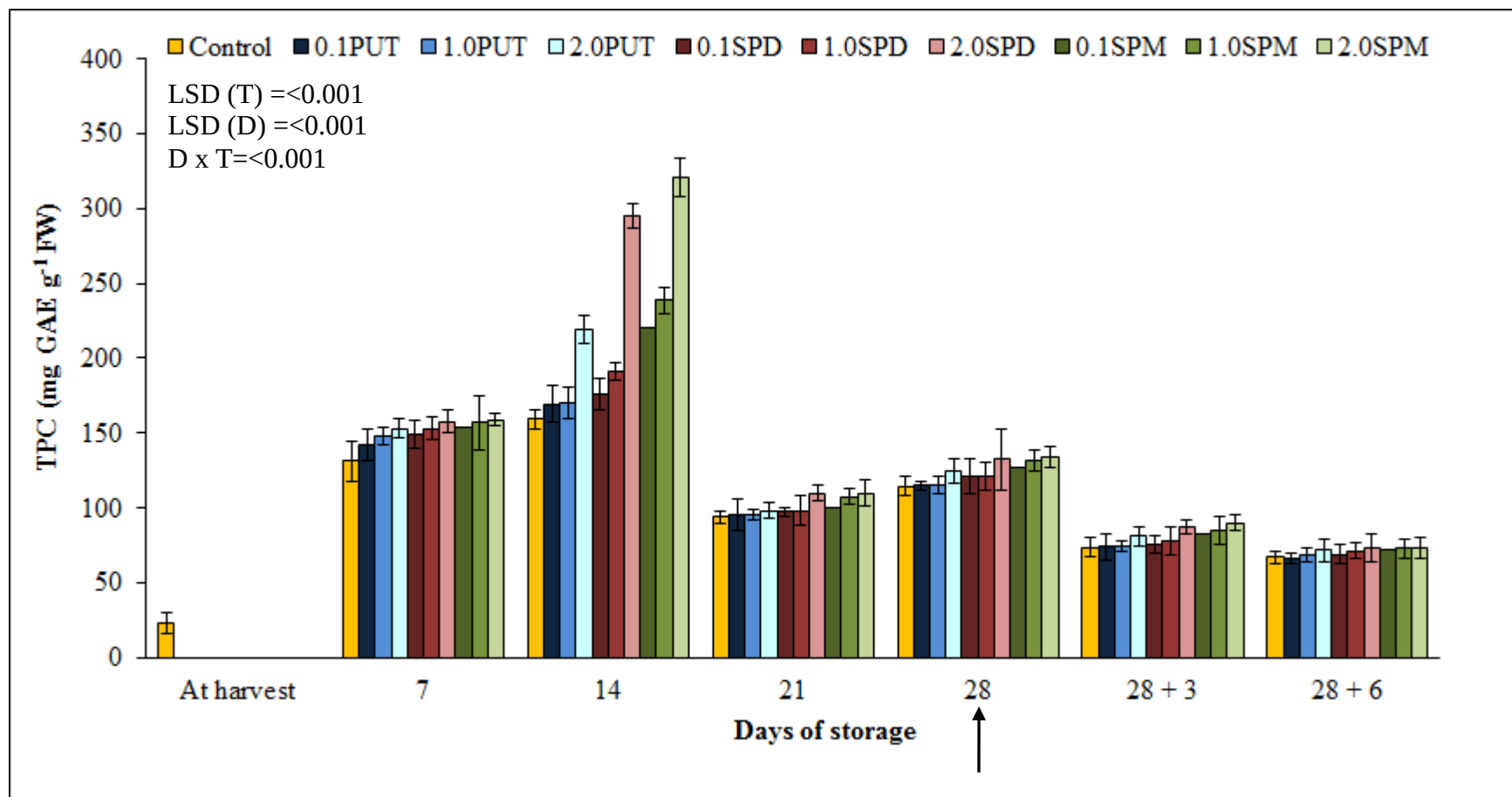


Figure 7.2: Effect of PUT, SPD and SPM treatment on total phenolic contents of B10 carambola during cold storage (3°C) for 28d followed by 6d at 22±1°C (shelf life). Vertical bars represent ±SE of pooled mean. n=15. An arrow (↑) indicates fruit was transferred from cold storage to ambient conditions for shelf life study. T=treatments, D=days, treatments x days = <0.001

7.3.3 Total antioxidants activity (TAA)

Irrespective treatments, TAA of carambola at first showed an increasing pattern from at harvest point up to 14 days of cold storage however, TAA declined gradually afterwards (Figure 7.3). During 14 days at cold storage, fruit treated by SPM, SPD and PUT at the highest concentration of 2.0 mM displayed highest TAA inhibition about 79.3, 77.5 and 66.7% respectively whilst control fruit showed 53.1% inhibition. The sharp reduction in TAA was observed on day 21 of cold storage irrespective of PAs treatments and concentrations used. By the end of cold storage, fruit treated with 2.0SPM, 2.0SPD and 2.0PUT demonstrated values of inhibition 45.8, 45.2 and 42.9% respectively while the control fruit had the lowest inhibition which was 37.09%.

TAA in both control and PAs-treated fruit declined at a rapid rate upon transferring to ambient for shelf life, where fruit treated with the highest concentration of SPM, SPD and PUT were effective to maintain TAA inhibition more than 23% at the end of the experiment. Similar to TAA, PAs application affected total antioxidant contents in dose dependent manner, where the higher activity of total antioxidant was maintained by the highest doses of SPM and SPD.

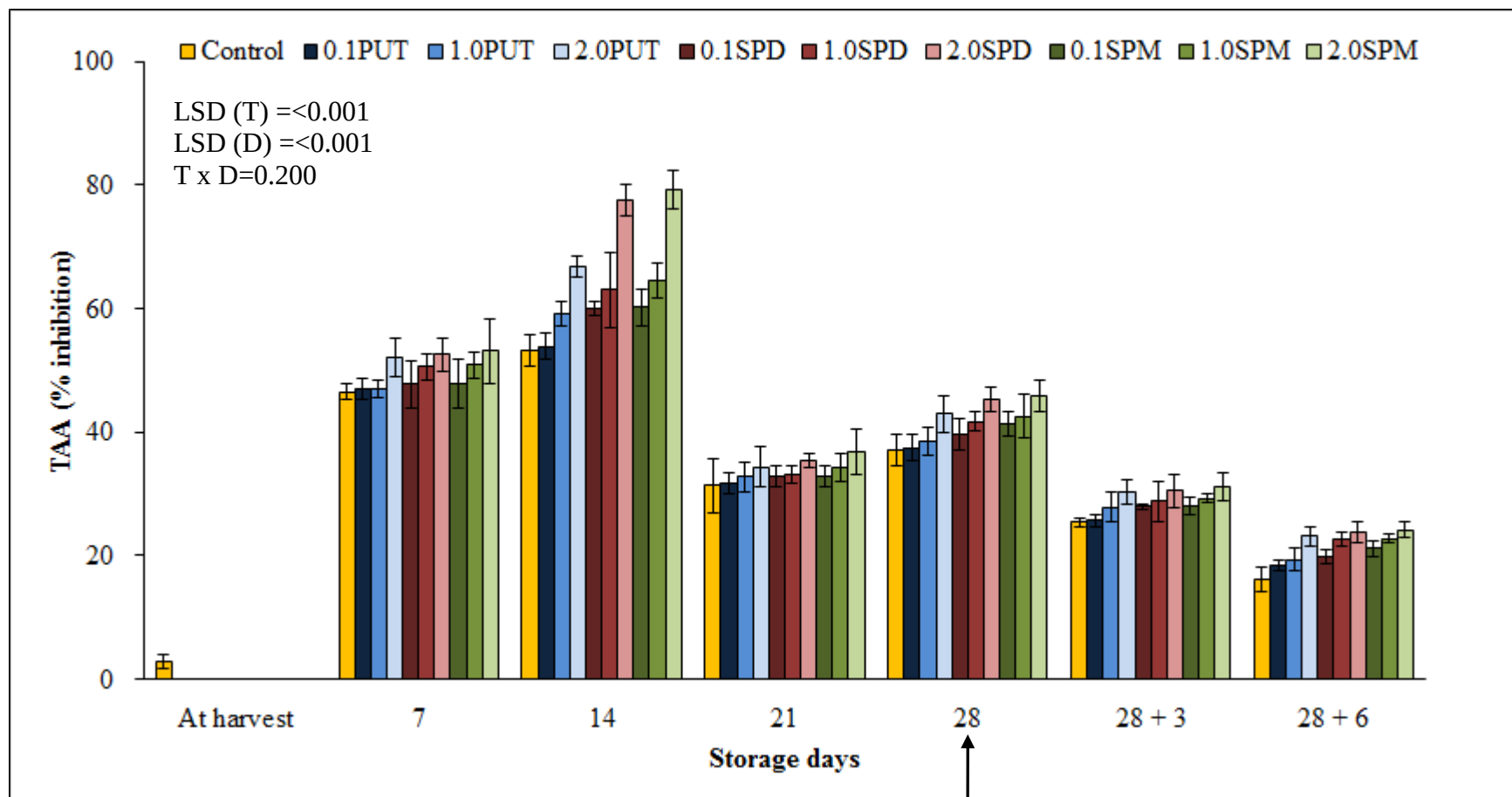


Figure 7.3: Effect of PUT, SPD and SPM treatment on the total antioxidant activity of B10 carambola during cold storage (3°C) for 28d followed by 6d at 22±1°C (shelf life). Vertical bars represent ±SE of pooled mean. n=15. An arrow (↑) indicates fruit was transferred from cold storage to ambient conditions for shelf life study. T=treatments, D=days, treatments x days = NS (p>0.05); NS = not significant

7.3.4 Membrane ion leakage

The rate and increment of membrane ion leakage was suppressed by treatment with PAs (Figure 7.4). Throughout cold storage, ion leakage rate demonstrated a gradual increases with control fruit exhibited highest percentage rate (23%) in contrast to fruit treated with PAs. Fruit treated with the highest doses of PUT and SPD (2.0 mM) showed similar percentage of ion leakage rate (21%). Parallel to PUT and SPD, lower doses of SPM (1.0 mM) also exhibited lower rate throughout cold storage with 22% of ion leakage.

However, a greater leakage percentage was observed upon shelf life in control fruit but the increasing rate was inhibited with PAs treatment. Overall, PUT treatment displayed a higher potential of inhibiting electrolyte leakage compared to other PAs. By the end of the experiment, control fruit showed maximum (49%) ion leakage rate in comparison to 2.0PUT which was the lowest (28%). The ion leakage rate in both 2.0SPD and 1.0SPM were relatively higher (30%) compared to 2.0PUT. At the end of experiment, there was a significant difference ($p < 0.05$) between all the treatments (Figure 7.4).

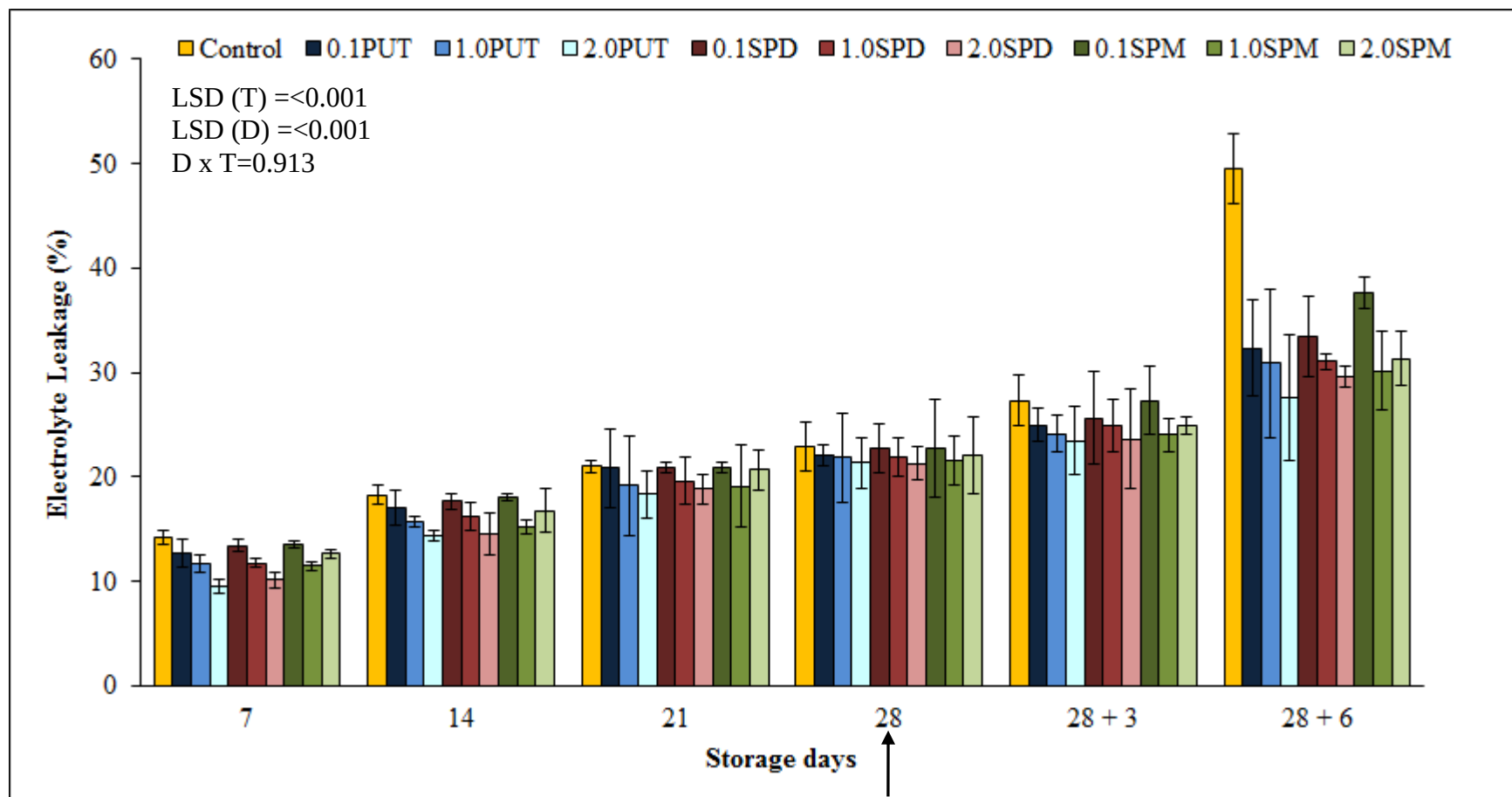


Figure 7.4: Effect of PUT, SPD and SPM treatment on ion leakage (%) of B10 carambola during cold storage (3°C) for 28d followed by 6d at 22±1°C (shelf life). Vertical bars represent ±SE of pooled mean. n=15. An arrow (↑) indicates fruit was transferred from cold storage to ambient conditions for shelf life study. T=treatments, D=days, treatments x days = NS (p>0.05); NS = not significant

7.3.5 Lipid peroxidation (MDA content)

The content of malondialdehyde (MDA), was significantly ($p<0.001$) suppressed by PAs treatment as compared to control fruit upon storage at 3°C and MDA content increased drastically thereafter during shelf life. In control fruit, MDA content increased steadily and 13-fold higher throughout 28 days of cold storage than at harvest. A similar trend was also showed in PAs-treated fruit however, the MDA content was much lower than that in control fruit. Both treatments by PUT and SPD at 2.0 mM dose equally effective in reducing MDA content increment throughout cold storage about 10-fold higher than at harvest (Figure 7.5).

It should be stressed that the treatment with the highest dose of PUT and SPD (2.0 mM) were the best treatments resulted in the highest reduction of MDA increase although there was no significance difference between two doses throughout the experiment. At the end of shelf life, the MDA level of 2.0PUT and 2.0SPD was 25 and 27-fold higher than at harvest respectively and the levels were far lower as opposed to control fruit (Figure 7.5). Compared with MDA content at the beginning of storage, control fruit displayed an incredibly high MDA content by 96.4% over time. The output suggested that exogenous PAs application accounted for lower lipid peroxidation due to chilling exposure.

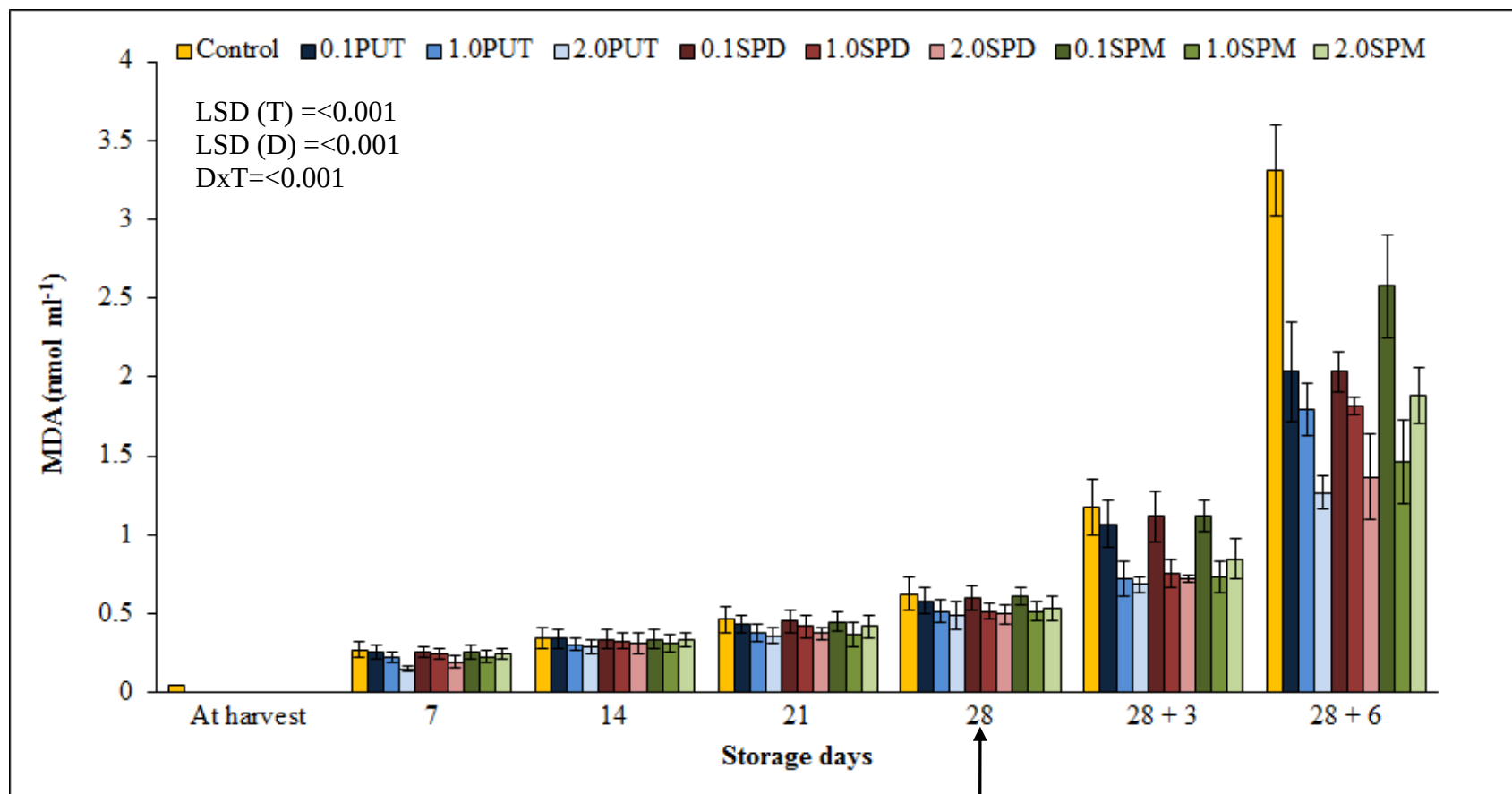


Figure 7.5: Effect of PUT, SPD and SPM treatment on MDA levels of B10 carambola during cold storage (3°C) for 28d followed by 6d at 22±1°C (shelf life). Vertical bars represent ±SE of pooled mean. n=15. An arrow (↑) indicates fruit was transferred from cold storage to ambient conditions for shelf life study. T=treatments, D=days, treatments x days = <0.001

7.4 Discussion

7.4.1 Impact of PAs application on functional properties with antioxidant activity in carambola during cold storage and ripening

Ascorbic acid (AA) has garnered huge attention due to their functional properties and is important from the standpoint of human health. This water-soluble antioxidant is capable in detoxification of active oxygen, in aiding removal of harmful H_2O_2 , $OH^\bullet$ and O_2^- levels in plant cell and has a strong protective effect against ROS oxidative activities (Valdenegro et al., 2012). Since, human body are not able to synthesize AA, more than 90% of this bioactive compound is supplied from human diets such as citrus fruit and leafy vegetables (Lee and Kader, 2000; Yahia et al., 2001).

Carambola fruit used in this study is a rich source of health promoting compounds because of the occurrence this bioactive compound. Application of PAs induced significantly higher ($p<0.001$) AA content, in contrast to the control fruit in which a considerable loss of this antioxidant was detected. Moreover, no significant loss of AA was detected during 3°C storage in all PAs-treated fruit. AA activity level was also coupled with rapid changes prior transferring to ambient with fruit treated with 1.0SPD contains up to 79.0 mg of AA (Figure 7.1).

In all cases, a small reduction of AA level has been observed during the final day of shelf life and this change could be connected to the advancement of ripening-related changes and resulted in fruit quality losses. In particular, decrease in endogenous PAs level during ripening also highly correlated with a reduction in AA content since PAs intimately involved in delaying ripening process (Valero et al., 2002). Another reason behind AA losses could be due to activation of ascorbate oxidase; a major enzyme responsible for enzymatic degradation of AA to dehydroascorbic acid (DHAA) and (Lee

and Kader, 2000; Mirdehghan et al., 2007c; Khan et al., 2008).

The exact mechanism of SPD and SPM affected ascorbic acid enhancement is still a mystery. However, higher AA content was found after the PAs treatment could be ascribed to the PAs nature to act as antisenescence which responsible for blocking the action between APX and AA therefore accountable in extended shelf life and retardation of senescence (Mirdehghan et al., 2007c). For instance, a study conducted by Mehta et al. (2002) proved the direct role of PAs as an antisenescence agent by attesting in engineered tomatoes under SAMDC1 gene promoter lead to accumulation of SPD and SPM, which enhanced juice quality and content of lycopene two-threefold higher than cultivated tomatoes.

Phenolics are secondary metabolites and are believed to be localized in the vacuole in different forms. The accumulation of this phytochemicals is attributed to the induction of phenylpropanoid metabolism in plant tissue (Balasundram et al., 2006; Kou et al., 2015). A group of phenolic compounds; flavonoids are the most common and widely distributed in plant kingdom with proanthocyanidins and epicatechin being the most abundant phenolics compound in carambola (Shui and Leong, 2006). Phenolic compounds are responsible for a large share of the antioxidant activity of fruits and vegetables.

Total phenolic contents (TPC) in the carambola pulp were greatly improved with PAs treatment compared to control and the maximum increase was observed with SPM treatment (27%) followed by SPD (17%) throughout experiment period. Application of PAs particularly SPM was effective in maintaining the concentration of phenolic compounds with 2.0SPM accumulates up to 22 mg phenolic content per 100 g fresh fruit during 14 days at cold storage (Figure 7.2). Apart from PAs effect, the increment of TPC

level during storage could also be attributable to the exposure to low temperature and under this conditions induce the activation of enzyme phenylalanine ammonia lyase (PAL) activity and stimulate the biosynthesis of phenolic compounds, and ultimately higher total phenols and flavonoids accumulation (Alothman et al., 2010).

A pronounced decrease in the level of TPC was observed in all fruit regardless the treatment with the advancement of storage period and this reduction in the values of total phenolics in fruit is probably due to the oxidation by polyphenol oxidase (PPO) (Shiri et al., 2013; Sayyari et al., 2016). Declining of TPC was also influenced by the storage conditions and depends on the fruit species even on the cultivar (Aguayo et al., 2010; Sdiri et al., 2012).

Total antioxidant activity (TAA) was significantly increased during storage and ripening irrespective of treatments. However, greatest activity in TAA during storage was more noticeable in highest concentrations of PAs-treated fruit particularly SPM and SPD at 2.0 mM. Averaged over PUT, SPD and SPM applications, mean total TAA about 38%, 41% and 42% inhibition respectively at the end of the experiment were higher compared to the controls with 35% inhibition. This present study revealed the TAA pattern mostly resembled the activity pattern in total phenolics throughout cold storage and subsequent 3 days at ambient (Figure 7.2 and 7.3).

The reason for the similar patterns between TAA and TPC is the activity of DPPH free radical scavenging largely contributed by abundant in presence of phenolics compounds, whereas a very little contribution by ascorbate and carotenoids (Diaz-Mula et al., 2009; Singh et al., 2012). The quenching ability of phenolic compounds against DPPH radical also contributed by a number of hydroxyl groups available in molecules

and on their electron donating property (Balasundram et al., 2006; Rapisarda et al., 2008).

Irrespective of PAs treatment concentrations, enhancement of total phenolic and antioxidant contents found in the fruit could be attributed to the PAs properties to delay the senescence process and to act as free radical scavengers (Mirdehghan et al., 2007c; Razzaq et al., 2014). In addition, this finding also indicated that the PAs treatment enhances remarkable changes in functional properties following the sequence of positive charges per molecules that is, $SPM^{4+} > SPD^{3+} > PUT^{2+}$ (Mirdehghan et al., 2007c). Moreover, it has been reported that PAs might have a part of the SOD/ascorbate-glutathione cycle that enables PAs to function as a protective antioxidant (Kim and Jin, 2006).

TPC and TAA, as well as the AA content in carambola exhibited significant responses to PAs treatment and that the concentration of bioactive compounds with antioxidant activity was maintained at higher levels than in control fruit throughout the experiment. Moreover, surges in activities or nonsignificant losses of these bioactive compounds were observed during cold storage after PAs application. PAs affected antioxidant activities in carambola in a dose-dependent manner, where higher values of TPC and TAA were found in the fruit treated with the highest concentration of SPM (2.0 mM). On the other hand, higher AA content was maintained by the lower doses of SPD (1.0 and 0.1 mM).

An interesting point of this study was treatment with a higher number of amino groups such as SPM and SPD showed higher efficacy in TPC and TAA aspect than PUT, in agreement with the previous work by Mirdehghan et al. (2007b) in pomegranate. Although most of the studies dealing with postharvest treatments of PUT to boost the

concentration of bioactive compounds in fruit as reported in plum (Khan et al., 2008), table grape (Shiri et al., 2013), mango (Razzaq et al., 2014) and orange (Habibi and Ramezani, 2017) this present result sheds light on the role of SPM and SPD on increasing the content of health-beneficial compounds in fruit.

7.4.2 Roles of PAs in reducing membrane ion leakage and lipid peroxidation level in carambola during cold storage and ripening

Chilling injury (CI) is an oxidative phenomenon in fruits and the primary effects of the oxidative injury are impairing energy state of the cell and/or alteration in the conformation of cell membranes (Hodges et al., 2004; Yahia et al., 2006; Imahori et al., 2008). Common feature accompanying CI and senescence is a significant increase in membrane permeability (Sayyari et al., 2016). Membrane ion leakage often used as an indicator for an early manifestation of tissue collapse following chilling storage and to evaluate treatment efficacy that modifies sensitive plants susceptibility towards chilling exposure (Wu et al., 2014).

Suppression of ion leakage increment by PAs application particularly PUT has been reported for tomato (Kim et al., 2002), squash (Martinez-Tellez et al., 2002), pomegranate (Mirdehghan et al., 2007a), pepper (Liu et al., 2013), lemon (Safizadeh et al., 2013) and table grape (Champa et al., 2014). Similarly, PAs treatment decreased ion leakage in cold-stored carambola and PUT treatment effectively reduced ion leakage 44.2% in comparison to control fruit. The mechanism by which PAs can reduce ion leakage is still vague, although it has been suggested that these compounds can reduce or delay the effects of chilling stress by its property to protect and stabilize cellular membranes integrity (Mirdehghan et al., 2007a; Bassard et al., 2010; Champa et al., 2014).

A study carried out by Gomez-Galindo et al. (2004) reported that storage at low temperatures altered membrane lipids composition resulting in membrane permeability subsequently causing ions leakage. In this sense, an increase in ion leakage was observed in the pulp of control fruit, indicating that their membrane had become damaged. By contrast, the application of PUT seems to enhance membrane integrity leading to membrane fluidity maintenance at cold storage, which was associated with the inhibition of ions leakage (Mirdehghan et al., 2007a; Liu et al., 2013).

Moreover, the content of MDA (malondialdehyde), a toxic and end product from oxidative degradation of polyunsaturated fatty acids (Luo et al., 2011), was also measured in cold-stored carambola to further evaluate the function of PAs treatment in chilling-induced membrane damage. MDA content is a biomarker for oxidative stress that can damage membranes structural integrity in the plant (Cao et al., 2010; Luo et al., 2011). Enhanced lipid peroxidation as indicated by MDA content causes damaging effect in the membranes barrier functions and decrease in membrane fluidity (Nukuntornprakit et al., 2015). This study revealed that MDA accumulation in control fruit increased throughout experiment but the levels was significantly inhibited by PAs treatment particularly PUT (Figure 7.5).

Reduction of MDA content in treated carambola could be ascribed to PAs may exhibit antioxidant capability in scavenging free radicals (Lester, 2000) or most likely did so by preventing activation of superoxide NADPH oxidase that allow PAs to protect cellular membrane from lipid oxidation during exposure to low temperatures (Mirdehghan et al., 2007a; Pang et al., 2007).

7.5 Conclusion

In conclusion, our data reveal the beneficial effect of PAs application on maintaining and enhancing functional properties with antioxidant activity in carambola throughout cold storage and ripening at shelf life. SPM and SPD at 2.0 mM were found to be the best treatments to maintain higher total phenolic content and total antioxidant activity. Meanwhile, the greatest increase in ascorbic acid level was demonstrated in fruit received SPD treatment at lower concentrations (1.0 and 0.1 mM). PUT application at 2.0 mM protects and provides membrane tolerance against chilling oxidative damage through significant reduction in membrane leakage and retardation in lipid peroxidation.

CHAPTER EIGHT

CHANGES IN ENDOGENOUS FREE POLYAMINES CONTENT IN RESPONSE TO POLYAMINES TREATMENT IN CARAMBOLA DURING COLD STORAGE AND RIPENING

8.1 Introduction

Polyamines (PAs) can exist as free soluble form, bound to macromolecules such as protein and conjugated to phenolic compounds (Bregoli et al., 2006; Nambeesan et al., 2008). It has been proposed that PAs in their free soluble form have profound effects as antisenescence agent and participate more actively in many biochemical and physiological processes (Valero et al., 2002). Conjugated PAs is thought to act as storage forms of PAs released into free forms during growth phase (Valero et al., 2002). PAs that are covalently linked to proteins involve in flower induction and reproduction (Kakkar and Sawhney, 2002).

The intracellular levels of PAs are tightly regulated and undergo substantial alterations during plant growth and development (Dios et al., 2006; Wimaleskera et al., 2011). The level of PAs is high during early fruit growth when intense cell division occurs but decrease at the later stage of fruit growth and ripening (Bregoli et al., 2002; Liu et al., 2006). Variations in cellular PAs level have been widely investigated in many plant systems and markly vary with plant species (Groppa and Benavides, 2008; Bassard et al., 2010).

Plants are sessile and considered as sensitive organisms therefore often subjected to a variety of abiotic stresses. Such unfavorable conditions are seriously affecting crop productivity and gradually become a severe threat to sustainable agriculture (Gill and

Tuteja, 2010). PAs have been associated closely with plant tolerance to adverse stress responses (Wimalasekera et al. 2011). In higher plant, elevation of intracellular PAs concentration appears to be adaptive mechanism to numerous environmental stresses with activation of antioxidant activity (Bassard et al., 2010), upregulating stress-related gene expression (Alcázar et al., 2010), directly altering cell membrane lipid properties (Königshofer and Lechner, 2002) and modulating the endogenous PAs metabolism (Pang et al., 2007). In addition to these protection modes, PAs in conjugation with other plant hormones such as GABA (γ -aminobutyric acid), ABA (abscisic acid), jasmonic acid (JA) and salicylic acid (SA), all known to mitigate negative effects of abiotic stresses (Gupta et al., 2013; Caldazilla et al., 2014; Palma et al., 2015).

Postharvest treatment with plant growth regulators such as PAs has been demonstrated to be effective tools in managing chilling injury in fruits by increasing endogenous levels of PAs in fruit cells (Bassard et al., 2010; Wimalasekera et al. 2011). Significant changes in PAs levels were positively correlated with the ability of fruit to withstand chilling stress (Valero et al., 2002). However, it is unclear if the increment in endogenous PAs levels is part of the adaptive mechanisms that protect and prevent tissue from CI or if they are general tissue responses towards cold storage (Abu-Kwapoh et al., 2002; Valero et al., 2002).

High performance liquid chromatography (HPLC) is a common technique used for endogenous PAs quantification. Previously, either HPLC or thin layer chromatography (TLC) were applied for between PAs determination and however, higher PAs content was detected in HPLC compared with those obtained by TLC (Rossetto et al., 2015). HPLC of benzoylpolyamines has been employed for analysis of higher plant extracts, without

significance interference from nonspecific compounds such as carbohydrate and phenolics. In addition, benzylation procedure allows extracts to be analyzed directly without undergoing purification procedures. Another advantage is benzyolated extracts are stable for several months without substantial losses of PAs compound (Flores and Galston, 1982).

In line with this, present work was undertaken to inspect the effects of postharvest treatment of PAs on the changes in endogenous PAs content, and to clarify PAs mode of action when carambola was subjected to postharvest cold storage, implying that these naturally occurring compounds maybe directly involved providing CI resistance in carambola during prolonged exposure at low temperatures.

8.2 Materials and methods

Carambola was subjected to the PAs treatment as described in sub-section 3.2.1 and freeze-dried fruit mesocarp kept at -80°C was used in this HPLC analysis prior cold storage and shelf life at ambient. For all PAs concentration including control fruit, three replicates each consisting of three fruit (n=9) were used in this experiment. The addition of benzoyl chloride into homogenate was done in a fume hood. Before starting each run, the HPLC system was warmed up and the baseline was allowed to stabilize.

8.2.1 Benzylation of free PAs

Free PAs content was analyzed according to Flores and Galston (1982) with some modifications. One gram of mesocarp tissue was extracted with 1 ml of cold 5% HClO₄ (Merck, Germany) and incubated on ice for 1 hour. The homogenate was centrifuged for 30 min at 12 000 rpm (Centrifuge 5810 R, Eppendorf, USA). 2 ml of free PAs in the

supernatant was benzoylated by adding 2 ml of 4N NaOH. 20 μ L benzoyl chloride (Merck, Germany) was added followed by vortexing for 15 sec and incubation for 20 min at RT. The supernatant was added with 4 ml of saturated NaCl. 4 ml of chilled diethyl ether (Merck, Germany) was added into supernatant thereafter and centrifuged at 12 000 rpm for 15 min. 2 ml of ether phase was pipetted out, evaporated to dryness under a stream of warm air. The dried residue was solubilized with 1 ml of methanol (HPLC grade) and filtered through 0.45 μ m nylon membrane filter (Millipore, Agilent, USA) before HPLC analysis (Agilent 1260, Foster City, CA, USA).

8.2.2 Analysis of free PAs by High Performance Liquid Chromatography (HPLC)

20 μ L of benzoyl-polyamines were eluted through a 250 x 4.6 mm reverse-phase C18 column packed with 5 μ m Hypersil ODS resin (Agilent, USA). The solvent system consisted of acetonitrile:water (HPLC grade), run isocratically at 52:48, at a flow rate of 1 ml/min and detected by absorbance at 254 nm. A relative calibration procedure was used to determine the PAs in the sample extracts, using 100 nmol of 1,6-hexadiazine (Sigma-Aldrich, USA) as the internal standards and PAs standard curves ranging from 1.25-40 μ M (Appendix E). The calibration curves were $y=4.409x-3.366$, $r^2=0.98$ for PUT, $y=1.648x$, $r^2=0.98$ for SPD and $y=2.1199x+0.8818$, $r^2=0.99$ for SPM. Results were expressed as nmol g⁻¹ fresh weight.

8.3 Results

8.3.1 Putrescine (PUT) content

In all cases, carambola subjected to 3°C storage for 28 days exhibited an initial increase in free PUT content during the first 7 days regardless the treatments. Later, the concentrations of PUT continued to increase gradually up to 21 days, giving rise to a major peak at the final day of cold storage. However, PUT content declined substantially prior shelf life at ambient for first 3 days and then maintained relatively constant PUT concentrations for 3 days later (Figure 8.1). In control fruit, a moderate increase was observed throughout cold storage and PUT content increased 16.2-fold higher than initial storage time. Similar but greater results were observed in fruit treated with 2.0PUT, 0.1SPM and 2.0SPD when the treatments lead to a striking increase in accumulation of free PUT content with values 44.2-fold, 28-fold and 26.3-fold higher than day 7 at cold storage respectively.

During shelf life at ambient, all fruit displayed a similar pattern of sharp decreasing in PUT content irrespective the treatments (Figure 8.1). At the end of the experiment, PUT content in fruit treated with 2.0PUT diminished progressively by about 17-fold lower than a final day at cold storage. Declining trends were also observed in fruit treated with 2.0SPD and 0.1SPM with values of 10.3-fold and 10-fold lower than day 28 respectively. Meanwhile, control fruit experienced 3.5-fold lower of PUT content at the end of the experiment. There was no significant difference ($p>0.05$) between PAs-treated and control fruit throughout experiment although changes in PUT content were remarkable.

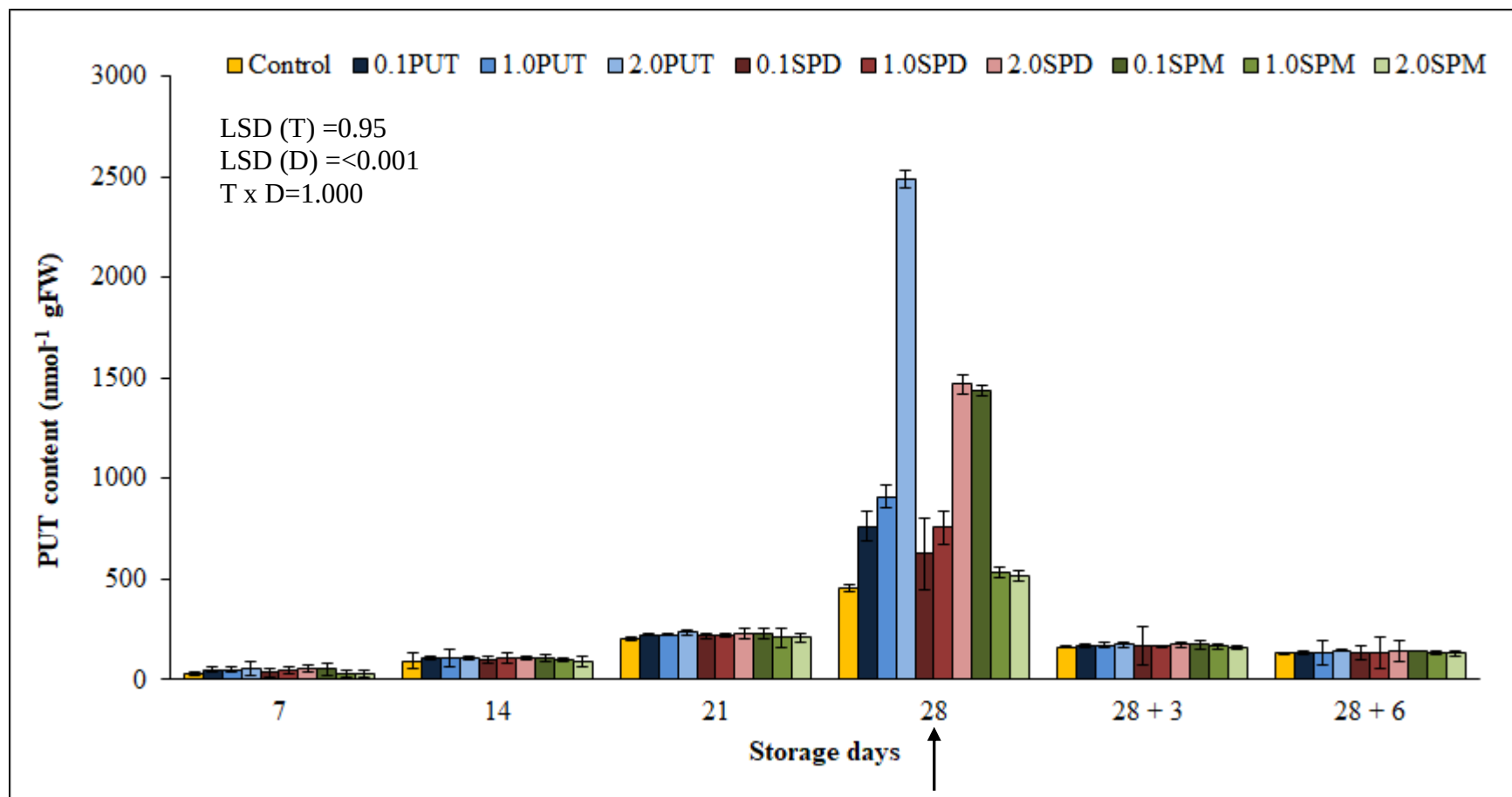


Figure 8.1: Effect of PUT, SPD and SPM treatment on endogenous free PUT content in B10 carambola during cold storage (3°C) for 28d followed by 6d at 22±1°C (shelf life). Vertical bars represent ±SE of pooled mean. n=9. An arrow (↑) indicates fruit was transferred from cold storage to ambient conditions for shelf life study. T=treatments, D=days, treatments x days= NS (p>0.05); NS = not significant. Value at harvest (Day 0): PUT content (nmol⁻¹ g FW)=0.00.

8.3.2 Spermidine (SPD) content

The result demonstrated that free endogenous SPD followed a pattern similar to those for free PUT content but with lower amplitude. One week (day 7) after PAs application, a steady and continuous increment in SPD levels throughout cold storage was observed in all fruit irrespective the treatments. Free SPD occurrence was the greatest on the final day of cold storage and thereafter the levels reduced prior transferring to ambient and remained approximately constant until the end of the experiment (Figure 8.2). Accumulation of endogenous SPD on day 28 at cold storage was further enhanced particularly in fruit subjected to the highest doses of PUT and SPD (2.0 mM) reaching maximum values of 17.4-fold and 16.2-fold increase than the initial storage time respectively.

Interestingly, control fruit demonstrated the lowest SPD concentration at day 7 but experienced dramatic changes on day 28 with free SPD value range from 4.4 to 287 nmol g⁻¹ FW (Figure 8.2). During shelf life at ambient for first 3 days, fruit that received 2.0PUT and 2.0SPD exhibited a considerable decrease in free SPD levels about 2.8-fold and 2.4-fold lower than the final day of cold storage respectively. In contrast, SPD levels in control fruit were also affected with less change (1.9-fold lower) as compared to PAs-treated fruit. Thereafter, a slight fall in SPD levels was observed in all fruit at the end of the experiment.

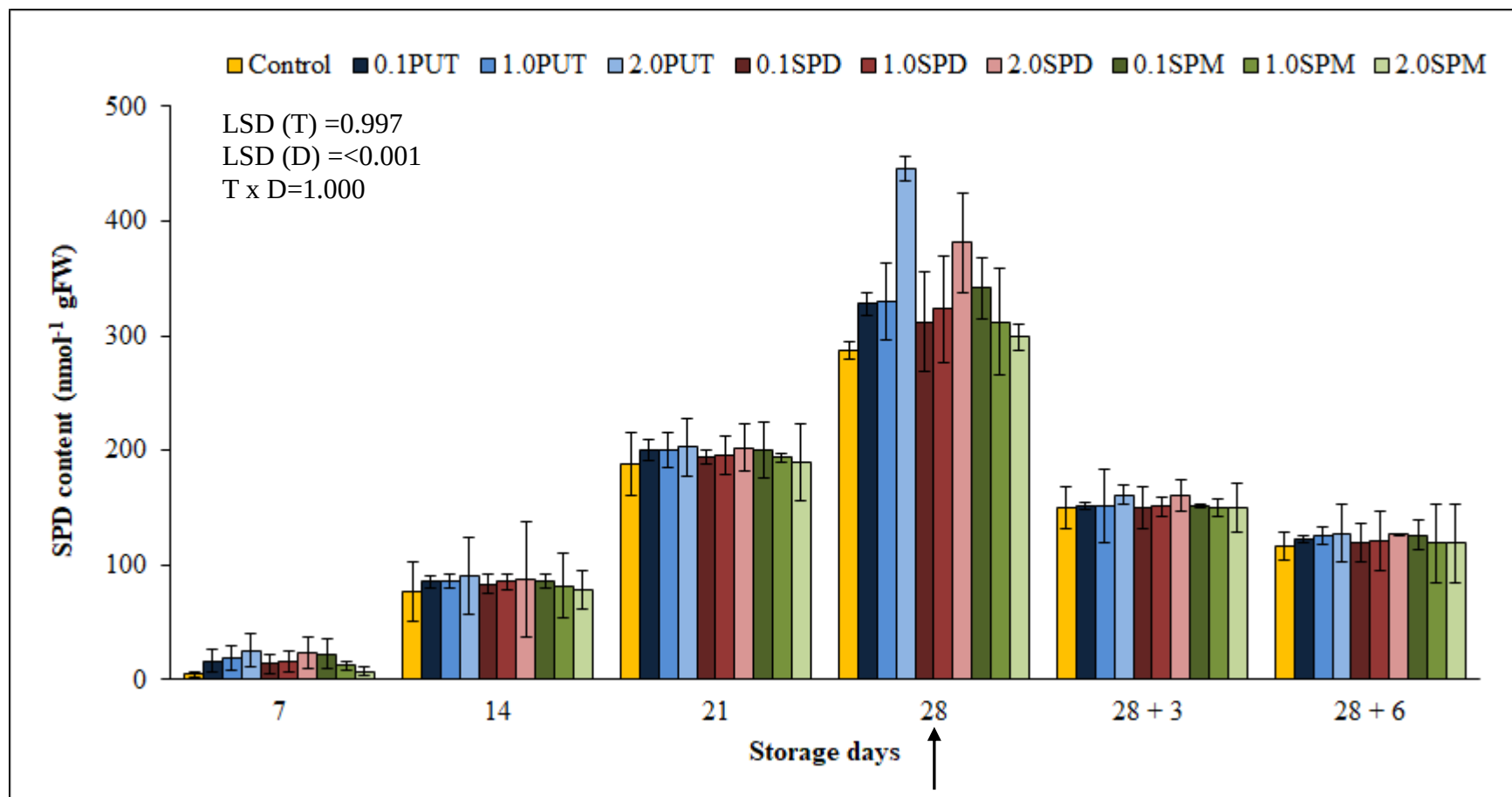


Figure 8.2: Effect of PUT, SPD and SPM treatment on endogenous free SPD content in B10 carambola during cold storage (3°C) for 28d followed by 6d at 22±1°C (shelf life). Vertical bars represent ±SE of pooled mean. n=9. An arrow (↑) indicates fruit was transferred from cold storage to ambient conditions for shelf life study. T=treatments, D=days, treatments x days= NS (p>0.05); NS = not significant. Value at harvest (Day 0): SPD content (nmol⁻¹ g FW)=0.00.

8.3.3 Spermine (SPM) content

As shown in Figures 8.3 in the tissue of carambola, SPM content was the least free PAs and accumulation pattern was in accord with of free PUT and SPD levels. Changes in free endogenous PAs significantly increased with the advancement of storage ($p<0.001$). SPM level was undetectable during first 7 days at 3°C storage however, a small amount was observed in fruit induced with 2.0PUT (3.8 nmol g⁻¹ FW) and 2.0SPD (1.7 nmol g⁻¹ FW). Later, free SPM content increased significantly and persisted up until the end of cold storage with fruit subjected to 2.0PUT and 2.0SPD exhibited the highest level of free SPM with mean values of 281.7 and 280.2 nmol g⁻¹ FW respectively. Similarly, a gradual rise was also observed in fruit received the lowest dose of SPM (0.1 mM) and control fruit with mean values of 265.5 and 243.2 nmol g⁻¹ FW respectively.

However, endogenous SPM levels declined during shelf life at ambient in both control and PAs-treated fruit, although the levels were always significantly higher in the tissue of treated fruit (Figure 8.3). At the end of the experiment, carambola treated with 2.0PUT and 2.0SPD experienced reductions when both treatments showed an about 2.4-fold decrease in SPM content final compared to when a final day at cold storage. Meanwhile, fruit subjected to 0.1SPM showed a decrease of SPM level about 2.3-fold lower than day 28. Parallel with treated fruit, control also displayed a decline in SPM accumulation with 2.2-fold lower.

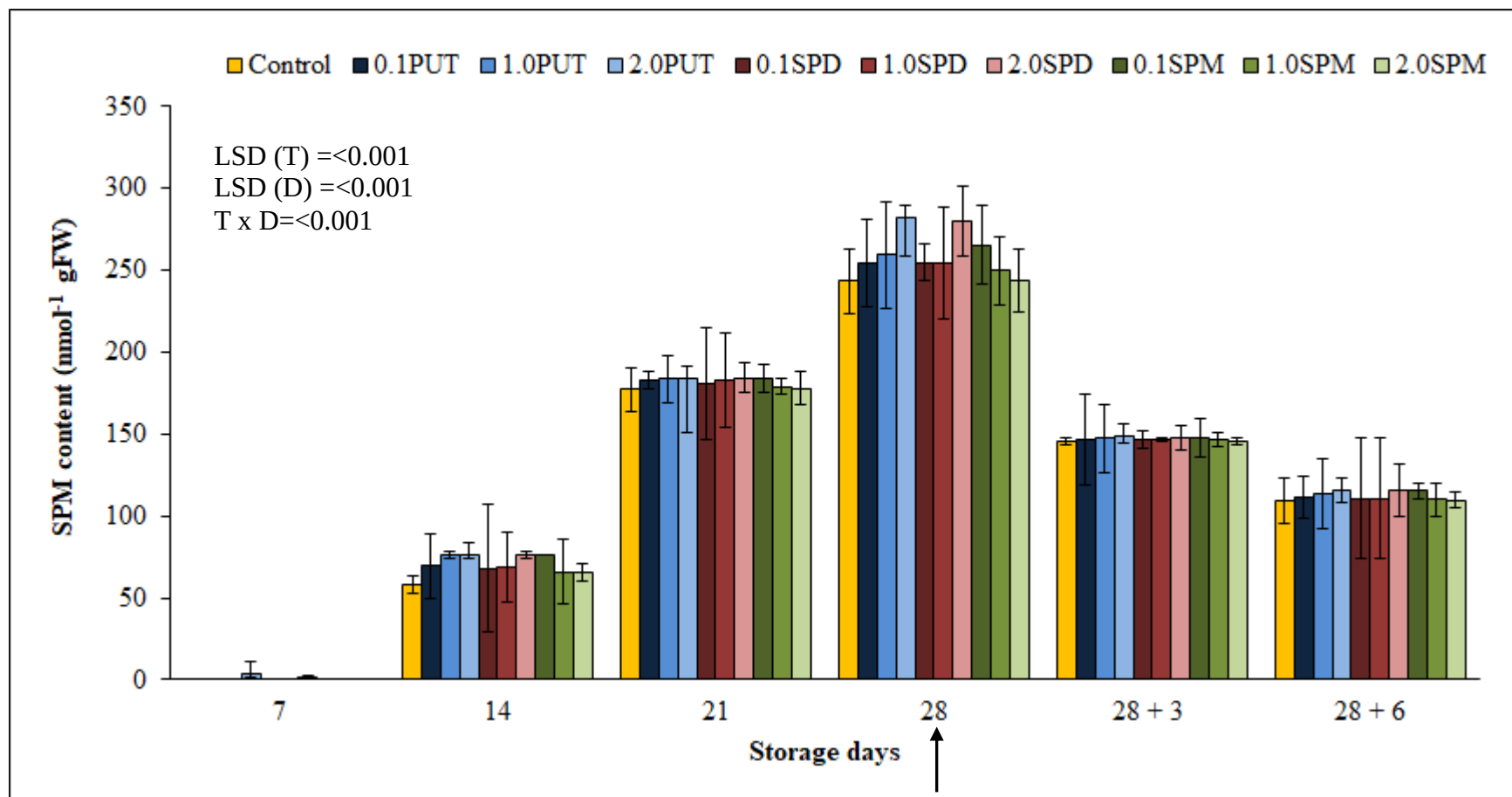


Figure 8.3: Effect of PUT, SPD and SPM postharvest treatment on endogenous free SPM content in B10 carambola during cold storage (3°C) for 28d followed by 6d at 22±1°C (shelf life). Vertical bars represent ±SE of pooled mean. n=9. An arrow (↑) indicates fruit was transferred from cold storage to ambient conditions for shelf life study. T=treatments, D=days, , treatments x days= <0.001. Value at harvest (Day 0): SPM content (nmol⁻¹ g FW)=3.50±0.01.

8.4 Discussion

8.4.1 Endogenous free PAs accumulation of carambola in relation to PAs postharvest treatment and alleviation of chilling stress

The concentration of endogenous free PAs in plant tissues, in brief, initiated with a peak level of PAs during fruit growth phase mainly characterized by rapid cell division. PAs levels drop at fruit development phase, ripening and senescence stage take place thereafter when free PAs levels are at their lowest (Liu et al., 2006; Torrigiani et al., 2012). Major free endogenous PAs in plant namely PUT, SPD and SPM were detected and quantified in carambola tissue during 28 days of cold storage and shelf life, with PUT being the most abundant free PAs followed by SPD and contain much less SPM (Figure 8.1 – 8.3).

Endogenous free PAs content particularly PUT and SPD in carambola tissues showed a gradual and remarkable increment throughout cold storage and the maximum accumulations occurred during the final day of cold storage. The results indicated that the increase in the endogenous pool of PAs could be due to exogenous supplied PAs could trigger stimulation of the PA biosynthesis pathway and inducing the synthesis of new PAs in fruit tissues (Alcazar et al., 2006). Previous reports suggested that exogenous PUT that is taken up in fruit tissue, a part of the PUT was synthesized to SPD then transformed into SPM using SAM decarboxylase (DCSAM) and another part of PUT bound to the cell wall (Perez-Vicente et al., 2002; Mirdehghan et al., 2007a).

In addition, a substantial increase in PUT levels has been correlated with up-regulation of ADC, an enzyme that involved in regulating PUT biosynthesis pathway (Mo and Pua, 2002; Groppa and Benavides, 2008). However, the increasing trend was coupled with reduced level once fruit transferred to ambient for shelf life. A drastic

decrease in PUT and SPD contents meanwhile a slight fall in SPM accumulations were observed during first 3 days of shelf life and an endogenous pool of PAs maintained constant afterwards. In this sense, decreases in PAs concentration during shelf life was closely related to the initiation of ripening process in carambola since intracellular PAs accumulation decreases concomitantly with the acceleration of tissue senescence as observed in a wide range of climacteric and non-climacteric fruit (Valero et al., 2002).

Furthermore, results further revealed that the level increment of individual PAs in carambola tissue was dependent upon the type and concentration of the PAs applied. PUT application seems to be more efficient since this molecule is taken up to a greater extent than SPD and SPM in carambola tissue. In all cases, intracellular PAs levels appear to be greater in fruit administered with highest doses of PUT or SPD (2.0 mM) and lowest concentration of SPM (0.1 mM). Given the strong effect on the endogenous pool of PAs, it was reasonable to assume that the enhanced PAs content in fruit tissue is modulated by PAs postharvest treatment.

In addition, analysis of the PAs content in the treated fruit presented important metabolic interactions. For instance, a significant concentration of PUT after 2.0SPD treatment was found and that the increase of PUT level could be attributed from inhibition of SPD synthase or could be due to SPD to PUT back-conversion or reverse reaction (Kramer et al., 1991). This back-conversion of PA dynamics is probably due to the action of a polyamine oxidase (PAO) (Neily et al., 2011; Torrigiani et al., 2012) and has been reported in Arabidopsis plant in order to maintain SPD homeostasis (Moschou et al., 2008). Treatment with 2.0SPD also appeared to increase SPM levels, probably a result of the conversion of the SPD to SPM via SPM synthase; also 0.1SPM treatment appeared to

increase PUT levels.

PAs are worthy of note because the accumulation of these signaling molecules may hold a crucial key to enhancing resistance towards a chilling condition in a wide variety of horticultural crops (Zhang et al., 2013; Palma et al., 2015). Earlier sub-chapter (8.4.1) confirm the implications of PAs postharvest treatment on the changes of endogenous PAs content, at the same time it is interesting to study and relate the connection between PAs and chilling injury (CI) in carambola fruit tissue subjected to chilling storage. There is increasing evidence that CI has been positively associated with membrane damage and deterioration and PAs are known to impart membrane tolerance and protection against CI. In line with this, the conflicting connection between CI and PAs has garnered great interest and inquire further investigations.

Endogenous PUT concentration increased greatly with respect to value at harvest throughout storage at 3°C, suggesting that this diamine accumulates upon long exposure to chilling temperature. As shown in Figure 8.1 endogenous free PUT increased in the progressive manner followed by free SPD with the similar trend but lower amplitude (Figure 8.2) and SPM with the modest accumulation (Figure 8.3). The result is consistent with previous studies clarifying that tremendous concentration of PAs in fruit tissue might be due to general response in fruit suffering CI (Wang, 1994b; Valero et al., 1997; Martinez-Romero et al., 2003). Taken together, this study suggests that increment of endogenous PAs level is a consequence of the stress-induced injury rather than protective mechanism against stress.

Also, HPLC analysis at harvest but before cold storage further revealed that the magnitude of changes in individual PAs accumulation was lower as compared to when

fruit subjected to cold stress. This observation suggests that endogenous PAs content particularly free PUT and SPD accumulate sharply during stress and not before the stress is initiated. Due to the behavior described above, increases in intracellular PUT and SPD are a response to injury stress (Valero et al., 1997; Serrano et al. 1997, 1998; Botella et al., 2000; Abu-Kwapoh et al., 2002). This result is in agreement with the previous studies in fruit that is highly susceptible to CI such as citrus fruits, grapefruit, pepper, and zucchini (Serrano et al., 1996, 1997, 1998; Gonzalez-Aguilar et al., 2000a).

With respect to the effect of the PAs treatment on changes of endogenous PAs level, this study also showed association between CI and PAs accumulation during cold storage. Accordingly, the reduction of CI scores in carambola have been found (sub-chapter 5.3.8) and correlated with the enhancement of endogenous PA concentrations (PUT, $r=0.34^*$, SPD, $r=0.55^*$ and SPM, $r=0.53^*$) (Appendix F). These finding was in accordance with other work in which synthesis of new PAs exerted by PAs treatment reduced CI scores in chilling sensitive fruit (Kramer et al., 1991; Martinez-Tellez et al., 2002; Nair and Singh, 2004; Mirdehghan et al. 2007a).

Moreover, PAs treatment also been proven to limit the severity of membrane ion leakage (sub-chapter 7.3.4) and influenced the reduction of lipid peroxidation level (sub-chapter 7.3.5) in carambola during cold storage. PUT accumulation exhibited positive correlation with the quality parameters evaluated (MDA content, $r=0.67^{**}$ and membrane ion leakage, $r=0.59^{**}$) during cold storage. Meanwhile, higher correlation values were observed in SPD (MDA content, $r=0.92^{**}$ and membrane ion leakage, $r=0.90^{**}$) and SPM levels (MDA content, $r=0.94^{**}$ and membrane ion leakage, $r=0.94^{**}$). These responses indicate that increases in intracellular PAs could be related to adaptation or cold

acclimation and endogenous PAs accumulation particularly free PUT content in carambola tissue resulted in direct effects on membrane integrity by lowering membrane permeability and retardation of lipid peroxidation levels (Gonzalez-Aguilar et al., 2000a; Mirdehgan et al., 2007b).

Accordingly, modest increases of intracellular PAs content in control fruit have been found concomitant with progressed development of CI. Hence, this study suggested that accumulation of endogenous PAs in the control fruit PAs is not high enough to induce cold adaptive mechanism and thus CI occurred in higher extend, accompanied with a high degree of membrane peroxidation level and electrolyte leakage.

Nevertheless, apart from PAs application another category that has been demonstrated to elevate PAs level is prestorage treatments such as high or low temperature conditioning. Such treatments are correlated to induction some factors involved in cold tolerance that alleviates CI symptoms. A study by Wang (1994b), proposed that these types of conditioning may trigger stimulation of tolerance mechanisms which in turn provide protection against another type of stress. For instance, the accumulation of heat shock proteins (HSP) by high temperature is closely related to increased tolerance to CI (Lurie, 1998).

Also, low but non-chilling temperature induced SAMDC activity and elevated endogenous PAs level thus diminished CI (Wang et al., 1994b). Prestorage at low temperature involved in hardening process by maintaining a high degree of unsaturated of membrane lipids (Campos et al., 2003). Thus, such conditioning treatment minimizes the initial stress effect on elevation of PAs concentration to withstand chilling stress (Gonzalez-Aguilar et al., 2000a, 2000b; Abu-Kpawoh et al., 2002; Xu et al., 2005;

Mirdehghan et al. 2007b; Cao et al., 2010b; Zhang et al., 2013).

Conversely, prestorage manipulation such as temperature conditioning also identified to induce PAs concentration. Conditioning treatments at a high or low temperature such as prestorage at 10°C in zucchini squash induce higher SAMDC activity and enhance the biosynthesis of PAs before storage at chilling ones (Wang, 1994b). A similar observation has been reported in mandarin (Gonzalez-Aguilar et al., 2000b) and peach (Xu et al., 2005). Accordingly, application of heat treatment in tomato (Zhang et al., 2013), lemon (Valero et al., 1998b) and hot water dips in plum (Abu-Kpawoh et al., 2002) and pomegranate (Mirdehghan et al. 2007b) led to an increase in PAs accumulation. In spite of this, the effect of heat treatment is poorly studied if the accumulation of intracellular PAs in fruit tissue is a consequence of the heat stress or as a result of beneficial effects in alleviating stress (Wang, 1994b).

8.5 Conclusion

Taken together, the results specify that exogenously supplied PAs led to stimulation of PAs biosynthesis pathway resulted in an increase in intracellular PAs and contribute a part role in the cold acclimation. The capacity of PAs-treated carambola to withstand CI might possibly through PUT and SPM accumulations as the consequence of the stress-induced injury. In addition, higher PUT and SPD concentrations could account for the greater resistance to CI incidence since the normal contents would not be sufficient enough to induce this adaptation to cold storage. Overall, the wide changes in endogenous PAs pool could contribute to improving postharvest quality and storability of carambola fruit storage at 3°C.

CHAPTER NINE

IDENTIFICATION OF HOMOLOGOUS FRAGMENTS OF GENES ENCODING POLYAMINES AND ETHYLENE BIOSYNTHETIC ENZYMES IN CARAMBOLA

9.1 Introduction

The plant hormones; ethylene and polyamines (PAs) particularly spermidine (SPD) and spermine (SPM) require S-adenosylmethionine (SAM) as a precursor in their biosynthesis route (Pang et al., 2007; Handa and Mattoo, 2010; Zhang and Wen, 2010). Due to their antagonist roles, these hormones may compete for the utilization of SAM pools in plant cells. In ethylene pathway, SAM evolves into ethylene through 1-aminocyclopropane-1-carboxylate (ACC) which catalyzed by the sequential action of ACC synthase (ACS) and ACC oxidase (ACO) (Lin et al., 2009).

Also, in PAs biosynthesis route SAM is decarboxylated into S-adenosylmethionine decarboxylase (SAMDC) which serves as a donor of the aminopropyl groups to SPD and SPM (Bassard et al., 2010). Generally in the plant, putrescine (PUT) is converted into SPD and SPM which are catalyzed by SPD synthase (SPDS) and SPM synthase (SPMS) respectively. Apart from ethylene, PAs metabolic pathway also seem interrelated with other important metabolic pathways such as nitric oxide (NO), γ -aminobutyric (GABA), abscisic acid (ABA) and Krebs cycle (Cona et al., 2006; Alcazar et al., 2010).

Molecular techniques are becoming an increasingly popular for exploring the important roles PAs play in plant biology. In the past decade, molecular evidence from plant have directed to isolation of a number of PA biosynthesis genes, the creation of plant mutants and genetic manipulation involving PA genes (Bassard et al., 2010; Gill and

Tuteja, 2010; Hussain et al., 2011). Much work has been carried out using Arabidopsis, tomato, tobacco and rice which are regarded as a transgenic model in order to probe the precise involvement of PAs in plants. Suppressing expression of PAs biosynthesis genes generally causes abnormal and often lethal phenotypes and overexpression of these genes resulted in stress tolerance improvement and a better overall quality (Alcazar et al., 2010; Torrigiani et al., 2012).

Overexpression of arginine decarboxylase (ADC) in PA biosynthesis pathway was shown to impart tolerance to various unfavorable environmental conditions including drought, salinity and minerals insufficiency (Pang et al., 2007; Wimalasekera et al. 2011; Caldazilla et al., 2014). In other case, Arabidopsis mutants deficient in ADC gene exhibited reduced tolerance level and more sensitive to abiotic stress than the wild type (Bassard et al., 2010; Hussain et al., 2011). Moreover, PA biosynthesis genes loss-of-function exhibited incomplete and aberrant morphology in floral organs and abnormal or significantly lower PAs titer (Liu et al., 2006).

Based on the fact that PAs is known to exert opposite biological functions with ethylene, an increasing number of molecular genetics manipulation attest to the function of PAs as modulator during plant senescence. Such studies on transgenics will help further understanding of how PAs biosynthesis is regulated and the processes that PAs influence with respect to ripening and senescence. For instance, ripening induced overexpression of SAMDC gene in tomato exhibited a two to three-fold increase in lycopene, which prolong vine life with higher SPD and SPM levels at the cost of PUT (Mehta et al., 2002). Similar observation was found in tomato with altered genes encoding for SPDS gene that significantly enhanced PAs content (1.5 to 2 fold) and lycopene

accumulation (1.2 to 1.6 fold) (Neily et al., 2011). Thus, it is desirable to create better crops with longer shelf life, higher nutrient content and traits conferring resistance to postharvest pathogens and environment fluctuations.

Throughout previous chapters, direct effects of PAs on postharvest aspects of carambola including quality, storage life, ripening, nutritional attributes and chilling resistance have been explored. With this information, it has become possible to dissect biochemical pathways involved in the PA metabolism (biosynthesis and catabolism) and their cross-talk with ethylene, along with the occurrence of physiological changes during ripening. Understanding the pathways and the signaling cascade associated with PAs and ethylene is essential. Thus, genes encoding for PAs and ethylene biosynthesis would be important candidates for exploring the ripening kinetics in carambola.

To the best of our knowledge, there is no any literature available on the molecular aspects in ripening of carambola particularly genes encoding for PAs and ethylene enzymes. The PAs complex pathway has been examined at biochemical and molecular level in the model plant *Arabidopsis* (dry), tomato (fleshy) and peach (drupe) (Hussain et al., 2011; Neily et al., 2011; Torrigiani et al., 2012) and less so in other fruit with agronomy importance.

Nonetheless, a large body of works of PAs and ethylene biosynthesis genes can be found in the NCBI database from numerous plants. Therefore, there is a requirement to obtain the partial fragment of the genes encoding PAs (ADC and SAMDC) and ethylene biosynthetic genes (ACS and ACO), which is parallel with a need to identify this gene in carambola. Furthermore, no established method is available for extracting total RNA from carambola mesocarp tissue. As a first step, isolation of total RNA and identification of

ADC, SAMDC, ACO and ACS genes of carambola were carried out accordingly.

9.2 Materials and methods

Freeze-dried fruit mesocarp kept at -80°C were used for this analysis. All apparatus used in the experiment for examples mortar, pestle, spatula, tips, centrifuge tubes, glassware and microcentrifuge tubes were immersed into distilled water treated with 0.1% (v/v) DEPC (diethyl pyrocarbonate) overnight before autoclaved (121°C for 15 min) to eliminate possible RNase presence. Reagents were prepared using water treated with 0.1% (v/v) DEPC and autoclaved thereafter except Tris-HCl solution without being autoclaved.

9.2.1 Total RNA isolation from carambola mesocarp tissue

Extraction of total RNA from carambola fruit tissue was carried out according to Zamboni et al. (2008) with slight modifications. Approximately 5 g of frozen mesocarp was pulverized into a fine powder using pestle and mortar in the presence of liquid nitrogen. Next, the fine powder was quickly transferred into 50 ml centrifuge tube and homogenized with 10 ml of preheated (65°C for 20 min) extraction buffer (2% CTAB, 2% PVP-40, 25 mM EDTA, 2 M NaCl, 0.05% spermidine trihydrochloride, 100 mM Tris-HCl pH 8). Then, freshly prepared β -mercaptoethanol (2%) was added into extraction buffer before use. Later, the homogenate was placed a water bath for 30 min at 65°C with occasional vigorous shaking to mix the homogenate completely.

The homogenate was placed at room temperature ($22\pm 1^{\circ}\text{C}$) for 10 min and allowed to cool down before addition of equal volume chloroform:isoamyl alcohol (24:1). The mixture was shaken vigorously and centrifuged at 12 000 rpm at 4°C for 10 min

(Centrifuge 5810 R, Eppendorf, USA). The clear supernatant was transferred to a new clean 50 ml centrifuge tube and re-extract with an equal volume of chloroform:isoamyl alcohol (24:1). The mixture was mixed vigorously and centrifuged at 12 000 rpm at 4°C for 10 min. The clear upper layer was collected carefully without disturbing chloroform and flocculent material through filaments forming between the interphase and tip.

The supernatant was added with 1/3 volumes of cold 8 M LiCl, the tube was then inverted several times to completely mix the solution and stored at -20°C overnight to allow RNA precipitation. On the following day, the RNA was recovered by means of centrifugation at 12 000 rpm, 4°C for 90 min. The supernatant was discarded and the translucent pellet at the bottom of the tube was collected and suspended in 500 µl suspension buffer (1 M NaCl, 0.5% SDS, 1 mM Tris HCL pH 8.0, 1.0 mM EDTA pH 8.0). 500µl of chloroform:isoamyl alcohol (24:1) was added to the mixture and then centrifuged at 12 000 rpm at 4°C for 20 min.

The upper aqueous phase was collected and added with 2 volumes of cold absolute undenatured ethanol and 1/10V of 3 M sodium acetate (pH 5.2). The RNA material was stored at -80°C for 2 hours for precipitation and then recovered by centrifugation at 12 000 rpm, 4°C for 20 min. The supernatant was pipetted out and the white pellet was washed with 70% cold undenatured ethanol by centrifugation at 12 000 rpm at 4°C for 10 min. Pellet was air-dried at room temperature for 20 min until all ethanol was completely evaporated off. Pellet was re-suspended in 100 µl DEPC-treated water and stored at -80°C until further use.

Total RNA was quantified with a nanodrop (ND-1000, Thermo Scientific Company, USA) at 230, 260, 280 and 320 nm for the possible contamination of

phenolic/polysaccharides and protein compounds. The integrity of RNA sample was analyzed by running on 1.0% (w/v) agarose gel to confirm the presence of RNA bands. The quality and yield of RNA sample were also analyzed using RNA lab chip analysis provided by the 1st base laboratory (First BASE, Malaysia).

9.2.2 cDNA first strand synthesis

The cDNA conversion mainly involves 2 main steps 1) elimination of genomic DNA 2) reverse transcription. Firstly, RNA undergoes DNase treatment and clean-up according to manufacturer's instruction (Qiagen RNase-Free DNase Set, Germany). The second step, the first strand cDNA was synthesized using the GoScript Reverse Transcriptase System (Promega, MA, USA). Approximately 500-1000 ng of purified RNA was added to oligo(dT) primer, 500 µg/ml (1 µl) and nuclease-free water with a total volume of 5 µl. The reaction was preheated at 70°C for 5 min and chilled immediately on ice for 5 min.

The master mix prepared from GoScript reaction buffer, 5x (4 µl), PCR nucleotide mix, 10 mM (1 µl), GoScript reverse transcriptase (1 µl), ribonuclease inhibitor (1 µl), MgCl₂, 25 mM (2 µl) and nuclease-free water (6 µl). 15 µl of master mix was added to RNA and primer mix (5 µl) for a final volume of 20 µl. Reaction tubes were placed in the temperature block at 25°C for 5 min for annealing, followed by incubation at 42°C for 1 hour for an extension. The reaction was stopped at 72°C for 15 min and cDNA products were kept in -20°C for further use.

9.2.3 Degenerate oligonucleotide design

Candidate genes were constructed using degenerate primers and were designed to amplify unknown members of a gene family. Primers were synthesized from conserved amino

acid regions of at least seven plant species from the NCBI protein database (Appendix G). Multiple alignments of conserved regions of target proteins were generated using MUSCLE (Multiple Sequence Comparison by Log-Expectation) (EMBL-EBI) (<https://www.ebi.ac.uk/Tools/msa/>). The sequence was then cut into the block using Block Maker (http://blocks.fhcrc.org/blocks/make_blocks.html) to identify conserved regions of protein sequences. Primers containing random nucleotide compositions were then designed from the protein blocks using CODEHOP (Consensus-DEgenerate Hybrid Oligonucleotide Primers) (<http://blocks.fhcrc.org/blocks/codehop.html>).

9.2.4 Polymerase chain reaction (PCR) and sequencing

PCR components consisted of 1 µg of cDNA template, 1 µl of 1 µM each primers (First BASE, Malaysia), 0.5 µl of 10 mM dNTPs mix, 2 µl of 25 mM MgCl₂, 2.5 µl of 5x PCR buffer, 0.5 µl of Taq polymerase (Promega, MA, USA) and the final volume was made up to 25 µl with RNase free water. For each degenerate primer PCR was initiated with gradient settings to determine the optimum annealing temperature (Gradient PCR, Eppendorf, Germany). PCR amplification was set at 95°C for 3 min for initial denaturing followed by 35 cycles of 95°C for 30 sec, optimized annealing temperature for 1 min and 72°C for 1 min. A final elongation step at 72°C for 10 min and PCR reaction was held at 10°C.

All genes used one pair of primers except ADC, which used two pairs of primers (Table 9.1). For ADC gene, the PCR product from primer pair I was not diluted and amplified with primer pair II using nested-PCR approach. The amplified partial fragments were checked on a 1.2% agarose gel stained with GelRed (Biotium, Inc.) and the desired products amplification was examined with a UV imager (InGenius 3 GeneSys, Syngene,

UK). A blank, reaction mix without cDNA template was included as controls. DNA fragments in the gel were extracted and purified using Qiaquick Gel extraction kit (Qiagen, Germany) according to the manufacturer's recommendations. The purified PCR products were sent for sequencing to the 1st base laboratory (First BASE, Malaysia).

Table 9.1: List of degenerate primers designed using CODEHOP

Gene	Forward and reverse (5'-3')	Product size
ACS	F-CATCGCCATCTTCCAGGAYTAYCAYGG R-AGCAGCCGCCGCARRTCCATCCA	800bp
ACO	F-TCAAGGACGCCTGCGARAAYTGGGG R-GCGTCGTTGCCGGGRTTRTARAA	747bp
SAMDC	F-ACCCCTACAAGGTCATCATCAARACNTGYGG R-CCGCAGGGGTCGAAC TCRAARTCRCA	456bp
ADC	Primer pair I F-AGGGCTACGGCTCCCAYTAYCARGG R-CGAACATCAGCTCGGGCTSRGTGTCAT	1542bp
	Primer pair II F-CGTGATCGTGCTGGAGCARGARGARGA R-TCAGCTCGGGCTCGTGYTGCATNAC	1200bp

The sequence analysis was analyzed with BLAST programme from NCBI databases to detect homologous sequence against protein sequence. Multiple sequence alignments and phylogenetic neighbor-joining diagrams were build using the MAFFT (Version 7) (<http://mafft.cbrc.jp/alignment/server/>) to show evolutionary relationships among genes of interest in carambola and its closest plant species.

9.3 Results

9.3.1 Isolation of total RNA from carambola mesocarp tissue

Modified RNA extraction from woody plants (Zamboni et al., 2008) was used for isolating total RNA from carambola mesocarp tissue (consisting of both flesh and peel). The method produced fairly good quality and sufficient yield of RNA. In all cases, the RNA was of high purity with minor contamination of polyphenols and polysaccharides at absorbances of 260/230 with ratio ranged from 2.1 to 2.3 (Table 9.2). Meanwhile, absorbances at 260/280 ratio ranged from 1.9 to 2.3, revealed a lack of protein and macromolecules interference.

Table 9.2: RNA quality and yield from carambola mesocarp quantified by nanodrop.

Sample	Absorbance		Yield (ng/ul)
Sample	OD260/230	OD260/280	
Fruit 1	1.91	2.16	278.3
Fruit 2	1.92	2.17	348.6
Fruit 3	2.29	2.31	460.0

The intactness of the RNA was confirmed by visualizing the sharpness of ribosomal RNA bands on 1.2% (w/v) agarose gel. Distinct 25S and 18S rRNA bands without degradation were observed in all RNA samples tested (Figure 9.1). Visualization of RNA bands on a conventional agarose gel is subjective due to the running electrophoresis condition as well as the amount of RNA loading. This method also depends on the assumption of RNA quality and quantity to reflect the presence of mRNA population.

Therefore, RNA was further quantified using the RNA lab chip instrument system to obtain a clear estimate to the major components that make up total RNA. The bioanalyzer was a microfluidics-based technology for a precise digital data with the electropherogram

showing the 28S:18S ratio, for quantitating and analyzing RNA amount and integrity. All the RNA yields obtained have 25S:18S ratio more than 1.0 indicating that samples reached an acceptance level for the downstream application.

RNA is considered of high quality when the ratio of 28S:18S ratio is about 2.0 and higher. However, the RNA integrity number (RIN) in all samples showed poor values that ranged from 1.8 to 4.1 which makes the samples less suitable for a downstream analysis for instance, real-time PCR. RIN value is one of important information for RNA integrity, it ranged from 1-10 where good RIN must have values higher than 7.0.

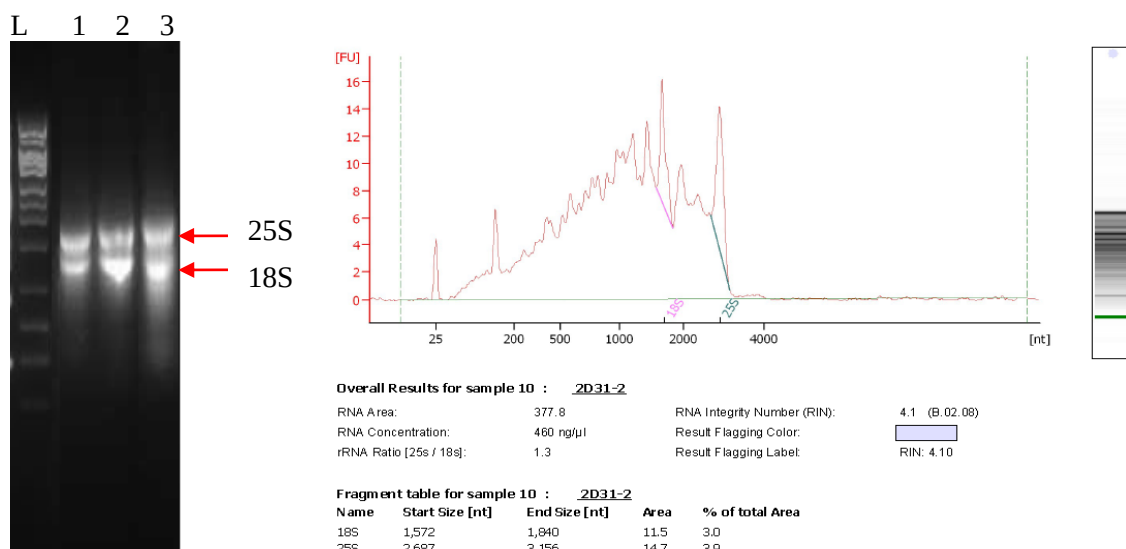


Figure 9.1: Visualization of total RNA on 1.2% agarose gel and RNA lab chip analysis. L: 1kb ladder. Lane 1-3: total RNA from carambola mesocarp tissues (Fruit 1-3).

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Cedric Notredame
CPU TIME:0 sec.
SCORE=99
*
  BAD AVG GOOD
*
Arabidopsis      : 99
Wildflax        : 99
Mustardgreen    : 99
Teashrub        : 99
Bittermelon    : 99
Muskmelon      : 99
Calamondin      : 99
cons            : 9

Arabidopsis      -----MALSAIGFEGYEKRLVETFFEPSIFQDSKGLGLRALTKSQLDEILTPAA
Wildflax         -----MALSAIGFEGYEKRLVSVFFEPSIFQDSKGLGLRALTKSQLDDILTPAA
Mustardgreen     -----MALSAIGFEGYEKRLVSVFFEPSIFQDSKGLGLRALTRSQLDEILTPAA
Teashrub         MALPVSPIGFEGYEKRLIISFFESSIFADPRSNGLRSLSKSQLDEILNPAECTIVSSLANDDVDSY
Bittermelon     MTFPTSAIGFEGYEKRLVSVFFEPGVFSDPRGMGLRALSKAQLDEILTLAECTIVDSLNDYLDSC
Muskmelon       MTCPTSAIGFEGYEKRLVSVFFEPSVFDPRGMGLRALSKAQLDEILTLAECTIVDSLNDYLDY
Calamondin      MALPVSAIGFEGYEKRLVSVFFEPGVFADPGGRGLRSLSKHQLDEILKPAECTIVSSLNVEYLDY

cons            :      . . . * . . . . * :

Arabidopsis      CTIVSSLSNDQLDSYVLSESSFFVYPYKVI IKTCGTTKLLLSIPPLLKLAGELSLSVKSVKYTRGS
Wildflax         CTIVSSLSNDHLDYSYVLSESSFFVYPYKVI IKTCGTTKLLLSIPPLLKLAGELSLSVKSVKYTRGS
Mustardgreen     CEIVSSLSNDHLDYSYVLSESSFFVYPYKVI IKTCGTTKLLLSIPPLLKLAGELSLSVKSVKYTRGS
Teashrub         VLSESSL-----FVYSYKII IKTCGTTKLLLSIPPLKFAEMLSLSVRSVRYTRGS
Bittermelon     VLSESSL-----FVYPYKFI IKTCGTTKLLLSIPALLKLADSLSLTVRSVRYTRGS
Muskmelon       VLSESSL-----FVYPYKFVIKTCGTTKLLLSIPALLKLADSLSLTVRSVRYTRGS
Calamondin      VLSESSL-----FVYPYKVI IKTCGTTKLLLSIPAILKLAESLSLSVRSVRTRGS

cons            ***          ***.*.:*****.:*:*  ***.:*:*:****

Arabidopsis      FLCPGGQPPFHRSFSEEVSVLDGHFTQLGL-NSVAYLMGNDDETKKWHVYAASA--QDSNC--NN-
Wildflax         FLCPGGQPPFHRSFSEEVSVLDGHFTQLGL-NSVAYLMGNDDETKQWHVYAASAQDSGNCNN--N--
Mustardgreen     FLCPGGQPPFHRSFSEEVSVLDGHFSGKLGL-NSVAYLMGNDDETRKWHVYAASA--QAPSN--NN-
Teashrub         FIFPGAQPPFHRDFSEEVAVLDGYFGKLGSGSKAYVMGSDDESRRKWHIYSASA---ES--T-NHL
Bittermelon     FIFPGAQSPFHRSFSEEVAVLDGYLGKLGSLDSTAYVMGSPDETKKWHVYSACA-NMGS-KS--NN-
Muskmelon       FIFPGAQSPFHRSFSEEVAVLDGYLAKLGLHGS-AYVMGSPDETKKWHVYSACA-NMGN-QS--NN-
Calamondin      FIFAGAQPPFHRSFSEEVAVLDGHFGKFGM-DSTAFVMGSPDNTKRWHVYSASA-EAGS-HV--N--

cons            *: .*.*.***.*****:*****.:*: . *.:*. *.:*:*.*:.*  *

Arabidopsis      -NVYTLEMCMTGLDREKAAVFYKDEADKTGSMTDNSGIRKILPKSEICDFEFPCGYSMNSIEGDA
Wildflax         --VYTLEMCMTGLDREKALVFYKDESDKTGSMTDNSGIRKILPKSQICDFEFPCGYSMNSIEGDA
Mustardgreen     -NVYTLEMCMTGLDREKASVFYKNETGENSGMTDNSGIRKILPKSQICDFEFPCGYSMNSIEGDA
Teashrub         RPVYTLEMCMTGLDREKVVVFYKTSASSAAAMTDLSGIRMI LPDSDICDFEFDPCGYSMNAIEGSA
Bittermelon     -PVYTLEMCMTGLDREKTSVFFKTDASSAAAMTKNSGIRKILPKSEICDFEFDPCGYSMNAIEGDA
Muskmelon       -PVYTLEICMTGLDKEKASVFFRTDASSAAAMTNSGIRKILPKSEICDFEFDPCGYSMNAIEGDA
Calamondin      -LVYTLEMCMTGLDREKASVFYKTNESSAAMTDSGIRKILPKSEICDFEFDPCGYSMNSIEGDA

cons            *****:*****.:. *: . . . . *. ***** *:*****:*****:***.*

Arabidopsis      ISTIHVTPEDGFSYASFEAVGYDFNTLDLSQLVTRVLSCFEPKQFSVAVHSSVGANSYK--EI-T
Wildflax         ISTIHVTPEDGFSYASFEAVGYDFNTMDLSQLVTRVLSCFEPKQFSVAVHSSV-GVNAIK-PEI-S
Mustardgreen     ISTIHVTPEDGFSYASFEAVGYDFNTLDLSQLVTRVLSCFEPKQFSVAVHSSV-GANAYK-PEI-S
Teashrub         ISTIHVTPEDGFSYASFEAVGYDFKSMNLTLLERVLACFPQSEFSLALHSDAVANKLSSD---MS
Bittermelon     ESTIHITPEDGFSYASFEASGYDFNEVNLSQLIGRVLACFPQSPDFSVLHSD-DCGENLED---LLC
Muskmelon       ESTIHVTPEDGFSYASFEAGYDFDDMNLSKLIIVRVLACFPQSPDFSVLHSD-VVGENLED---LLC
Calamondin      VSTIHVTPEDGFSYASFEAVGYDFEVVKLTSLLERVLACFPKPAEFSVALHSD-IVGD--EHGDTFT

cons            *****:***** *****.:*: *: *****:*. *:*.*:.*  .

Arabidopsis      VDLEDYGCERTFESLGEESGTVMYQTFEKLGKYCGSPRSTLKCEWSSNNSCSSEDEKDEGI
Wildflax         VDLEDYGCERTFESLGEESGTVMYQTFEKLGKYCGSPRSTLKCEWSSNNSCSSEDEKDEGI
Mustardgreen     VDLEDYGCERTFESLGEESGTVMYQTFEKLGKYCGSPRSTLKCEWSSNNSCSSEDEKDEGI
Teashrub         LDVQGYCGERSFEVLGKEGGSVYHNFMAKAGCGSPRSLHCCWSEDEK-----
Bittermelon     VELKGYEGGEKSCMLG-ENGTVIYQSPFMKTGDYASSPRSLHCCWSEDEK-----
Muskmelon       LELKGYEGGEKSCMLG-ENGTVIYQSPFMKTGGDYASSPRSTLKCEWSEDEKDEEVGKY---
Calamondin      LDFKGYSCGEKIYEELG-NNGSLIYFSRSTDCSTSPRSLHCCWSEDEKDEEVGK-----

cons            :.:.* *: * * .:*.*:.* *: . . . . .

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Figure 9.2: The multiple sequence alignment of the deduced amino acid sequences for various plant SAMDC genes as produced by T-coffee (<http://tcoffee.crg.cat/apps/tcoffee/index.html>). An asterisk (*) below any amino acid indicated that position is conserved in all sequences; colon (:) means that conserved substitutions have been observed; dot (.) means that semi-conserved substitutions are observed.

9.3.2 Degenerate primer design

A PCR primer sequence is called degenerate if the sequence to be amplified is not actually known. Therefore, primers must be designed in regions of amino acids sequence that is highly conserved within known sequences for the gene of interest and then design the primers within those regions. One can, thus, use MUSCLE to look for sequences of the target protein of conserved regions from multiple related species as shown in Figure 9.2. Given a series of blocks of the protein of interest from the highly conserved region of homologous proteins, CODEHOP will transform amino acid sequence into a DNA consensus sequence which later on will be used to design degenerate primers (Table 9.1).

The sequences of each protein were selected with a minimum value of 80% similarity and carambola is a member of the family Oxalidaceae, part of the order Oxalidales or the wood sorrel family, and it is the most prominent member in its order. There is no available data on DNA or protein consensus sequence of PAs and ethylene either from carambola or Oxalidaceae family. Malpighiales and Celastrales are closely related to Oxalidales, and members of the same COM clade (Figure 9.3). Malpighiales is the closest and large order and the most common plant species is *Hevea brasiliensis* (rubber tree). However, only ethylene biosynthetic gene (ACS and ACO) sequence has been reported and deposited into NCBI protein database but no polyamine gene sequences have been explored.

Fabids is a group of a large nitrogen-fixing clade which consisted of four families; Fabales, Rosales, Fagales and Cucurbitales. They are an order of eudicots that include flowering plants included in the Eurosids group in the Angiosperm Phylogeny Group III (APG III). Hence, it is necessary to choose closer members from this group in order to

obtained conserved protein regions.

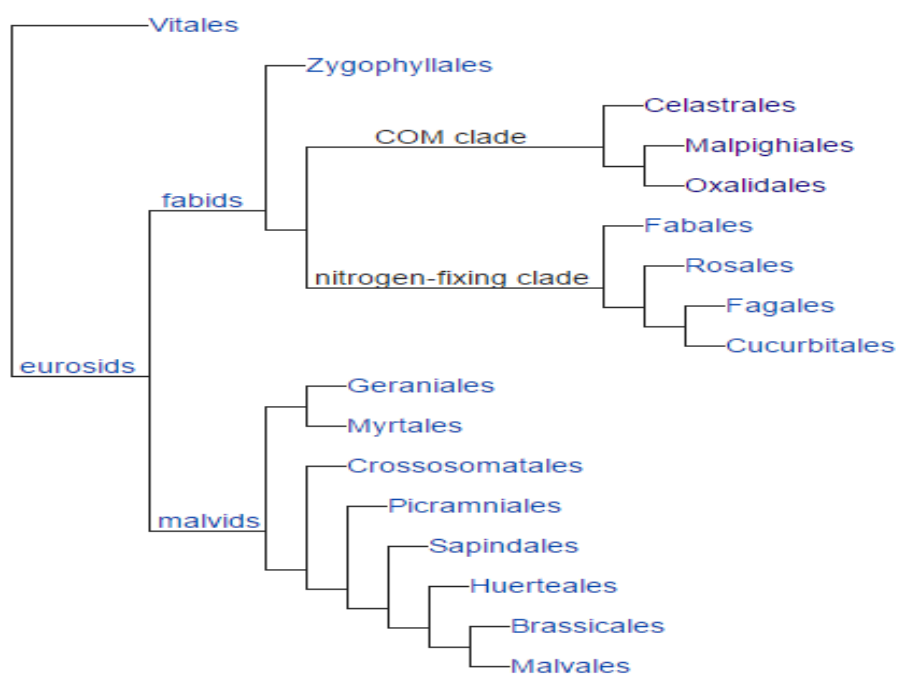


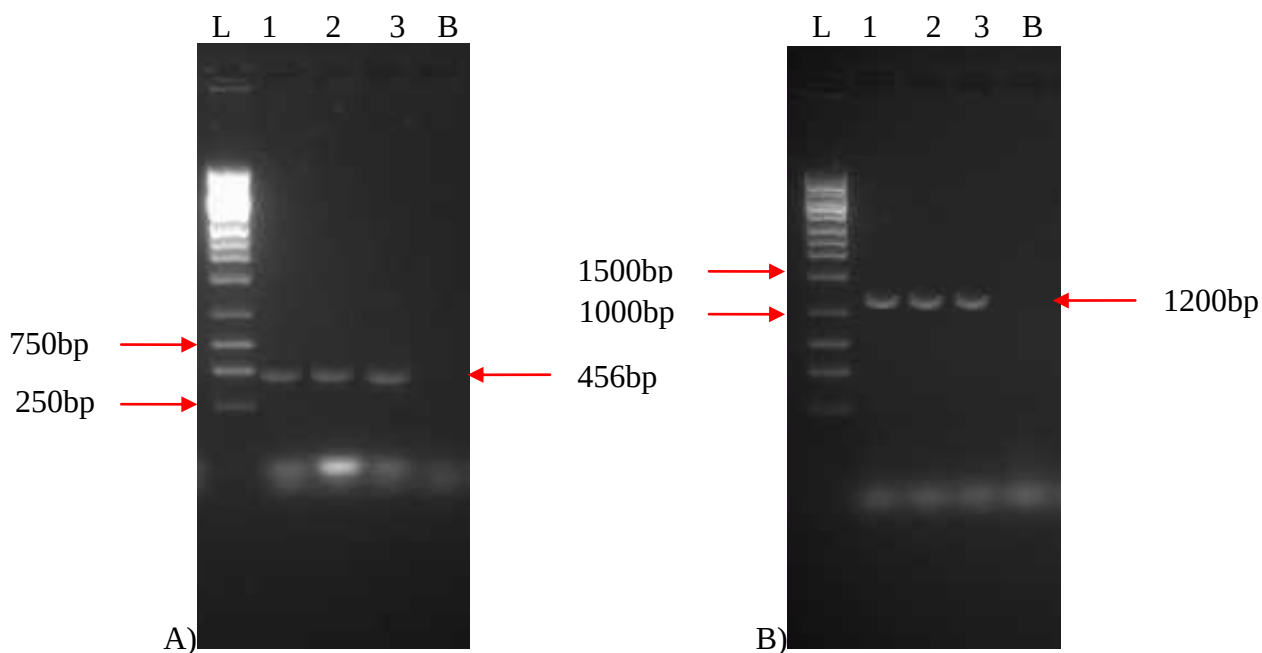
Figure 9.3: Illustration of phylogenetic tree exhibiting the Oxalidales evolutionary relationship in the orders and families of flowering plants (Angiosperm Phylogeny Group, 2009).

9.3.3 Polymerase chain reaction (PCR)

As shown in Figure 9.4, a single band of the expected size was produced during amplification with degenerate primers in SAMDC (456bp), ACS (800bp) and ACO (747bp) genes. However, amplification with the first pair of degenerate primers designed for ADC consistently resulted in no product bands in the agarose gel. Thus, the second round of PCR (nested-PCR) for ADC was carried out with second primers pair under the same PCR conditions and resulted clear single bands of ADC (1200bp). Without a doubt, the most critical parameter to vary when establishing a new PCR assay is to determine the optimal primer annealing temperature.

SAMDC (55.1°C), ACS (60.6°C), ACO (55.6°C) and the second pair of primers for

ADC (51.9 °C) successfully produced clear bands but weaker signals were observed in SAMDC, ACS and ADC as compared to ACO. Apparently ADC first pair of degenerate primers failed to produce bands although the annealing temperature was initially optimized. The different MgCl_2 concentrations (0.25, 1.0 and 2.5 mM) did not exhibit significant effects on the PCR reaction, thus the concentration was maintained at 2.5 mM. Different primer concentrations (0.1, 0.5 and 1 μM) was tested in this reaction and 1 μM of primers produced clear and intense bands however, the primer-dimer background was observed on the agarose gel.



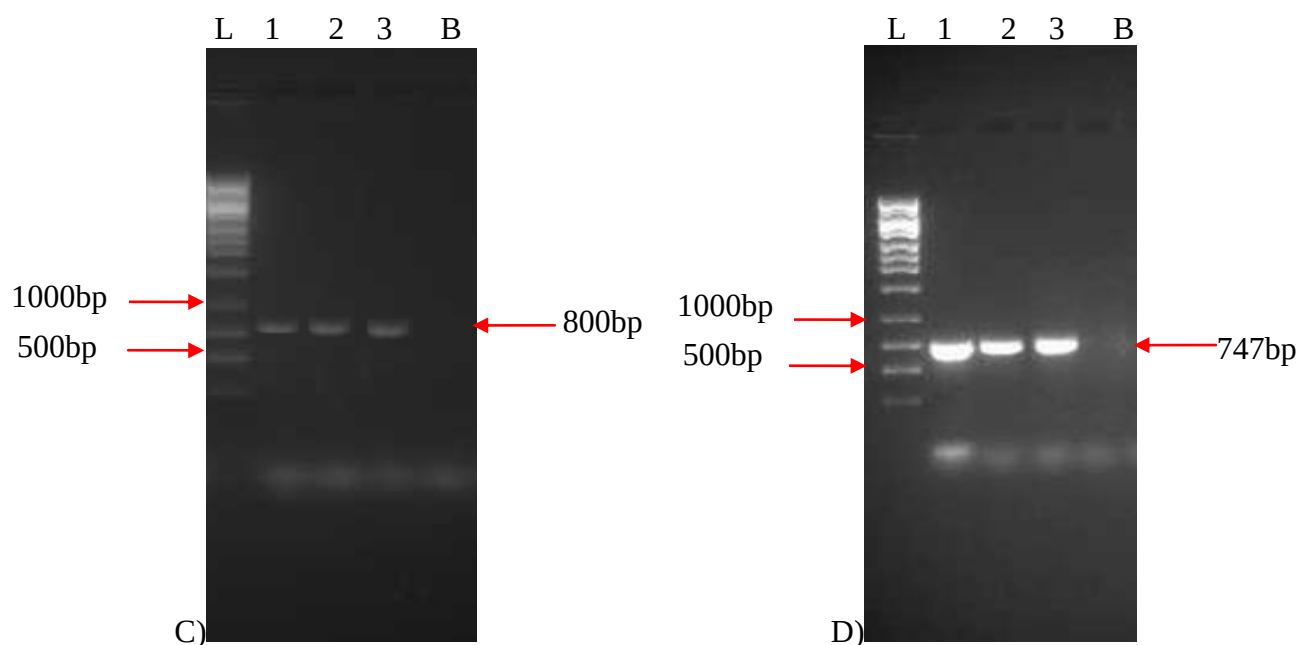


Figure 9.4: PCR reaction for each of the degenerate primers used for A) SAMDC B) ADC C) ACS D) ACO. L: 1kb ladder. Lane 1-3: Amplified DNA bands from cDNA B: Blank (without cDNA template).

9.3.4 Sequencing and validation of primers A)

Range 1: 16 to 699			GenBank	Graphics	▼ Next Match ▲ Previous Match	
Score	Expect	Method	Identities	Positives	Gaps	Frame
417 bits(1071)	2e-144	Compositional matrix adjust.	198/228(87%)	213/228(93%)	0/228(0%)	+1
Query 1	SAIGFEGYEKRLEVTFEFSIFQDSKGLGLRALTKSQLDDILTPAACTIVSSLSNDHLDS	60				
Sbjct 16	SAIGFEGYEKRLEV+FFEPS+F D +G+GLRAL+K+QLD+ILT A CTIV SLSND+LDS	195				
Query 61	YVLSESSFFVYPYKVIKTCGTTKLLLSIPPILKFAEMLSLSVRSVRYTRGSFIFPGAQP	120				
Sbjct 196	YVLSESS FVYPYK +IKTCGTTKLLLSIP +LK A+ LSL+V+SVRYTRGSFIFPGAQ	375				
Query 121	FPHRDFSEEVAVLDGYLGKLGLDSTAYVMGSPDETKKWHVYSACANMGNQSNPNVYTLEI	180				
Sbjct 376	FPHRSFSEEVAVLDGYLAKLGLHGSAYVMGSPDETRKWHVYSACANMGNQSNPNVYTLEI	555				
Query 181	CMTGLDKEKASVFFRTDASSAAAMTENS GIRKILPKSEICDFEFDPCG	228				
Sbjct 556	CMTGLDKEKASVFFRTDASSAAAMTENS GIRKILPKSEICDFEFDPCG	699				

B)

Range 1: 330 to 548 [GenPept](#) [Graphics](#) ▼ Next Match ▲ Previous Match

Score	Expect	Method	Identities	Positives	Gaps	Frame
370 bits(949)	8e-122	Compositional matrix adjust.	179/219(82%)	196/219(89%)	0/219(0%)	+2
Query 1	GANMRVIDIGGGLGIDYDGSKSCSDISVGYGLEEYAHAVVQAVKFVCDRKNIKHPVLCS					60
Sbjct 330	GA+M+ IDIGGGLGIDYDGSKS DS+ISV Y LEEYA AVV+AV VCDRK++KHPV+CS					389
Query 61	ESGRAIVSHSVLIFEAVSASKQHETPNMSAVGLQYFVENLSEEARADYRNLSAAASCGE					120
Sbjct 390	ESGRA+VSHHSV+IFEA+S+S + P MSA LQYF+E L+EEARADYRNLSAAA GE					449
Query 121	SHTCLVYADVLRKQRCVEQFKEGSGVIEQLATVDGLCDMVSKAIGA FDPVRTYHVNLSVFT					180
Sbjct 450	CL YAD LKQRC++QFKEGS+GIEQLATVDGLCDMVSKAIGA DPVRTYHVNLSVFT					509
Query 181	SIPDFMGIGQTFPIVPIHRLDQRPVARGILSDLTCDSDG					219
Sbjct 510	SIPDFMGIGQTFPIVPIHRLDQRPVARGILSDLTCDSDG					548

C)

Range 1: 13 to 141 [GenPept](#) [Graphics](#) ▼ Next Match ▲ Previous Match

Score	Expect	Method	Identities	Positives	Gaps	Frame
219 bits(557)	4e-67	Compositional matrix adjust.	97/129(75%)	112/129(86%)	0/129(0%)	+2
Query 12	LLSKIATSEGHGENSPYFDGWNKAYDRNPFHPTKNPEGVIQMGLEAENQLSFDLIEDWIKKN					71
Sbjct 13	LLSKIAT++GHGENS YFDGWNKAY+ +PFH + NP GVIQMGLEAENQL FDLI++W+ N					72
Query 72	PKASICTPEGVEEFKNVAIFQDYHGFPEFRKAVAMFMSKARGGRVTFDPNRVVMSSGGATG					131
Sbjct 73	PKASICT EG E+FK++AIFQDYHG PEFR+AVA FM K RG RVTDPNR+VMSSGGATG					132
Query 132	ANELVMFCL					140
Sbjct 133	A+E + FCL					141

D)

Range 1: 40 to 146 [GenPept](#) [Graphics](#) ▼ Next Match ▲ Previous Match

Score	Expect	Method	Identities	Positives	Gaps	Frame
137 bits(346)	8e-38	Compositional matrix adjust.	84/107(79%)	99/107(92%)	0/107(0%)	+2
Query 2	SIPVELLDTEKMTKGHYRKCMEQRFKELVASKALEGVQTEIKMDWESTFYLKHLPEST					181
Sbjct 40	I E +DTVE++TK HYRKCMEQRFKELVAS+ALEG+QTE+ DMDWESTFY++HLP+ST					99
Query 182	VFQVPDLDDYRKMKEFALKleqlaeellldllcenlgleKGFLKNV					322
Sbjct 100	+ +VPDLD+EYRKMKEFALKLE+LAEELLDLLCENLG+EKG+LK V					146

Figure 9.5: Sequencing results for A) SAMDC B) ADC C) ACS and D) ACO from NCBI database. The query was referred to carambola sequence and the subject was referred to muskmelon (*Cucumis melo*), peach (*Prunus persica*), tobacco (*Nicotiana tabacum*) and sweet oranges (*Citrus sinensis*) sequences respectively.

Next, a phylogenetic tree was constructed from sequence alignment of related plants with highest scores in similarity and query cover to exhibit evolutionary relationship. From the phylogenetic diagram, there was a varying level of evolutionary relationship of PAs and ethylene biosynthetic genes in carambola and its related plant species. For instance, a few groups were observed in SAMDC gene within the tree, the closest plant that displayed highly homologous region of SAMDC was muskmelon (*Cucumis melo*) followed by sweet oranges (*Citrus sinensis*) (Figure 9.6). SAMDC gene from black cotton wood (*Populus trichocarpa*) was closely related meanwhile, tomato (*Solanum lycopersicum*), castor bean (*Ricinus communis*) and grapevine (*Vitis vinifera*) showed a lesser degree of similarity.

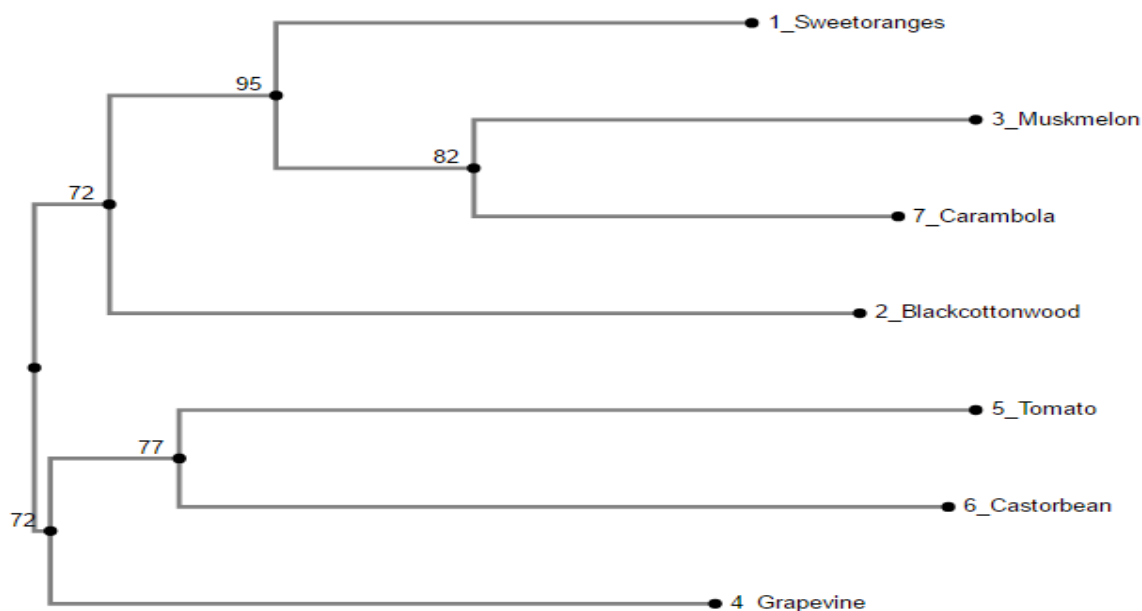


Figure 9.6: Phylogenetic tree analysis of SAMDC sequence alignment of carambola and its related plant species. The deduced amino acids were constructed using the MAFFT programme. Results for ADC, ACS and ACO phylogenetic analysis were presented in Appendix H.

Table 9.3: Putative sequences of the partial fragments of genes involved in polyamines and ethylene biosynthesis in carambola.

Gene	BLASTX			
	Ident (%)	Query cover (%)	Accession no.	Species
SAMDC	87%	95%	NP_001315394.1	<i>Cucumis melo</i>
ADC	82%	93%	XP_007200307.1	<i>Prunus persica</i>
ACS	75%	94%	NP_001313190.1	<i>Nicotiana tabacum</i>
ACO	79%	97%	NP_001275812.1	<i>Citrus sinensis</i>

9.4 Discussion

9.4.1 Isolation of total RNA from carambola mesocarp tissue

Several protocols exist for isolating RNA from plant tissues rich in polysaccharides including phenol-chloroform (Lopez-Gomez and Gomez-Lim, 1992), hot borate method (Wan and Wilkins, 1994), strong protein denaturants such as guanidium thiocyanate-phenol-chloroform (Vareli and Frangou-Lazaridis, 1996), PVP and ethanol precipitation (Salzman et al., 1999) and insoluble PVP, potassium acetate and ethanol (Hu et al., 2002). However, these methods failed to produce acceptable yields and high-quality of RNA (Hu et al., 2002; Tao et al., 2004). To note, the effectiveness of a protocol was subjected to fruit type, mesocarp tissue location and maturity stage (Shellie et al., 1997).

Carambola is rich in both phenolic and pectins and as the fruit ripens mesocarp tissue contained a high level of water, polysaccharides and other unidentified compounds. Extraction of RNA from the mature tissue is likely to be more difficult than from immature tissue because of biosynthesis of potentially interfering substances such as polyphenols, polysaccharides and other secondary metabolites (Shellie et al., 1997). RNA isolated from carambola tissue using the commercial kit (RNeasy plant kit, Qiagen), extraction method for papaya mesocarp by Lopez-Gomez and Gomez-Lim (1992), and

Trizol failed to present two intact ribosomal RNA bands upon gel electrophoresis (data not shown). Moreover, these methods encountered three main difficulties: 1) RNA was degraded 2) very low yields were obtained 3) high polyphenols and polysaccharides contamination. Hence, isolation of RNA was carried out with slight modifications of the CTAB-based method (Zamboni et al., 2008).

The main disadvantages of working with carambola fruit are the high content of phenolics (Lim et al., 2007) and polysaccharides as well as other secondary metabolites (Paull and Duarte, 2012) which render RNA usable for downstream work. These interfering compounds are known to affect both the quality and quantity of RNA by co-precipitating and contaminating the isolated RNA during the extraction (Asif et al. 2000). Polyphenols are readily oxidized and strongly bind to nucleic acid, which would complicate nucleic acid extraction (Xu et al., 2004). Therefore, the addition of soluble PVP (Salzman et al., 1999), EDTA and β -mercaptoethanol are necessary to remove polyphenols (Asif et al., 2000). Nevertheless, other methods did not include the use of PVP due to its capability to bind to nucleic acids and PVP is incompatible with extraction buffer containing phenol (Asif et al., 2000).

Additionally, polysaccharides also interfere with downstream applications since the compound exhibit similar physicochemical characteristics to RNA (Lopez-Gomez and Gomez-Lim, 1992). Polysaccharides also make genomic material become viscous and fail to amplify by inhibiting *Taq* enzyme activity in the PCR reaction (Xu et al., 2004). One of the most effective countermeasures to this problem is to have precipitation step with 100% ethanol and 3 M of sodium acetate that allows RNA to remains in the solution (Asif et al. 2000; Tao et al., 2004). Sodium acetate during this step is also efficient in removing

secondary metabolites from nucleic acid (Xu et al., 2004). Ethanol was widely used in precipitation step as it does not contaminate and reduce the loss of the RNA. Residual ethanol also partially removes the polysaccharides (Tao et al., 2004).

High concentrations of CTAB (2%), together with a high salt concentration (2 M), greatly assisted elimination of polysaccharides and lithium chloride precipitation also partially removed some polysaccharides (Lopez-Gomez and Gomez-Lim, 1992). Carambola contains high water content (more than 90%) in pulp during ripening (Paull and Duarte, 2012). The presence of water will affect the effectiveness of the extraction process by diluting the concentration of reagents buffer and the yield of RNA. Obtaining high quality RNA often proves to be a daunting work due to RNA degradation. By its structure, RNA is inherently weaker than DNA which makes it chemically labile and prone to ribonucleases (RNases).

RNases are ubiquitous and hardy, that cannot be easily eliminated (Miesfeld, 1999). RNases contamination can come from exogenous sources and from the samples themselves as fruit tissues contain endogenous RNases particularly during ripening (Lopez-Gomez and Gomez-Lim, 1992). DEPC solution (diethyl pyrocarbonate) is a common inhibitor used to inactivate RNases by covalent modification. However, this reagent is considered carcinogenic. There are also other types of specific RNase inhibitors to protect against RNA degradation: 1) angiogenin binding protein isolated from human placenta called Rnasin® 2) mixture of vanadyl-ribonucleoside complexes that function as inhibitory transition state analogues of RNase activity (Miesfeld, 1999).

9.4.2 Degenerate primer design and PCR reactions

Genes of interest (SAMDC, ADC, ACS and ACO) are directly involved in PAs and ethylene biosynthesis in plants. However, there is no information available to probe the relationship between PAs-ethylene in carambola fruit particularly during development and ripening. The cross-talk between PAs-ethylene still remains obscure, especially from a molecular point of view. Hence, there is a need to explore and identify PAs and ethylene biosynthetic homologous genes in carambola. As the first step, degenerate primers were employed to obtain a partial fragment of the gene encoding for SAMDC, ADC, ACS and ACO in carambola fruit.

A PCR primer sequence is called degenerate if some of one or more of its position have several possible nucleotides/bases (Wei et al., 2003). In this case, designing degenerate primers is probably the most decisive factor for the success of this study. Operatively, the primers must amplify generally conserved regions and yet operate on very divergent sequences (Iserte et al., 2013). Therefore, we aligned amino acid sequence from at least seven plants with more than 80% similarity. This technique is commonly used to pursue phylogenetic studies in related species or identification of new gene family members (Lang and Orgogozo, 2011; Iserte et al., 2013).

The chosen primers satisfy several rules of thumbs for primer design. These include low degeneracy, feasible annealing temperature, the optimal distance between primers (400bp to <1kb), duplex stability (45-60% of GC content) and each primers length approximately 24-31 nucleotides long. The selection of degenerate primers is constrained by limiting the maximum degeneracy. High degeneracy rises chances of annealing to nonspecific sites, amplified unrelated sequences and losing specificity (Jabado et al., 2006;

Lang and Orgogozo, 2011). A gain in degeneracy number will also increase difficulties to find optimum annealing temperatures (Rose et al., 1998). Therefore, the lowest degenerate number that is presented in primers has the highest probability of PCR success.

Technically, CODEHOP primers are typically made up of a long 5' non-degenerate consensus clamp along with a shorter 3' degenerate core region. The 5' consensus clamp (about 18-25bp) allows stability of the 3' degenerate core annealing to target template without high degeneracy. Meanwhile, a shorter length of 3' degenerate region (about 11-12bp) minimizes the number of individual primers. Even though, the amplification using degenerate primers provides the flexibility of PCR applications but at the same time it also increases the difficulty of primer design (Rose et al., 1998; 2003).

However, the process for designing this type of primer presents more challenge that isn't encountered during the amplification of known sequence. At earlier stage of this study, numerous PCR reactions attempt have been carried out using designed degenerate primers however no amplifications were observed for all the genes and this could be attributed to 1) inadequate primer design i.e. unspecific annealing of the designed primer or insufficient sequence specificity 2) high dissimilarity between consensus primer and unknown target that hinders annealing efficiency to the template 3) great dissimilarity in both forward and reverse primers to efficiently amplified the desired PCR product (Rose et al., 2003; Wei et al., 2003).

9.4.3 Gene sequencing

The PCR products of SAMDC, ADC, ACS and ACO were sent for sequencing to ensure the perfect match of the amplified sequences with the gene sequences that used for primer designation. In all cases, the homology searches showed that sequences analysis matched

the known protein sequences with more than 80% similarity and query cover range from 90 to 95% from other plant species. Such results indicated that the designed degenerate primers were working well by amplifying DNA sequences in the samples. The sequenced products was highly homologous with the protein sequence present in the NCBI database, SAMDC (*Cucumis melo*, NP_001315394.1), ADC (*Prunus persica*, XP_007200307.1), ACS (*Nicotiana tabacum*, NP_001313190.1) and ACO (*Citrus sinensis*, NP_001275812.1) (Table 9.3).

9.5 Conclusion

The main purpose of this work was to established an efficient, rapid and yet simple RNA extraction method from carambola mesocarp tissue. CTAB method was optimized to increase RNA yields and quality. RNA obtained was suitable for cDNA synthesis and further analysis such as reverse transcription PCR. We successfully identified partial fragments of gene sequences involved in PAs biosynthesis pathway by exploiting CODEHOP features to design degenerate oligonucleotide primers. However, further study into gene expressions analysis is recommended in order to gain a deeper insight into PAs biosynthesis genes and their interrelation with ethylene exerted by PAs postharvest treatment during ripening and cold storage of carambola.

GENERAL DISCUSSION AND CONCLUSION

PAs as postharvest application emerges as safer alternative to other chemical treatments because PAs is rather simple biological molecules with well-known metabolic and possess no toxicological residues in the fruit and environment. In this current work, carambola exhibited significant responses to exogenous treatment of PAs. All these responses contribute improvement of carambola postharvest quality during long term storage at 3°C and subsequent shelf life at ambient. Thus, as can be observed in Table 10.1, PAs treatment confers positive attributes on carambola physicochemical quality, blocked the concerted actions of softening enzymes, maintained higher concentration of bioactive compounds, alleviated CI symptoms severity and attenuated lipid peroxidation and membrane ion leakage increment throughout experiment period.

Table 10.1: Demonstrated effects of PAs application in carambola during cold storage and shelf life.

Parameter	Status	PAs beneficial effect
Physicochemical attributes		
a) Weight loss	↓	2.0PUT exhibited positive effects on overall physicochemical quality
b) Colour changes	↓	
c) Firmness	↑	
d) Soluble solid contents	↓	
e) Titrable acidity	↑	
f) Respiration rate	↓	
g) Ethylene production	↓	
h) Sensory evaluation	↑	
Cell wall degrading enzymes activity		
a) PG	↓	2.0PUT effectively suppressed advancement of enzymes activity
b) PME	↓	
c) β-gal	↓	

Bioactive compounds with antioxidant activity

a) Ascorbic acid (AA)	↑	1.0SPD prevented AA losses meanwhile higher
b) Total phenolic content (TPC)	↑	TPC and TAA were enhanced by 2.0SPM
c) Total antioxidant activity (TAA)	↑	

CI evaluation, lipid peroxidation and membrane ion leakage level

a) CI symptoms	↓	2.0PUT stimulated tolerance to chilling stress and retarded cell membrane deterioration
b) MDA content	↓	
c) Ion leakage	↓	

↑=increase, improve, induce ↓=decrease, retard, inhibit

PAs in their free forms have been pictured as antisenescence agent and exerted their beneficial effect by slowing down ripening-related changes, postponed senescence commencement and maintain carambola's sensory quality. Moreover, PAs strongly cross-linked to pectic substances in the cell wall preventing from crumbling down. PAs also blocked the action of softening enzymes hence reducing the softening rate during storage. Immersion of PAs boosted bioactive compounds level in carambola which attributed to PAs properties to act as free radical scavengers and a delayed ripening course. Mainly due to the polycationic nature and membrane-binding capacity, substantial reduction of CI symptoms was observed in fruit treated with PAs. Accordingly, the ionic interaction between PAs and membrane phospholipids serves to trigger membrane rigidification and stabilization thus inhibit membrane permeability.

Our data showed that PAs immersion resulted in an increase of endogenous PAs pool in carambola at 3°C and subsequent storage at ambient (Table 10.2). It appears that exogenously added PAs could induce higher SAMDC activity which favored an activation of the PA biosynthesis pathway and led to an increase in new endogenous PAs in fruit

tissues. Significant rate of PAs accumulation particularly free endogenous PUT could be a protective mechanism against chilling stress. In addition, this current study revealed that there was close correlation between CI scores and enhancements of PAs concentration in carambola tissues.

Table 10.2: Changes in PA contents during storage at 3°C and shelf life at ambient in carambola

PAs type	3°C + 28 days	22±1°C + 6 days
PUT	↑	↓
SPD	↑	↓
SPM	↑	↓
↑=increases ↓=decreases		

A simple and effective RNA isolation from carambola mesocarp tissue was established for the first time. The molecular approach was further studied by identification of partial fragments of PAs and ethylene biosynthetic genes. An optimized PCR amplification with degenerate primers successfully produced single band with expected size. Sequencing results revealed that putative sequences of the partial fragments of ADC, SAMDC, ACS and ACO in carambola were highly homologous with the genes region present in peach, muskmelon, tobacco and sweet oranges respectively.

Yet, an expression of genes encoding PAs enzymes, and their interrelation with ethylene, along their transcripts occurrence exerted by exogenous PAs treatments remain unknown in carambola. Hence, further molecular studies are highly recommended in order to probe and elucidate role of PAs in modulating fruit ripening and senescence. Finally, an attempt to explore into gene expression can assist in providing a clear understanding of the

variations in the PAs signaling cascade and metabolic pathways in response to stress in the plant.

Sweet and tart flavoured carambola are usually consumed in the country they are produced, except for high-quality carambola grown in Malaysia that are exported to European countries which covered almost 90% of the total exports. This research is considered significantly important in order to control or reduce postharvest losses in Malaysian carambola and to achieve clear understanding for the use of PAs as a method of extending shelf life and retaining fruit keeping quality, thus assisting the global aim of food security and sustainable agriculture.

In conclusion, among exogenous PAs studied, PUT at the highest dose (2.0 mM) was found to be the best treatment to enhance postharvest keeping quality in carambola. Carambola treated with 2.0PUT was of better physicochemical performance and longer shelf life, and in general during cold storage, they reduced chilling damage, increased health enhancing properties, diminished activities of cell wall degrading enzymes, retarded membrane leakage and lipid peroxidation level. Moreover, the 2.0PUT application could boost endogenous PAs accumulation particularly free PUT content in carambola tissue resulted in better improvement of postharvest features such as quality and storability of carambola at 3°C.

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APPENDICES

Appendix A

Starfruit/carambola maturity index



Index I



Index V



Index II



Index VI



Index III



Index VII



Index IV

Source: Malaysia Agriculture Research and Development Institute (MARDI).

Appendix B

Hedonic Scale Rating

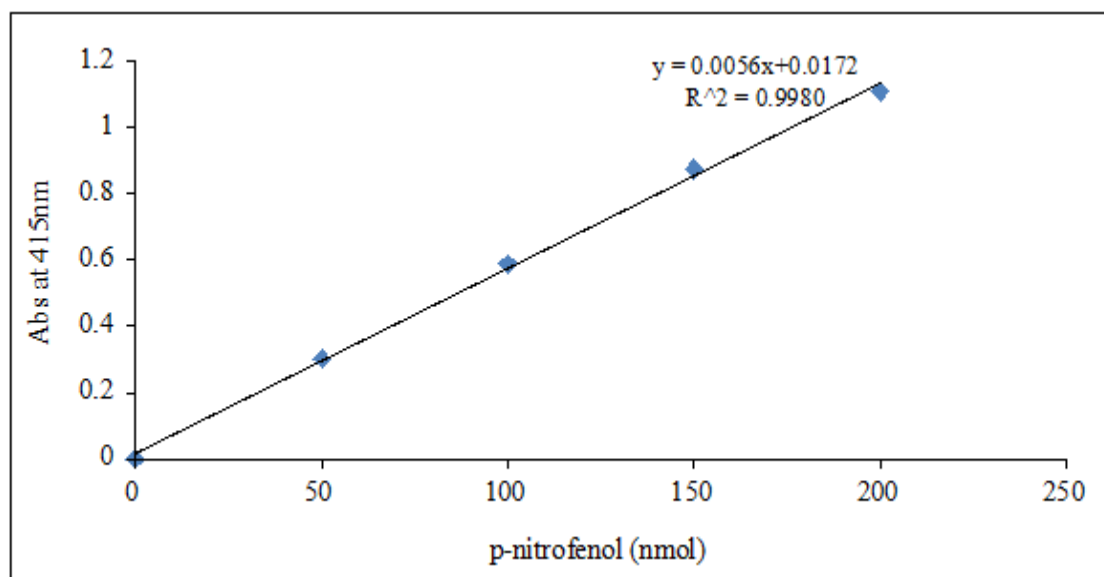
Product: _____ Variety: _____ Date: _____
Name of panelist: _____ Signature: _____

Instructions: (Please read the instructions carefully before filling blanks.)

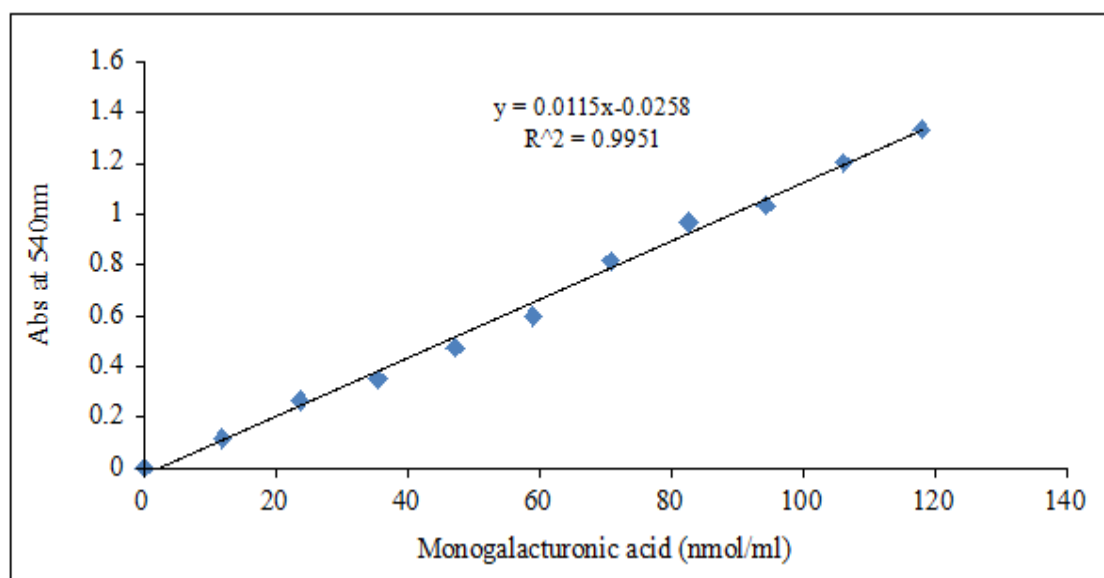
1. This is sensory analysis form for the evaluation of different treatments.
2. Please follow the numerical system for scoring the samples.
1..... Extreme dislike
3..... Dislike
5..... Acceptable
7..... Good
9..... Excellent
3. Please do not disturb the sequence of the samples provided.
4. Please rinse your tongue before testing next sample, with the water provided.

Sample	Appearance	Pulp colour	Aroma	Sweetness	Texture	Overall acceptability
#530						
#174						
#412						
#160						
#802						
#185						
#162						
#282						
#206						
#287						
#219						
#251						

Appendix C

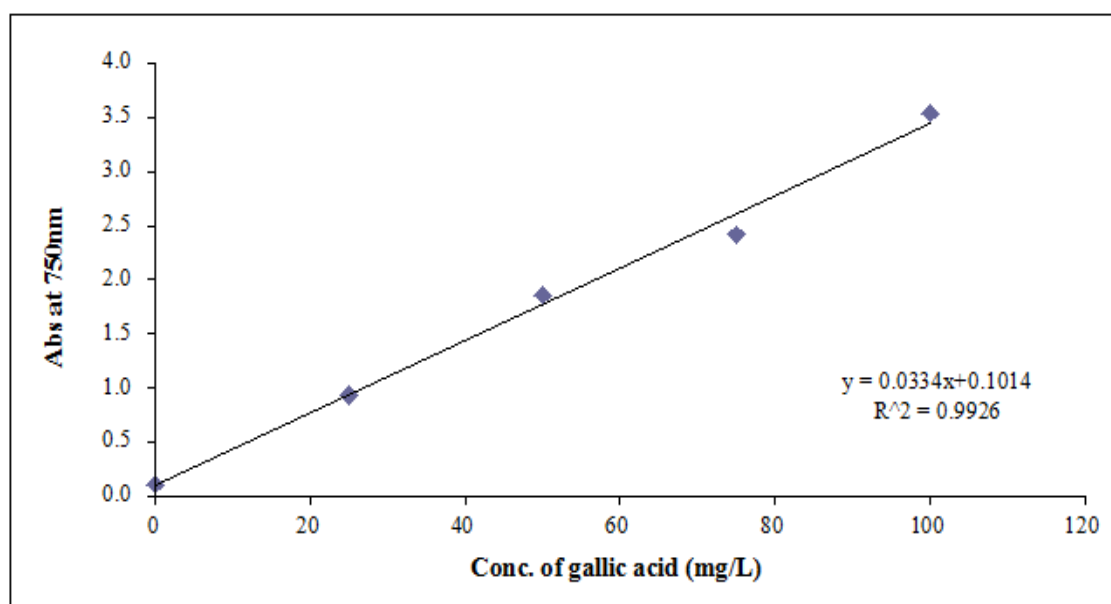


p-nitrophenol standard curve for β -galactosidase assay



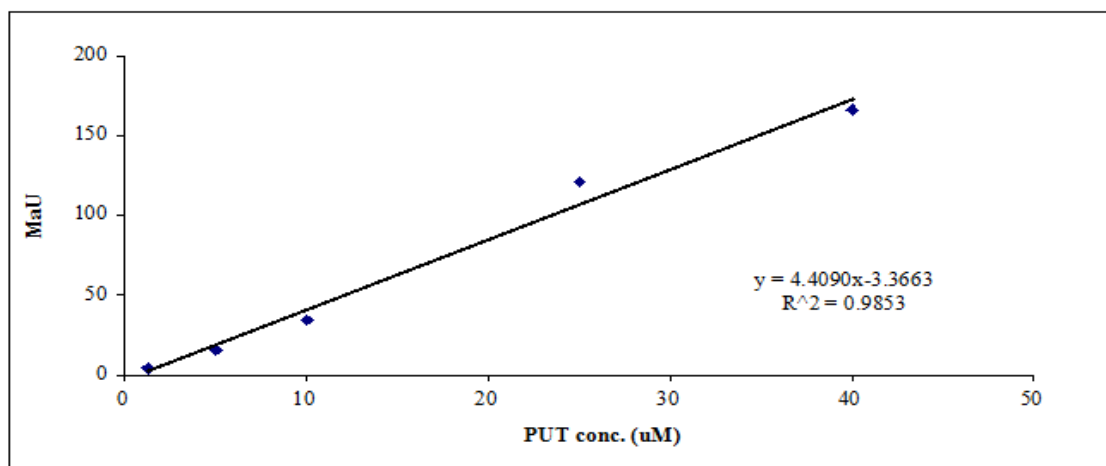
Monogalacturonic acid standard curve for polygalacturonase assay

Appendix D

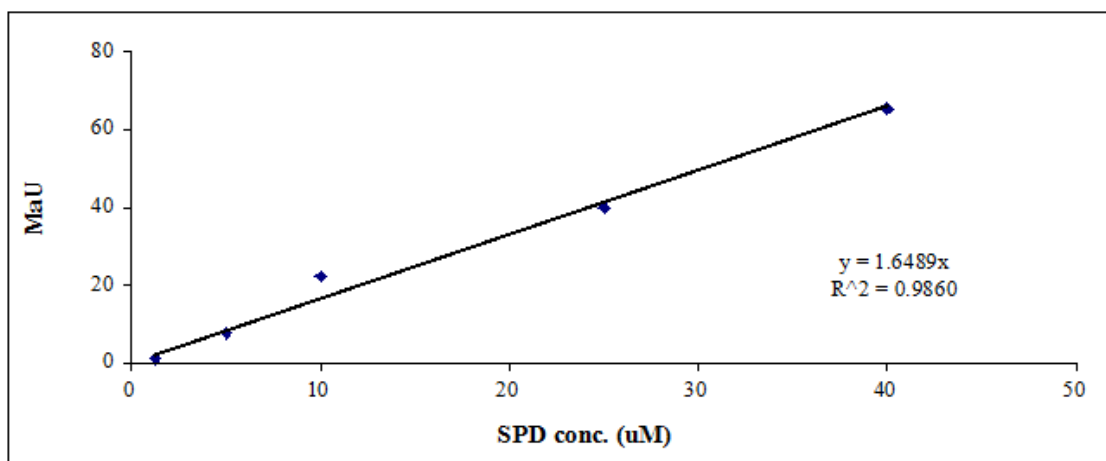


Gallic acid standard curve for total phenolic content (TPC)

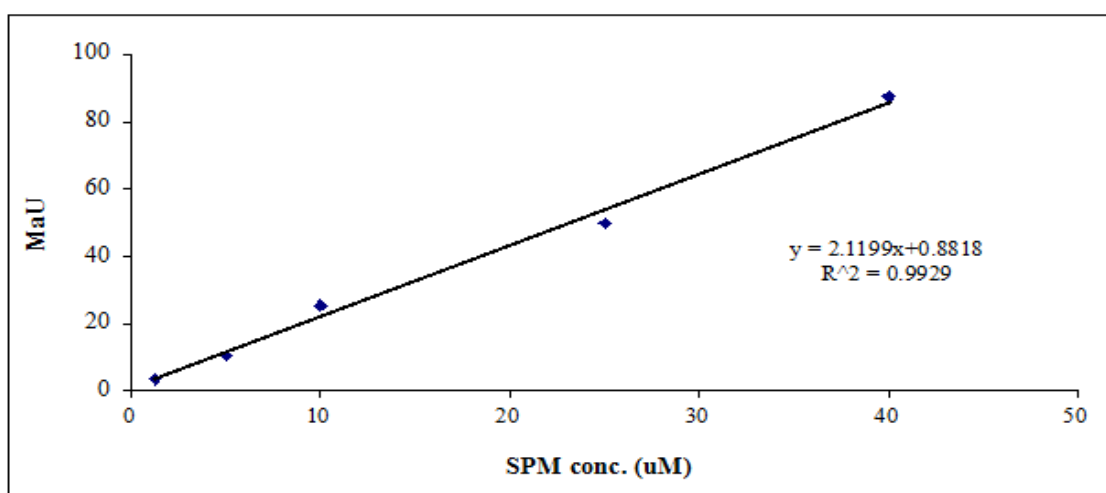
Appendix E



Putrescine (PUT) calibration curve

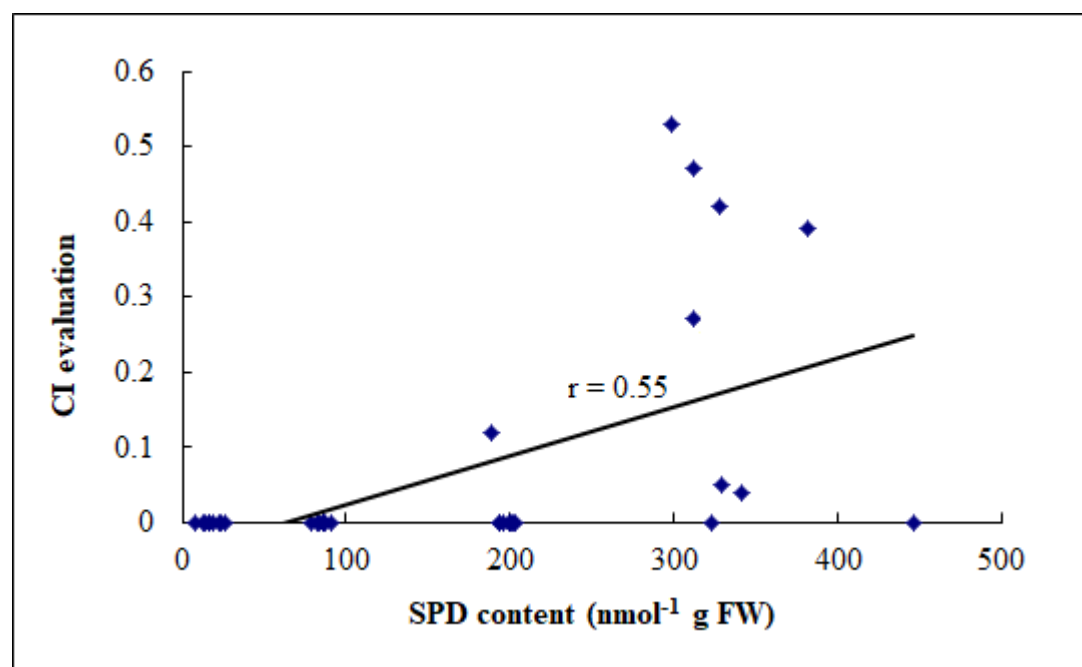
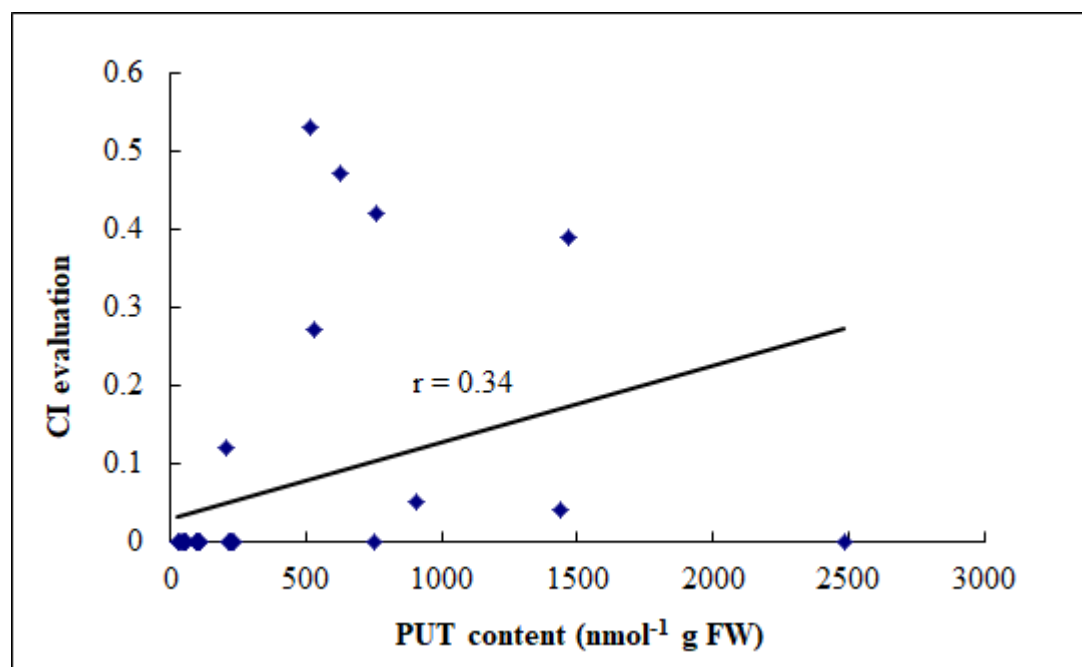


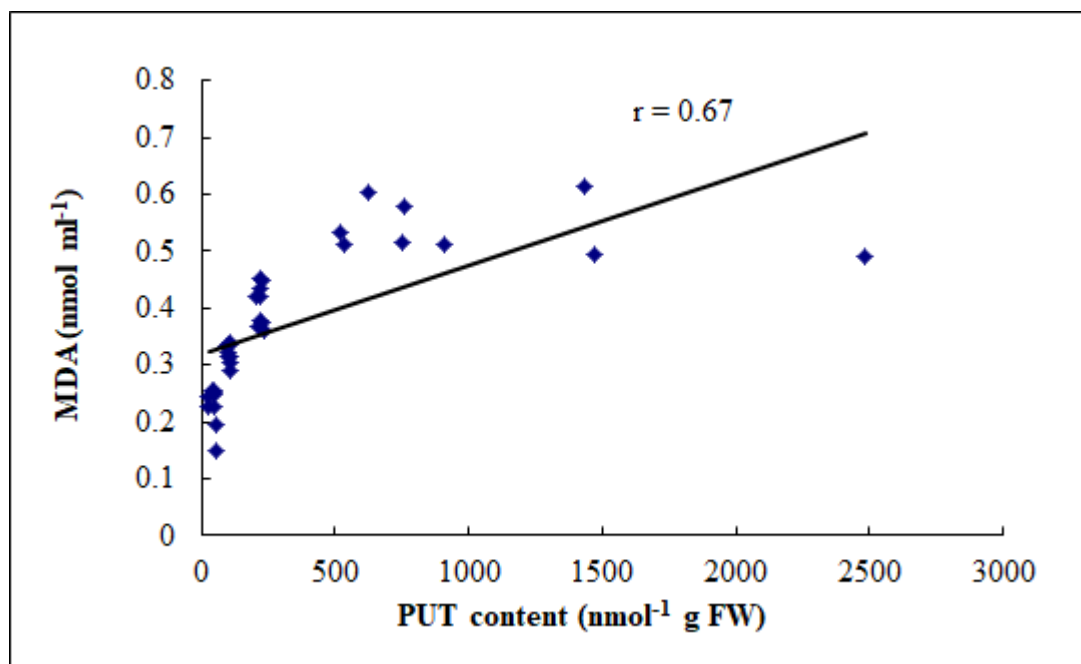
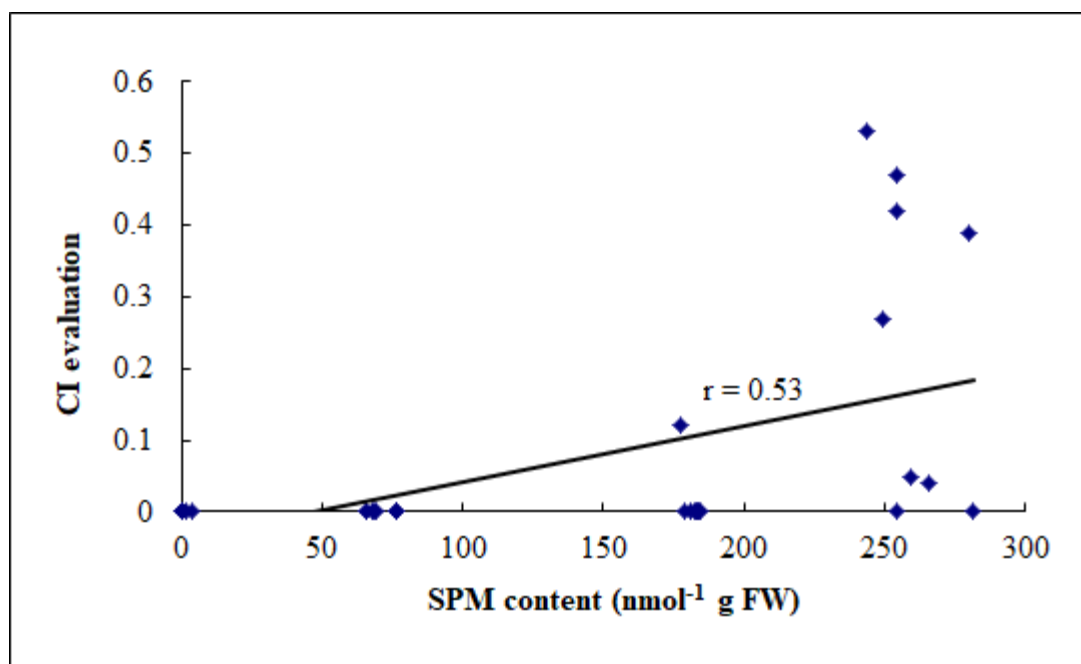
Spermidine (SPD) calibration curve

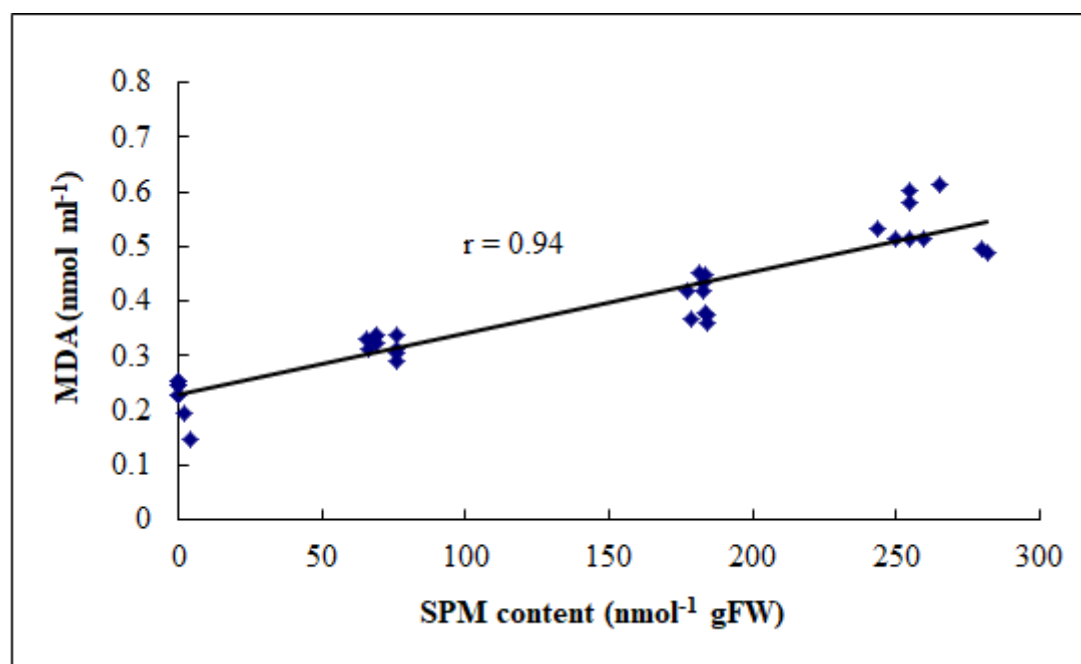
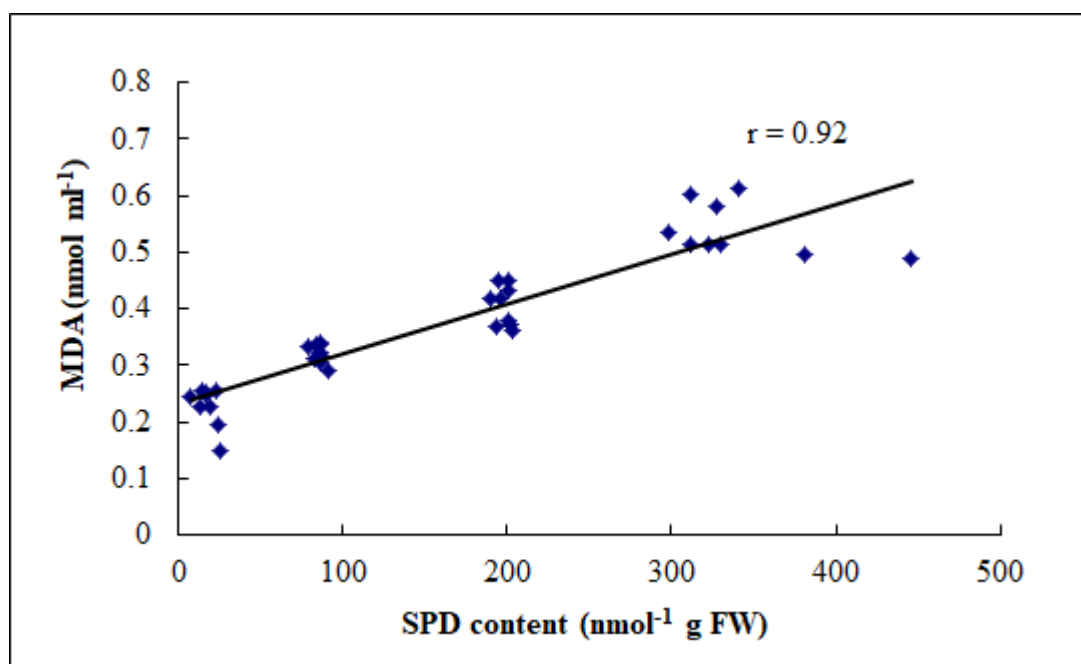


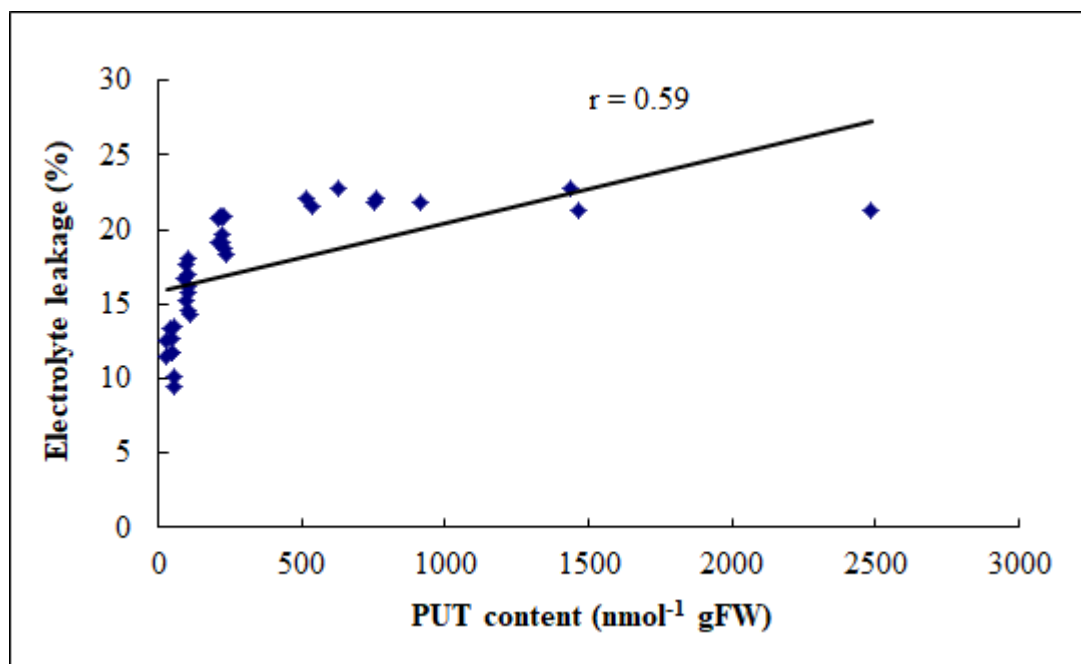
Spermine (SPM) calibration curve

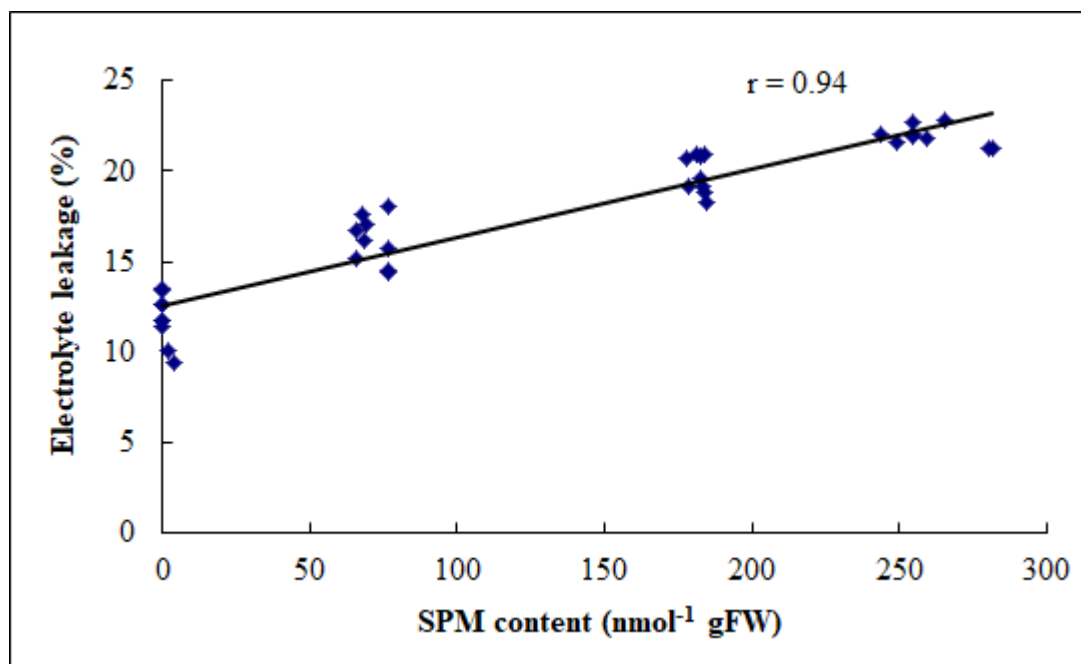
Appendix F











Results from Pearson correlation coefficients of some selected parameters

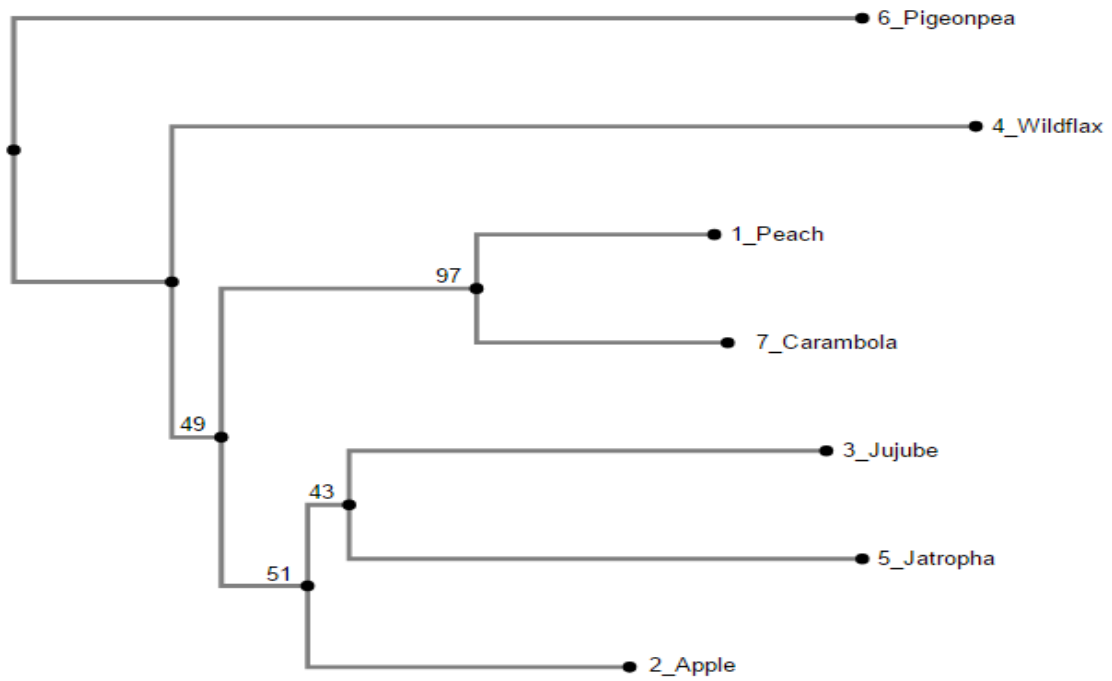
Appendix G

List of protein sequences used for creating protein blocks sourced from the Protein Database on NCBI.

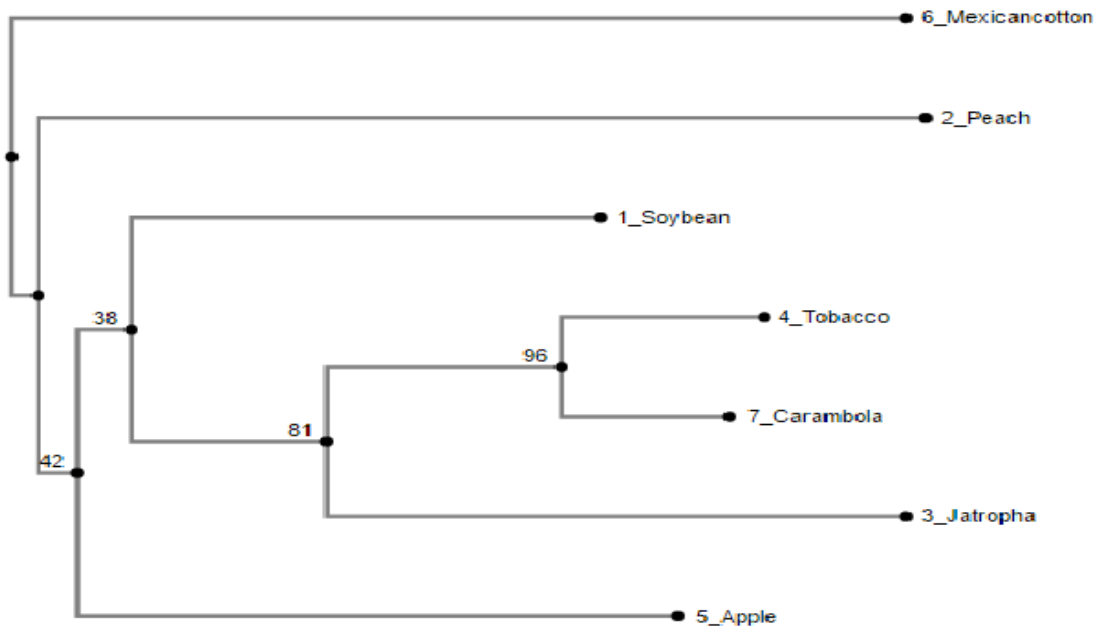
Acc number	Plants species	Gene
NP_186896.1	<i>Arabidopsis thaliana</i> (arabidopsis)	SAMDC
XP_010463654	<i>Camelina sativa</i> (wild flax)	
AAS45435	<i>Brassica juncea</i> (mustard green)	
AGT80621	<i>Camellia sinensis</i> (tea shrub)	
AGL95795	<i>Momordica charantia</i> (bitter melon)	
AGV68760	<i>Cucumis melo</i> (muskmelon)	
AAM44307	<i>Citrus x microcarpa</i> (calamondin)	
NP_001238359	<i>Glycine max</i> (soybean)	ADC
XP_014501343	<i>Vigna radiate</i> (mug bean)	
XP_017422047	<i>Vigna angularis</i> (adzuki bean)	
XP_020218693	<i>Cajanus cajan</i> (pigeon pea)	
XP_016186925	<i>Arachis hypogaea</i> (peanut)	
XP_008461023	<i>Cucumis melo</i> (muskmelon)	
BAD74163	<i>Malus domestica</i> (apple)	
AJF23499	<i>Pyrus communis</i> (pear)	
NP_001295829	<i>Cucumis sativus</i> (cucumber)	
XP_002306141	<i>Populus trichocarpa</i> (cottonwood)	
NP_001236141.2	<i>Glycine max</i> (soybean)	ACS
XP_017440792.1	<i>Vigna angularis</i> (adzuki bean)	
NP_001280870.1	<i>Malus domestica</i> (apple)	
AAD04199.1	<i>Pisum sativum</i> (pea)	
ACT54524.2	<i>Eriobotrya japonica</i> (loquat)	
CAB60721.1	<i>Citrus sinensis</i> (sweet orange)	
AGB96761.1	<i>Ricinus communis</i> (castor bean)	
BAA94599.1	<i>Populus euphratica</i> (desert poplar)	
XP_012086824.1	<i>Jatropha curcas</i> (jatropha)	
AJT35543.1	<i>Hevea brasiliensis</i> (rubber tree)	
NP_001234024.1	<i>Solanum lycopersicum</i> (tomato)	ACO
XP_016554275.1	<i>Capsicum annuum</i> (pepper)	
AHN13883.1	<i>Coffea Arabica</i> (coffea)	
AEC32837.1	<i>Psidium guajava</i> (guava)	
XP_009781832.1	<i>Nicotiana tabacum</i> (tabacco)	
AAP41850.1	<i>Hevea brasiliensis</i> (rubber tree)	
AJW28690.1	<i>Rubus idaeus</i> (raspberry)	
AGF95124.1	<i>Prunus mume</i> (plum)	
XP_008348143.1	<i>Malus domestica</i> (apple)	
AAK57516.1	<i>Carica papaya</i> (papaya)	

Appendix H

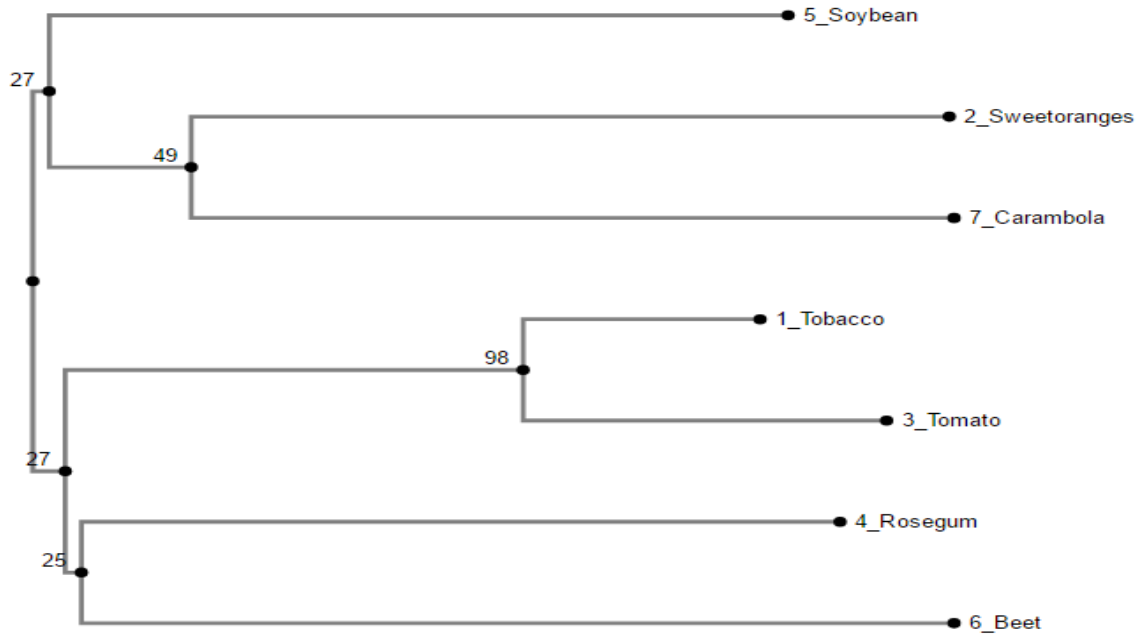
A)



B)



C)



Phylogenetic trees analysis of A) ADC B) ACS C) ACO sequence alignments of carambola and its related plant species. The deduced amino acids were constructed using the MAFFT program

Appendix I

SOV	d.f	s.s	m.s	v.r	F pr
Trt	9	517.93	57.54	23.96	<.001
Day	5	7328.26	1465.65	610.27	<.001
Trt.Day	45	157.33	3.49	1.46	0.037
Residual	300	720.49	2.40		
Total	359	8724.03			

Figure 5.1: Analysis of variance for weight loss (%) of control and polyamine-treated carambola during cold storage (3°C) for 28d (4 weeks) followed by 6d at 22°C (shelf life).

SOV	d.f	s.s	m.s	v.r	F pr
Trt	9	2742.38	304.71	21.84	<.001
Day	6	66106.87	11017.81	789.8	<.001
Trt.Day	54	1183.96	21.93	1.57	0.009
Residual	350	4882.53	13.95		
Total	419	74915.75			

Figure 5.2: Analysis of variance for firmness (Newton) of control and polyamine-treated carambola during cold storage (3°C) for 28d (4 weeks) followed by 6d at 22°C (shelf life).

SOV	d.f	s.s	m.s	v.r	F pr
Trt	9	1849.54	205.5	3.81	<.001
Day	6	85944.6	14324.1	265.46	<.001
Trt.Day	54	1361.26	25.21	0.47	1.000
Residual	630	33994.79	53.96		
Total	699	123150.2			

Figure 5.3: Analysis of variance for effects of PAs applications of different concentrations of PAs on the respiration rate during cold storage (3°C) followed by 6 days at 22°C (shelf life).

SOV	d.f	s.s	m.s	v.r	F pr
Trt	9	10.22	1.13	0.39	0.942
Day	6	532.01	88.67	30.18	<.001
Trt.Day	54	13.82	0.25	0.09	1.000
Residual	630	1851.15	2.93		
Total	699	2407.21			

Figure 5.4: Analysis of variance for effects of PAs applications of different concentrations of PAs on the ethylene production during cold storage (3°C) followed by 6 days at 22°C (shelf life).

SOV	d.f	s.s	m.s	v.r	F pr
Trt	9	7.51	0.83	66.92	<.001
Day	6	348.22	58.03	4654.14	<.001
Trt.Day	54	10.77	0.19	16.00	<.001
Residual	350	4.36	0.01		
Total	419	370.87			

Figure 5.5: Chilling injury index of control and polyamine-treated carambola during cold storage (3°C) for 28d (4 weeks) followed by 6d at 22°C (shelf life).

SOV	d.f	s.s	m.s	v.r	F pr
Trt	9	1263.01	140.33	28.25	<.001
Day	6	4184.12	697.35	140.40	<.001
Trt.Day	54	163.78	3.03	0.61	0.986
Residual	350	1738.47	4.96		
Total	419	7349.39			

Table 5.1: Analysis of variance for variation in luminosity (L^*) of carambola during cold storage (3°C) +6d at 22°C (shelf life) in relation to postharvest treatment with different concentrations of PAs.

SOV	d.f	s.s	m.s	v.r	F pr
Trt	9	740.96	82.33	24.43	<.001
Day	6	1415.13	235.85	69.99	<.001
Trt.Day	54	129.97	2.40	0.71	0.934
Residual	350	1179.45	3.37		
Total	419	3465.53			

Table 5.2: Analysis of variance for variation in saturation (C^*) of carambola during cold storage (3°C) +6d at 22°C (shelf life) in relation to postharvest treatment with different concentrations of PAs.

SOV	d.f	s.s	m.s	v.r	F pr
Trt	9	10246.37	1138.49	60.03	<.001
Day	6	129204.63	21534.10	1135.45	<.001
Trt.Day	54	9384.99	173.80	9.16	<.001
Residual	980	18585.91	18.97		
Total	1049	167421.90			

Table 5.3: Analysis of variance for variation in hue angle (h^o) of carambola during cold storage (3°C) +6d at 22°C (shelf life) in relation to postharvest treatment with different concentrations of PAs.

SOV	d.f	s.s	m.s	v.r	F pr
Trt	9	0.49	0.06	8.15	<.001
Day	6	21.69	3.61	533.63	<.001
Trt.Day	54	0.48	0.009	1.33	0.070
Residual	350	2.37	0.006		
Total	419	25.05			

Table 5.4: Analysis of variance for variation in soluble solids content (SSC) in percentage of carambola during cold storage (3°C) followed by 6 days at 22°C (shelf life) in relation to postharvest treatment with different concentrations of PAs.

SOV	d.f	s.s	m.s	v.r	F pr
Trt	9	0.49	0.06	8.15	<.001
Day	6	21.69	3.61	533.63	<.001
Trt.Day	54	0.48	0.009	1.33	0.070
Residual	350	2.37	0.006		
Total	419	25.05			

Table 5.5: Analysis of variance for variation in soluble solids content (SSC) in percentage of carambola during cold storage (3°C) followed by 6 days at 22°C (shelf life) in relation to postharvest treatment with different concentrations of PAs.

SOV	d.f	s.s	m.s	v.r	F pr
Trt	9	0.61	0.068	13.67	<.001
Day	6	19.25	3.21	642.84	<.001
Trt.Day	54	0.56	0.0104	2.09	<.001
Residual	350	1.74	0.0049		
Total	419	22.18			

Table 5.6: Analysis of variance for variation in titrable acidity (TA) in the percentage of oxalic acids of carambola during cold storage (3°C) followed by 6 days at 22°C (shelf life) in relation to postharvest treatment with different concentrations of PAs.

SOV	d.f	s.s	m.s	v.r	F pr
Trt	9	571.22	63.47	4.21	<0.001
Day	6	17654.49	2942.42	194.98	<0.001
Trt.Day	54	120.38	2.23	0.15	1.000
Residual	980	14788.98	15.09		
Total	1049	33135.06			

Figure 6.1: Analysis of variance for polygalacturonase (PG) activities in control and polyamine-treated carambola during cold storage (3°C) for 28d (4 weeks) followed by 6d at 22°C (shelf life).

SOV	d.f	s.s	m.s	v.r	F pr
Trt	9	8392.4	932.5	4.47	<0.001
Day	6	351788.0	58631.3	281.10	<0.001
Trt.Day	54	1811.9	33.6	0.16	1.000
Residual	980	204403.6	208.6		
Total	1049	566396.0			

Figure 6.2: Analysis of variance for pectin methylesterase (PME) activities in control and polyamine-treated carambola during cold storage (3°C) for 28d (4 weeks) followed by 6d at 22°C (shelf life).

SOV	d.f	s.s	m.s	v.r	F pr
Trt	9	794.84	88.32	6.21	<0.001
Day	6	46848.82	7808.14	548.78	<0.001
Trt.Day	54	1250.30	2.32	0.16	1.000
Residual	980	13943.49	14.23		
Total	1049	61712.45			

Figure 6.3: Analysis of variance for beta-galactosidase (β -gal) activities in control and polyamine-treated carambola during cold storage (3°C) for 28d (4 weeks) followed by 6d at 22°C (shelf life).

SOV	d.f	s.s	m.s	v.r	F pr
Trt	9	13293.5	1477.1	11.14	<0.001
Day	5	229872.8	45974.6	346.74	<0.001
Trt.Day	54	6421.2	142.7	1.08	0.342
Residual	840	111376.0	132.6		
Total	899	360963.4			

Figure 7.1: Analysis of variance for ascorbic acid levels in control and polyamine-treated carambola during cold storage (3°C) for 28d (4 weeks) followed by 6d at 22°C (shelf life).

SOV	d.f	s.s	m.s	v.r	F pr
Trt	9	167584.9	18620.5	18.71	<0.001
Day	6	2970997.2	495166.2	497.56	<0.001
Trt.Day	54	288138.3	5335.9	5.36	<.001
Residual	980	975285.2	995.2		
Total	1049	4402005.7			

Figure 7.2: Analysis of variance for total phenolic contents in control and polyamine-treated carambola during cold storage (3°C) for 28d (4 weeks) followed by 6d at 22°C (shelf life).

SOV	d.f	s.s	m.s	v.r	F pr
Trt	9	9944.93	1104.99	12.57	<0.001
Day	6	274098.98	45683.16	519.68	<0.001
Trt.Day	54	5523.49	102.29	1.16	0.200
Residual	980	86148.09	87.91		
Total	1049	375715.49			

Figure 7.3: Analysis of variance for DPPH inhibition in control and polyamine-treated carambola during cold storage (3°C) for 28d (4 weeks) followed by 6d at 22°C (shelf life).

SOV	d.f	s.s	m.s	v.r	F pr
Trt	9	3.23677	0.359	3.92	<0.001
Day	6	71.63	11.93	130.13	<0.001
Trt.Day	54	8.08	0.149	1.63	0.012
Residual	140	12.84	0.091		
Total	209	95.78			

Figure 7.4: Analysis of variance for MDA levels in control and polyamine-treated carambola during cold storage (3°C) for 28d (4 weeks) followed by 6d at 22°C (shelf life).

SOV	d.f	s.s	m.s	v.r	F pr
Trt	9	1736.82	192.98	4.59	<0.001
Day	6	34699.83	5783.31	137.42	<0.001
Trt.Day	54	1678.24	31.08	0.74	0.913
Residual	350	14729.54	42.08		
Total	419	52844.44			

Figure 7.5: Analysis of variance for electrolyte leakage (%) of control and polyamine-treated carambola during cold storage (3°C) for 28d (4 weeks) followed by 6d at 22°C (shelf life).

SOV	d.f	s.s	m.s	v.r	F pr
Trt	9	1716711	190746	0.37	<0.001
Day	6	21034474	3505746	6.72	<0.001
Trt.Day	54	9172038	169853	0.33	1.000
Residual	140	73067753	521913		
Total	209	104990977			

Figure 8.1: Analysis of variance for PUT contents in control and polyamine-treated carambola during cold storage (3°C) for 28d (4 weeks) followed by 6d at 22°C (shelf life).

SOV	d.f	s.s	m.s	v.r	F pr
Trt	9	17823	1980	0.16	0.997
Day	6	2381288	396881	31.98	<.001
Trt.Day	54	43846	812	0.07	1.000
Residual	140	1737486	12411		
Total	209	4180444			

Figure 8.2: Analysis of variance for SPD contents in control and polyamine-treated carambola during cold storage (3°C) for 28d (4 weeks) followed by 6d at 22°C (shelf life).

SOV	d.f	s.s	m.s	v.r	F pr
Trt	9	88054493	9783833	9.54	<.001
Day	5	261106416	52221283	50.92	<.001
Trt.Day	37	194003562	5243340	5.11	<.001
Residual	88	90253326	1025606		
Total	139	633417798			

Figure 8.3: Analysis of variance for SPM contents in control and polyamine-treated carambola during cold storage (3°C) for 28d (4 weeks) followed by 6d at 22°C (shelf life).

PUBLICATIONS

Submissions to Refereed Journals

Nur Fatin Afifah Hashim, **Azhane Ahmad**, Paa Kwesi Bordoh. Sains Malaysiana (2017). Chilling tolerance, antioxidant status and postharvest storability in chitosan coated Japanese cucumber. (In press).

Nur Aina Adlan Mustafa, Paa Kwesi Bordoh, **Azhane Ahmad**. Pertanika (2018). *Garcinia atroviridis* crude extracts incorporated with gum arabic controlled postharvest anthracnose and extend shelf-life of dragon fruits. (In progress).

Azhane Ahmad, Asgar Ali (2018). Improvement of postharvest quality, regulation of softening enzymes and antioxidant capacity during cold storage of carambola in response to polyamines postharvest treatment. (In progress)

Azhane Ahmad, Asgar Ali (2018). Enhanced polyamines accumulation alleviates chilling disorders in carambola treated with exogenous polyamines during cold storage. (In progress).

Conference Presentations

Biosciences School Postgraduate Symposium 10 June 2014-Oral Presenter, UNMC

Monash Science Symposium 2014 17-18 July 2014-Poster Presenter, Monash University

Research Talk School of Biosciences 3 September 2014-Oral Presenter, UNMC

Research Showcase 2015 8 May 2015-Poster Presenter, UNMC

Biosciences School Postgraduate Symposium 16 June 2015-Oral Presenter, UNMC

International Conference on Crop Improvement 2-3 Dec 2015-Speaker, Universiti Putra Malaysia

21st Biological Sciences Graduate Congress (BSGC) 15-16 Dec 2016-Poster Presenter, Universiti Malaya