Improving production and quality of Gac (*Momordica cochinchinensis* Spreng.) fruit

By

Thi Xuan Tran

Bachelor of Food Processing and Preservation

Master of Biotechnology

Supervisors:

Dr Sophie E. Parks

Dr Paul D. Roach

Associate Professor Minh H. Nguyen

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School of Environmental and Life Sciences

Faculty of Science and Information Technology

University of Newcastle

Australia

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STATEMENT OF ORIGINALITY

This thesis contains no material which previously has been accepted for the award of any other degree or diploma in any universities or tertiary institution. Further, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference has been made in the text.

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Thi Xuan Tran

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PUBLICATIONS

Peer-reviewed journal papers:

1. Tran, X. T., Parks, S. E., Roach, P. D., Golding, J. B., & Nguyen, M. H. (2016). Effects of maturity on physicochemical properties of Gac fruit (*Momordica cochinchinensis* Spreng.). *Food Science & Nutrition*, 4(2), 305-314.

2. Xuan T Tran, Sophie E Parks, Paul D Roach, Minh H Nguyen and Tuyen C Kha (2017). Changes in physicochemical properties of Gac fruit (*Momordica cochinchinensis* Spreng.) during storage. *Australian Journal of Crop Science*, 11(4), 447-452.

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Conference papers:

1. Xuan T Tran, Sophie E Parks, Paul D Roach and Minh H Nguyen (2015). Changes in physical and chemical properties of Gac (*Momordica cochinchinensis* Spreng.) fruit at five stages of maturity. The 48th Annual AIFST convention, 11-13th August, 2015, Sydney, Australia.

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3. Xuan T Tran, Sophie E Parks, Paul D Roach and Minh H Nguyen (2014). Effects of temperature and storage time on pollen viability and pollen tube length in Gac (*Momordica cochinchinensis Spreng*.). The 47th Annual AIFST convention, 22-25th June, 2014, Melbourne, Australia.

LIST OF ABBREVIATIONS AND UNITS OF MEASUREMENT

Abbreviations

a*	Red/green coordinate
ACN	Acetonitrile
b*	Yellow/blue coordinate
BK	Brewbaker and Kwack medium
°Bx	°Brix
$Ca(NO_3)_2\ 4H_2O$	Calcium Nitrate Tetrahydrate
CA	Citric acid
C	Chroma
CO_2	Carbon dioxide
C_2H_4	Ethylene
DCM	Dichloromethane
DW	Dry weight
EC	Electrical conductivity
Fig	Figure
FW	Fresh weight
HPLC	High performance liquid chromatography
H_3BO_3	Boric acid
H^o	Hue angle
IBA	Indole-3- butyric-acid
Kgf	Kilograms force
KNO_3	Potassium nitrate
GSI	Germination speed index
L	Lightness
LAI	Leaf area index
MeOH	Methanol
$MgSO_4\ 7H_2O$	Magnesium Sulfate Heptahydrate
mL	Milliliter
mm	Millimeter

N	Moles of solute in mol
NaOH	Sodium hydroxide
PMC	Pollen moisture content
RH	Relative humidity
R^2	Coefficient of determination
SE	Standard error
SEM	Scanning electron microscope
SD	Standard deviation
TA	Titratable acidity
TSS	Total soluble solids
WAP	Weeks after pollination

Units of measurement

%	Percentage
°C	Degree Celsius
dS/m	Siemens per meter
g	Gram
g/L	Gram per liter
kg	Kilogram
$kg^{-1}h^{-1}$	Kilogram per hour
cm	Centimeter
dS m^{-1}	Siemens per meter
g g^{-1}	Gram per gram
mL.min^{-1}	Milliliter per minute
mg g^{-1}	Milligram per gram
mm	Millimeter
nm	Nanometer
µl	Microliter
µm	Micromole
µmol $m^{-2}s^{-1}$	Micromole per metre per second
v/v	Volume per volume
v/v/v	Volume per volume per volume

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ABSTRACT

The focus of the work undertaken in this study on the Gac plant was to develop production and postharvest practices that would increase yield and maximize fruit quality. The plant Gac is dioecious. The fruit has high nutritional value, and is used in traditional medicine and in processed foods. However, the agronomic and postharvest practices for this crop are not well developed. Specifically, improved ways of germinating seeds and propagating seedlings, methods of assisted pollination, managing fruit size, and controlling fruit quality are required. The methods used for other crops in agronomy and postharvest to improve practices were expected to provide suitable models for Gac. Greenhouse and hydroponic methods were used to grow experimental crops and postharvest techniques were also used to analyse the quality of fruit in this study.

In Chapter 2, the effect of temperature and seed age on seed germination, the effect of auxin concentration on the rooting and survival rate of cuttings and the effects of three rootstock ages and two grafting methods on the survival of grafts were investigated. Seed germinated well at 25-35 °C, IBA (indole-butyric acid) concentrations of 3-5gL^{-1} were suitable for propagating softwood cuttings and top-wedge and slice type grafts were successful with 4 and 8 weeks old rootstocks. These methods could be widely used to propagate Gac with the purposes of increasing the number of female plants. Ways to prolong the viability of seed in storage is a recommend area for further research.

Chapter 3 included developing an *in vitro* method to evaluate pollen viability. The effect of pollen storage on pollen viability and on Gac fruit quality was also investigated. Following this, the effect of temperature on pollen germinability was investigated. The medium including 1% agar, 0.01% H_3BO_3, 0.01% KNO_3 15% sucrose, 250mg $MgSO_4.7H_2O$ and 700mg $Ca(NO_3)_2.4H_2O$ was found to be suitable for Gac pollen

germination at 35 °C. Although pollen germination declined with storage time, hand pollination with stored pollen (for up to four weeks at 4 °C and eight weeks at -20 °C) showed a high fruit set (>73%) and no differences in fruit quality (lycopene and β-carotene concentrations) compared with fresh pollen. Better storage regimes will require an understanding of the desiccation sensitivity of Gac pollen.

In Chapter 4, the effect of fruit load and fruit-set order on fruit weight and quality of Gac was evaluated. The resource allocation among leaves and fruits was also explored. With increased fruit load and fruit-set order, declines in fruit weight and aril quality were found in fruit highlighting the important effect that fruit load can have on fruit quality. This study highlights that leaf area index (LAI) provided a non-destructive indicator of canopy area, having a positive relationship with leaf dry weight ($r^2 = 0.56$), and it may be suitable for use in future studies requiring canopy area estimates.

Chapter 5 evaluated the impact of postharvest storage on some physiochemical characteristics of mature Gac fruit in Vietnam and Australia. Gac fruit harvested prior to full maturity continues to ripen, increasing nutritional quality, in terms of oil, lycopene and β-carotene concentrations in aril. Fruit firmness, skin colour and the TSS (total soluble solids) of aril were identified as potential indicators of the lycopene and β-carotene concentrations. The postharvest research on Gac showed that it may be possible to use a simple measurement of TSS (total soluble solids) in aril juice, or whole-fruit firmness to indicate fruit quality during ripening.

In Chapter 6, Gac fruit harvested at five maturity stages were described in terms of their physicochemical characteristics. Fruit maturity stages M4 and M5 showed the highest quality characteristics in terms of aril oil, lycopene and β-carotene concentrations. The respiration rates and ethylene produced from fruits during storage suggested a climacteric

nature which needs further investigation. This will assist in determining the appropriate storage conditions for Gac fruit.

In conclusion**,** methods of propagation, pollination, canopy management and postharvest practices have been improved as a result of this study and provide information that can be used as a base for further developments in the commercialization and conservation of this species.

CHAPTER 1

TOWARDS IMPROVED CULTIVATION OF GAC

The increased demand for processed products made from Gac has highlighted the need for improved production practices and management of fruit quality. In this study, the practices selected for improvement include propagation, pollination, fruit load management, fruit storage and indices of fruit quality with commercial potentiality are explored.

1.1 Overview of Gac and its uses

The species *Momordica cochinchinensis* Spreng., (*Cucurbitaceae*) called Gac, is a perennial climber, and is dioecious, having male and female flowers on separate plants. It is a variable species and is widely distributed, occurring in Southeast Asia, India and the Cape York Peninsula of Australia (Telford, 1982, Wilde and Duyfjes, 2002) with morphological variation of fruit shape and seed and leaf morphology among variants (Wimalasiri et al., 2016).

Gac fruits are utilized in traditional medicine as anticancer, antidiabetic and antioxidant agents (Somporn et al., 2009) and for treating liver and spleen disorders and sores (De Shan et al., 2001, Xiao et al., 2007). The extract from the Gac leaf is also used to treat skin disease (Somporn et al., 2009) but the benefits of Gac for these conditions have not been verified in clinical trials. Ripe Gac fruits are highly concentrated in carotenoids including lycopene and beta carotene and a greater use of Gac in the diet was shown to alleviate vitamin A deficiency in poor Vietnamese children (Vuong et al., 2002). Furthermore, other carotenoids are also present in Gac, including lutein, zeaxanthin and β-cryptoxanthin (Kubola and Siriamornpun, 2011, Kha et al., 2013a). In particular, lutein

is a treatment taken for the prevention of macular degradation (Roberts et al., 2009) but currently is not extracted from Gac for this purpose. A review of the bioactive compounds in Gac and their potential health benefit has been conducted by Chuyen et al. (2015).

The high levels of carotenoids of Gac fruit was recorded in previous studies, especially carotenes and lycopene compared to other fruits and vegetables. The lycopene concentration in Gac fruit is at least five times higher than in some fruits (grapefruit, papaya, guava and watermelon) (Aoki et al., 2002, Rao and Rao, 2007). This content in the Gac aril has been recognized as high as 70 times that in tomatoes (Burke et al., 2005) which are the major source of lycopene in the Western diet. Gac aril contains the highest concentration of β-carotene in comparison to other fruits and vegetables (Vuong, 2000). For example, this content is 16 times higher than that in yellow pumpkin or eight times higher than the level in carrots, which are recognized as being high in β-carotene (Vuong et al., 2002).

Gac is a popular fruit in Vietnam with reputation of having a high nutritional value (Vuong, 2000) and it is used in food industries. A review of processing options for Gac has been conducted by Kha et al. (2013a). The Vietnamese use the ripened flesh surrounding the Gac seeds (aril) as a colorant for cooking sticky rice to made “xoi Gac” a dish for weddings and New Year celebrations. It also has been used as a vegetable in India (Vijay et al., 1977), Thailand (Kubola and Siriamornpun, 2011), China and Japan (Jeffrey, 2001). The pulp can be mixed with red aril and sticky rice to make “xoi Gac” dish. When the Gac fruit is immature, the pulp is used as green vegetable. For example, the Vietnamese use the pulp to cook with fish or pork brine. In Thailand, Gac is cultivated at a commercial scale to produce functional foods including health drinks (Somporn et

al., 2009). The carotenoids in Gac fruits including lycopene and β-carotene have the potential to be used as natural colorants in the food industry (Bateman et al., 2004). Furthermore, some Vietnamese and international companies have invested in commercial production of Gac and have developed health products including Gac seed alcohol and drinks and capsules made from the aril of the fruit.

1.2 Characteristics of the Gac plant

1.2.1 Species diversity

Momordica cochinchinensis is a variable species and is particularly heterophyllous (Wilde and Duyfjes, 2002). There are two types of wild species in North East India and the Andaman islands (Joseph and Bharathi, 2008). Also, in Vietnam, Gac is divided into three types: ordinary Gac (Gac te), sticky Gac (Gac nep) and hybrid Gac (Gac lai). The sticky Gac has a big fruit with sparse and big spines and deep red aril. The ordinary Gac has a medium or small fruit size, thick skin, shaped spine, light red aril and the hybrid Gac has not been described to date. The morphological variation of M. *cochinchinensis* was described with 42 accessions collected from Vietnam, Thailand and Australia. The highest diversity of Gac was observed in accessions in Vietnam and Australia. Australian samples had a similar morphology to those of the Southern Vietnam accessions (Wimalasiri et al., 2016).

New varieties may be established to promote varieties with bisexual flowers. This may improve the yield of Gac (Sanwal et al., 2011). Cross pollination between *M. dioica* Roxb. and *M. cochichinensis* Spreng. has been attempted to improve fruit quality. The size of crossed fruit increased when *M. cochichinensis* was used to pollinate *M. dioica* (Mohanty

et al., 1994). However, these studies are preliminary in the development of new Gac varieties.

1.2.2 Botanical characteristics

1.2.2.1 Flowers

Gac is dioecious in that it has male and female flowers on separate plants and flower is solitary. The female flower can be detected by a bulge at the base of the flower, which is the unformed fruit (Singh and Vawara, 1988). The bract is small, sepals is oblong with 4-10mm long (Bharathi and John, 2013). The first flower bloom after growing 90-120 days (Bharathi and John, 2013). The female flowers grow in less time (19-22 days) than male flowers (20-24 days) from bud to full blossom phase (Vijay et al., 1977). Male flowers tend to appear prior to the main flush of female flowers (Parks et al., 2013).

The Gac male and female flowers are imperfect flowers which contain either a stamen or a pistil only (Fig 1.1). The Gac flower is large (7.5 centimeters across) and the color is cream with five petals and three inner petals (Joseph and Bharathi, 2008). The flower is solitary with a dark bract on the base. Anthesis (flower opening) is in the morning. It takes more than two hours to open (Maharana and Sahoo, 1995) and the receptive time for female flower is 24 hours.

(a) (b)

Fig 1.1 The Gac flowers are imperfect flowers (a): Male flower shows three stamens; (b): Female flower shows an ovary with three cells.

1.2.2.2 Fruit morphology

Gac fruits are green coloured when immature and ripen to orange or red at maturity. In general, they are harvested at maturity (90-100 days after pollination) (Bharathi and John, 2013). The components of Gac fruit include the skin, yellow pulp, aril and seed. Gac is a large fruit, ovoid, oblong or globular shaped, with the weight depending on type (350-2500 g), its length is 15-30 cm and its diameter is 8- 25 cm (Fig 1.2) (Wilde and Duyfjes, 2002). The fruit stalk is 5-12 cm long (Bharathi and John, 2013).

Fig 1.2 The Gac fruit.

The Gac fruit skin is densely covered in soft spines, the length of a spine being 10 mm. The skin is green when immature and turns orange or red when ripening. The skin contains carotenoids including lutein, with higher concentrations when compared with the aril or pulp (Kubola and Siriamornpun, 2011).

The yellow pulp or mesocarp makes up the highest component of Gac fruit (49% total of weight) (Fig 1.3) (Kha, 2010). It includes some carotenoids but with a lower concentration when compared with the skin or the aril (Kubola and Siriamornpun, 2011, Aoki et al., 2002).

Fig 1.3 The Gac pulp.

The Gac seeds are covered by aril of a deep red colour when mature (Fig 1.4). The mature red aril has the highest nutritional value of all fruit parts with lycopene and carotene and other bioactive compounds at high concentrations. The proportion of aril has been recorded previously as 10%, 18% and 24.6% of fresh fruit weight (Ishida et al., 2004, Kha, 2010, Nhung et al., 2010). Gac also contains bioactive substances such as protein that may inhibit tumor development of some cancers (Sarma et al., 2011). Furthermore, vitamin E, fatty acid, flavonoid glycosides have also been found in Gac fruits (Sarma et

al., 2011). The carotenoids in Gac aril are responsible for the orange or red colour of the dish “xoi Gac”.

Fig 1.4 The Gac aril.

The number of seeds per fruit varies with between about 10-50 seeds per fruit. The size and shape of seeds are circular, ovate or elliptic, 2.6-2.8 cm diameter and 5-6 mm thickness (Fig 1.5). When fruits are young, the seed coat is white and floppy and it develops to a brown or black colour. In generally, the number of Gac seed did not change during fruit development (Tran et al., 2016). The seed coat is lumpy and has undulated edges (Wilde and Duyfjes, 2002, Handique, 1988, Somporn et al., 2009). Gac seeds can be stored for six months in the refrigerator (Singh and Vawara, 1988). The grower must consider the unpredictable ratio of male and female plants when Gac seeds are utilized as a source for propagation with the ratio male and female 1:10 (Maharana and Sahoo, 1995). Gac seed is used known as “Mubiezi”, a traditional medicine in China with cooling properties for the treatment of liver and spleen disorders, wounds, bruises and swelling (Zhi-Yan et al., 2012). The Vietnamese use Gac seeds blended with alcohol to treat swelling, mastitis and mixed with vinegar to cure hemorrhoids.

Fig 1.5 The Gac seed.

1.3 Traditional cultivation

Gac fruits are collected from the wild and are cultivated in home gardens and grow over lattices. The plants are traditionally cultivated by seed or hardwood cuttings. The Gac plant is perennial and after harvesting the fruit, the main stem is cut back to 20 cm above the ground and regrows, producing fruit in the next year. One major challenge when growing from seed is not knowing the gender of the plant until the flowers have opened. Vegetative propagation by vine cuttings is an effective alternative. In one study, propagation by cuttings required less time than cultivation from seed (Joseph and Bharathi, 2008) but information is limited in this method .

1.4 Potential crop practices to improve production

1.4.1 Propagation

The improvement of propagation conditions is a potential practice to enhance production for Gac. Previously, cooking the seed in “xoi Gac” is thought to overcome dormancy but a study has indicated that dormancy is not a problem with seed germination (Parks et al., 2013). The information on standardization of germination parameters is important for any seed propagated crop (Kumar et al., 2011) and these parameters have not been

investigated for Gac. Hardwood cuttings of Gac are only used in traditional cultivation and available material is usually limited. There is a poor understanding of production using other cutting types including semi-hardwood and softwood. Moreover, the effect of other propagation conditions such as the use of rooting hormones and grafting methods are limited for Gac. The application of suitable propagation conditions would allow growers to better manage propagation and improve production.

1.4.2 Pollination

The Gac flower is normally pollinated by insects and traditional cultivation has shown poor control of pollination. A previous study indicates that hand pollination can be used to significantly improve fruit set (Maharana and Sahoo, 1995). Male flowers tend to bloom earlier than female flowers (Parks et al., 2013) and since their flowering times may not always coincide, pollen availability may be scarce. Therefore, storage of pollen may be useful to provide a pollen source when male flowers are not available. Furthermore, these practices may be used to produce Gac in greenhouses that lack insect pollinators. Ultimately, stored pollen would reduce the need for unproductive male plants within the crop and maximize the number of female plants.

1.4.3 Fruit load management

Gac is a perennial plant with large fruits and an unrestricted fruit load is commonly used in traditional cultivation. This may lead to non-uniformity in fruit size and quality. The crop load and fruit position has been shown to affect fruit size and fruit quality in a number of species. For example, in cucumber, increasing the number of fruits per plant reduced fruit weight due to decreasing dry-matter assimilation per fruit (Marcelis, 1994) and in olive, a high crop load was shown to reduce fruit oil concentration (Gucci et al.,

2007, Trentacoste et al., 2010). A lower leaf-fruit ratio in persimmon showed a high yield but fruit weight, soluble solid and fruit colour declined highlighting the effect on quality (Choi et al., 2010). Therefore, the investigation of effect of fruit load on Gac fruit quality may provide to select the suitable fruit load and improve the quality for Gac fruit.

1.4.4 Fruit quality control

The nutritional quality of Gac fruit is likely to be affected by many factors including stage of maturity, growing conditions and variety. The stage of maturity has a strong effect on aril quality. For example, Gac fruit harvested at a fully ripe stage were of a higher quality than less ripe fruits in term of their carotenoid contents (Nhung et al., 2010, Kubola and Siriamornpun, 2011). Gac fruits sourced from the field contained higher levels of carotenoids than fruits sourced from the markets (Nhung et al., 2010, Ishida et al., 2004, Vuong et al., 2006) perhaps reflecting that the market fruits were not fresh and had reduced quality.

The effect of fruit maturity and storage of fruit on fruit quality of Gac is not well understood. Traditionally, in Vietnam, Gac fruit are harvested by farmers according to fruit firmness, and by how much of the skin has turned orange and the fruit are stored for between one and four weeks. Quality of fruit can be affected by a number of factors such as storage time and stage of fruit maturity at harvest. For example, during storage for two weeks, carotenoid contents had significantly declined (Nhung et al., 2010). Also, Gac fruit harvested at a fully ripened stage were of a higher quality than less ripe fruits in terms of their carotenoid concentrations (Nhung et al., 2010, Kubola and Siriamornpun, 2011). It is possible that indices of fruit quality could be developed to monitor Gac fruit quality. For example, the indices of firmness in European plums (Usenik et al., 2014),

skin colour in apples (Łysiak et al., 2014) and TSS in mangoes (Amin et al., 2013) are currently being used as indicators of fruit quality. Understanding the factors that affect fruit quality will lead to better postharvest methods.

The aim of this study was to develop production and postharvest practices that would increase yield and maximize fruit quality. Specifically, improved ways of seed germinating, cuttings and grafting, methods of assisted pollination, managing fruit size, and controlling fruit quality are required. The methods used for other crops in agronomy and postharvest to improve practices were expected to provide appropriate models for Gac. Greenhouse and hydroponic methods were used to grow experimental crops and postharvest techniques were also used to analyse the quality of fruit in this study.

CHAPTER 2

IMPROVING PROPAGATION OF GAC

2.1 Introduction

Traditionally, Gac is propagated by seed or female cuttings as Gac is a dioecious species. Currently, the plant gender of seedlings can only be identified at the development of flowers (Parks et al., 2013) presenting a challenge when propagating by seed. Other methods could be used to propagate Gac including the use of cuttings and grafting techniques but little information is available on these.

2.1.1 Seed germination

Temperature is an important factor affecting seed germination (Verma et al., 2010) and its effect on Gac seed has not been investigated. The optimum temperature guideline for germination for any species is based on obtaining a maximum germination rate. Temperatures that are excessively high or low can inhibit germination of vegetable seed (Wagenvoort and Bierhuizen, 1977). A previous study has investigated the optimum temperature of some seeds that belong to *Curcubitaceae* including cucumber (22 °C), muskmelon (24-26 °C), squash (22 °C) and watermelon (21-22 °C) (Nau, 1991). Traditionally in Vietnam, fresh Gac seed is germinated after steaming it with the sticky rice dish, xoi Gac. However, seed germination was demonstrated at a temperature of 25°C without steaming in a previous study (Parks et al., 2013).

Seed germination may also be greatly influenced by seed age (Oziegbe et al., 2010). In *Ludwigia* species, older seeds germinate earlier than fresh seed due a 2 years dormancy (Oziegbe et al., 2010). The Gac seed is coarse and is covered by a thick hard seed coat and when removed has been shown to overcome dormancy of Gac (Pandey et al., 2013). However, Parks et al. (2013) have demonstrated that Gac seed do not appear to have a

dormancy phase. Other species such as grain amaranth have germination rates rapidly decline for seeds older than one year (Aufhammer et al., 1998). The rate of germination can also relate directly to seed weight, with heavier seeds germinating first, for example as occurs in tamarind (Olufunke and Gbadamosi, 2009). The Gac seed has a large weight and any relationship between seed weight and germination rate may be useful in seed selection for propagation.

2.1.2 Cuttings

Traditionally, Vietnamese farmers strike Gac cuttings from plants that have proven to yield well for 5-6 years as mother plants. Cuttings or "hom" are prepared from semi-hardwood or hardwood stems that are 0.5-2 cm thick. They are cut into 30-40cm lengths having 4-5 nodes and are de-leafed. The cuttings are planted directly into the soil or a plastic tub containing soil or sand. The stems root within about 20-30 days. This propagation method is successful for increasing the number of female plants but it requires a large biomass of mother rootstock and may not be as economical for commercial propagation as striking softwood cuttings.

The survival and rooting rate of cuttings of a range of species depends on several factors including the selection of cutting materials, environmental conditions during propagation and the use of rooting hormones (Hartmann et al., 2002). Commercially, auxins are commonly applied to stimulate the rooting of cuttings in liquid, powder or gel formulations, containing indole-3-butyric acid (IBA), 1-naphthaleneacetic acid (NAA), or a combination of the two (Blythe et al., 2004). A previous study on Gac reported used rooting hormone to promote rooting of cuttings (Bharathi and John, 2013) but the application of auxin for Gac propagation has not fully been described. In a closely related species, *Momordica dioica* Roxb., the rooting of cuttings increased when they were

treated with IBA hormone powder (Mohammad et al., 1991). In a preliminary study of Gac propagation by cuttings, with IBA (powder and gel) the benefit of auxin treatment was inconclusive and further investigation was recommended (Parks et al., 2013).

2.1.3 Grafting

Grafting is a potential technique to salvage unwanted male Gac plants by using them as mature rootstock material. Grafting of the Gac plant has been demonstrated previously using the insertion grafting technique with male rootstocks and female scions (Joseph and Bharathi, 2008). The study was limited to a small number of plants but the rate of success the graft was high and 25 fruits were harvested per grafted plant compared with 16 fruits for seed-grown and 8 fruits for plants propagated from cuttings. The grafted plants also required less space (4-5 m^2) and less time for growing compared with plants that were grown from seed or cuttings (Joseph and Bharathi, 2008).

Seedlings have potential as a ready source of rootstocks for union with female scions. Seedlings are used as a rootstock source for grafting of many related species including cucumber, watermelon and squash (Colla et al., 2013, Mohamed et al., 2012, Traka-Mavrona et al., 2000). This method can save time and increase the number of fruiting plants in production as well as providing other benefits including disease resistance. However, the age of the rootstock and the methods of grafting will affect the success of the grafts (Medagoda I. , 2007, Solomon Jr et al., 2012) and these factors need to be investigated for Gac propagation.

This chapter aimed to improve propagation techniques for Gac using several approaches. The effect of temperature and seed age on the germination of Gac seed, the effect of auxin (indol butyric acid-IBA) concentration on the rooting and survival rate of Gac cuttings,

the effects of three rootstock ages (4, 8 and 12 weeks) and two grafting methods (slice and wedge) on survival rate of grafting combination were investigated.

2.2 Materials and Methods

2.2.1 Seed germination experiment

Mature Gac fruits were harvested in May during three consecutive seasons (2012, 2013 and 2014) from greenhouse crops grown at the NSW Department of Central Coast Primary Industries Centre, Ourimbah, NSW, Australia (151° 22'E, 33° 21'S). The air temperature and the relative humidity of the greenhouses were maintained within bands of 18 °C to 25 °C and 60% to 80 %, respectively. The seeds were removed from fruit and washed with distilled water. They were dried at room temperature (21± 1°C, 60% relative humidity) on plastic trays for one week until they reached approximately 10% moisture content. These seeds were placed in open paper bags and stored at room temperature (21 ± 1°C). Each individual seed was labelled and weighed before conducting the experiment. The moisture content of a sample of seed was determined using constant temperature oven dry method as described in ISTA rules (ISTA, 1993).

The Gac seeds produced in three seasons (2014, 2013 and 2012) were extracted and stored for 6 months, 18 months and 30 months, respectively and sown into coco peat trays (35cm × 30cm × 6cm in size). Trays were placed into five incubators at constant temperatures of 20 ºC, 25 ºC, 30 °C, 35 °C and 40 ºC. All treatments were kept on a 12h light/12h dark (approximately 50 µmol photons $m^{-2}s^{-1}$) photoperiod provided by cool white fluorescent lights for 20 days using a factorial design with five replicates. Each replicate contained 15 seeds which were sown at a depth of 5 cm in the trays. The trays containing the seeds were sprayed with 100ml distilled water, daily per tray, to ensure adequate moisture for

germination. A seed was considered to have germinated when the radicle had emerged from the seed coat.

Germination speed index (GSI) estimates the mean number of seeds germinated per day and was calculated using the formula (1) as described by (Ribeiro and Costa, 2015):

$$GSI = G1/N1 + G2/N2 + \ldots + G20/N20 \quad (1)$$

where G1, G2,.. G20- is the number of seeds germinated every other day and N1, N2,.., N20- is the number of days after seed incubation began.

2.2.2 Cuttings experiment

Cuttings were collected from three- year old greenhouse- grown female plants. Two types of cuttings were collected: semi-hardwood (0.6-0.8cm diameter) and softwood (0.3-0.5cm diameter), 7-10cm long with one node for both. The cuttings were dipped 0.5cm deep for 1s into commercial indole-3-butyric acid (IBA) gel (Yates Clonix Purple, Padstow, NSW 2211, Australia) at four treatment concentrations (1.5g/L, 3.0g/L, 5g/L and 8.0g/L) and a water control (0g/L). The cuttings in all treatments were placed in moistened rock wool on the trays in a randomized complete block design with 10 treatment combinations and eight replications. Each experimental unit contained 10 cuttings. The trays with the cuttings treated with IBA were placed in a bench (2m × 1m × 1m). Air temperature at the cutting height ranged between 16 ºC- 25 ºC and relative humidity was maintained above 90% with overhead sprinklers. A propagation bench was covered with 80% shade in a polyethylene house. The propagation house temperature and relative humidity were maintained at 18-30ºC and 60-100%, respectively. The survival rate (cuttings with roots), root number and leaf number were measured after six weeks.

2.2.3 Grafting experiment

The seedling rootstocks were germinated in trays before being transfered to plastic pots (150 mm diameter, 200 mm deep). The pots were filled with potting mix (coir, vermiculite, perlite in 1:1:1 v/v/v). These pots were placed in the greenhouse and irrigated with overhead sprinklers. The seeds were sown at 4 week intervals and grafted at the same time (4, 8 and 12 weeks old). New softwood scions were collected from three-year old female plants (6-8 cm with one bud in length). The mother stocks had been pruned after fruiting and the new scions were selected from softwood shoots. The stem diameter of scions was matched with the rootstocks at grafting.

The experiment combined three seedling rootstock ages (4, 8 and 12 weeks after germination) and two grafting methods: slice and wedge (Lee et al., 2010). Seedling rootstocks were cut at 10-15 cm above ground level at grafting. Grafts were wrapped with plastic buddy tape (25mm wide) (Aglis Co., Ltd, Fukuoka, Japan). A completely randomized design was used for 6 treatment combinations; each treatment included 10 rootstocks with 6 replicates. Grafted plants were placed in a growth room with temperature maintained at 22 (±1°C), with humidity 90 % and a 12-h photoperiod (approximately 100 µmol $m^{-2}s^{-1}$). The number of surviving grafts was counted weekly and the experiment was monitored for 6 weeks.

2.2.4 Statistical analysis

The statistical analysis was done using SPSS software version 24.0. Analysis of variance (ANOVA) was used to test for seed age, temperature effects, IBA concentration and rootstock age effects. The least significant difference (LSD) at a 5% level was used to compare the means of different test parameters under treatment temperatures, IBA concentrations and rootstock ages. Post hoc tests for all analyses were made with LSD ($p< 0.05$). Graphs were made by using GraphPad Prism 7.

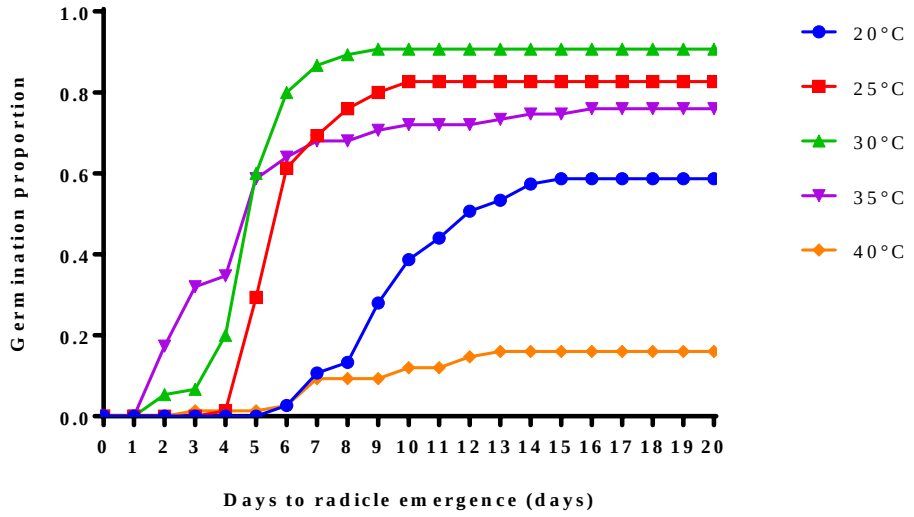
2.3 Results

2.3.1 Effects of temperature, seed age and seed weight on the germination of seeds

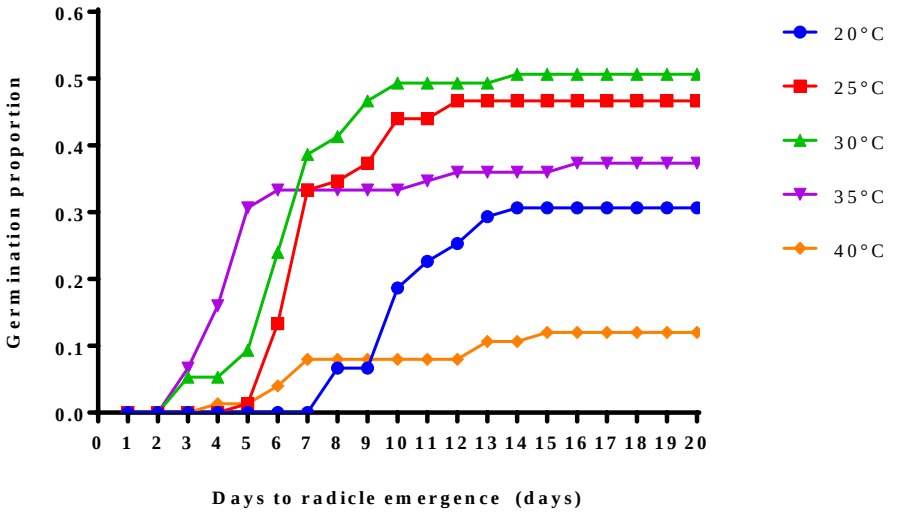
Effect of temperatures

No germination was observed for seed that was 30 old months at any temperature. The effect of temperature on the germination proportion of 6 months and 18 months old Gac seed is presented in Fig 2.1 (a and b). The highest seed germination proportion was obtained for 6 months old seed at 30 °C. In comparison 18 month old seed, the germination rate was less than 45% at a similar temperature. At a low (20°C) or at a high (40°C) temperature, the germination of all seed was significantly reduced compared to those at 25, 30 and 35°C for seed age at 6 or 18 months (Fig 2.2). A multiple regression was run to predict germination proportion from seed age and temperature. These variables significantly predicted germination proportion, $F(2,197) = 18.852$, $p < 0.05$), there was no difference in germination proportion between 25, 30 and 35°C for both seed ages. A two way ANOVA was conducted to examined the effect of seed age and temperature on germination proportion. There was a significant interaction between the effect of seed age and temperature on germination proportion of Gac seed, $F(4, 190) = 3.781$, $p = 0.006$.

The mean germination speed index (GSI) of 6 months old seed incubated at 30 and 35 °C was significantly higher than the other temperatures. GSI at 20 and 40 °C were significant lower than other temperatures for both seed ages (Table 2.1).



(a)



(b)

Fig 2.1 Mean germination percentage of the 6 month old (a) and 18 month old (b) Gac seed at five temperatures (n=15).

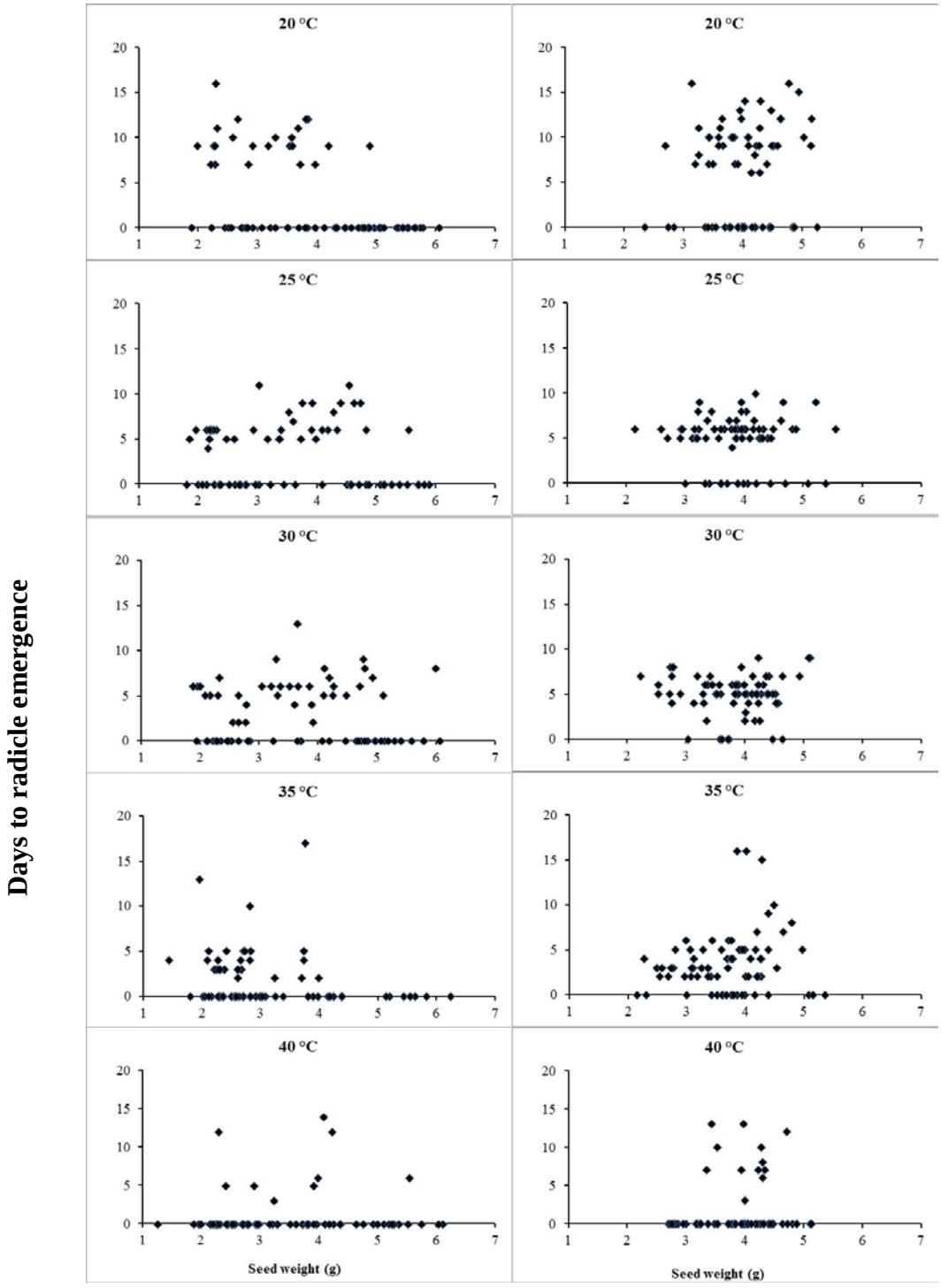


Fig 2.2 The effect of seed weight on the number of days to germination for Gac seed stored at 18 months (a) and 6 months (b) (n=75) at five different temperatures (20, 25, 30, 35 and 40°C).

Table 2.1 Mean value (± SD) of the germination speed index (GSI) after 20 days of incubation of *Momordica cochinchinensis* Spreng. Values represent means of five replicates of seeds (15 seeds per replicate). Means with different letters within columns are significantly different ($p<0.05$), according to the LSD test.

Temperature (°C)	GSI (seeds/day)	
	2013 (18 months)	2014 (6 months)
20	1.2 ± 0.9^{a}	2.3 ± 1.6^{b}
25	2.8 ± 1.4^{b}	5.2 ± 2.7^{c}
30	3.5 ± 1.4^{b}	6.6 ± 2.9^{d}
35	3.1 ± 1.2^{b}	6.4 ± 2.5^{d}
40	0.7 ± 0.3^{a}	0.8 ± 0.4^{a}

Effect of seed ages

Seed age strongly affected final germination proportion. Older seed had lower germination rates compared with fresh seed at the same temperature (Fig 2.1 and 2.2). The extreme temperatures (20°C and 40°C) took longer to reach the maximum germination proportion. The mean seed germination proportion at two seed ages (6 and 18 months) at all temperatures over 20 days is presented in Fig 2.1 (a and b). The radicle emergence was faster at 25-35 °C and for 6 months (7-9 days) old seed compared with 18 months old seed (5-11 days). At 30 °C, the emergence was faster than at other temperatures with exception of 18 months old seed at 35 °C. The seed radicles emerged at 5 days but maximum germination achieved was less than 0.4.

Table 2.2 The mean weight (±SD) of Gac seed, of three ages (30, 18 and 6 months), (n = 374).

Seed ages	Mean weight before storing (g)	Mean weight after storing (g)	Weight range after storing (g)
30 months (2012)	4.1 ± 0.3	2.8 ± 0.5	0.9 - 4.6
18 months (2013)	4.1 ± 0.3	3.5 ± 1.0	1.8 – 6.2
6 months (2014)	4.2 ± 0.2	3.9 ± 0.5	2.2 – 5.6

Effect of seed weight

The average weight of Gac seed at three seed ages is presented in Table 2.2. The seed weight before storing showed that the mean weight did not differ among the three seasons (2012, 2013 and 2014). However, the weight of the older seed age stored unsealed at ambient temperature had declined significantly after storing. The seed aged 6 months lost 7% weight while seed at 18 months lost 14% and seed at 30 months lost 32% weight.

The correlation Pearson analysis ($p<0.01$) indicated that the weight of Gac seed germinated at three seed ages (30, 18 and 6 months) had no relationship to the germination proportion. In addition, the seed weight did not appear to have an effect on the time to radicle emergence.

2.3.2 Effect of cutting types and plant hormone (IBA) on the survival rate and rooting of Gac cuttings

The effects of two cutting types and five IBA concentrations on survival are presented in Table 2.3. The analysis of variance showed significant differences in survival rate ($p < 0.0005$) and root number ($p < 0.0005$) between softwood and semi-hardwood cuttings, but there were no differences on survival rate, leaves number and root number among different IBA concentrations. The interaction between cuttings types and IBA concentrations only had statistically significant to survival rate ($p = 0.029$).

Table 2.3 The effect of five IBA concentrations on survival rate and rooting of two types of Gac cuttings. Data were recorded after 6 weeks of planting. The means are based on eight replicates, each with 10 cuttings. Means with different letters within columns are significantly different ($p<0.05$).

IBA treatment ($g\ L^{-1}$)	Softwood			Semi-hardwood		
	Survival rate (%)	Number of new leaves	Number of roots	Survival rate* (%)	Number of new leaves	Number of roots
0	53.1 ± 10.2^{a}	1.6 ± 0.7	5.6 ± 1.9	81.3 ± 14.1	1.4 ± 0.8	2.8 ± 1.2
1.5	57.8 ± 7.8^{a}	1.5 ± 0.8	3.9 ± 2.1	82.8 ± 10.9	1.8 ± 0.9	3.3 ± 1.2
3.0	73.4 ± 11.3^{b}	2.0 ± 1.1	6.5 ± 3.1	87.5 ± 12.5	1.7 ± 0.6	4.1 ± 1.2
5.0	76.6 ± 11.3^{b}	2.3 ± 0.8	6.6 ± 2.6	71.9 ± 16.4	1.7 ± 1.1	2.8 ± 0.6
8.0	65.6 ± 7.0^{ab}	1.7 ± 1.1	6.2 ± 4.2	78.1 ± 12.5	1.2 ± 0.6	3.5 ± 1.3

*survival rate did not differ significantly

2.3.3 Effects of rootstock age and grafting methods on the survival of the grafting combination

The effects of rootstock ages and grafting methods on survival rate of grafting combination are presented in Table 2.4. The linear regression was run to predict survival rate and shoot length of grafting combination from rootstock ages. These variables significantly predicted survival rate, $F(1,34) = 31.152$, $p < 0.0005$, $R^2 = 0.478$ and shoot length, $F(1,34) = 52.393$, $p < 0.0005$, $R^2 = 0.606$. The analysis of variance showed significant difference in shoot length among grafting methods ($p<0.0005$) but no differences on survival rate was observed between slice and wedge method ($p = 0.553$). There was no difference on survival rate between rootstock age 4 weeks old and 8 weeks old ($p = 0.23$) but the analysis of variance indicated significant differences in survival rate ($p = 0.17$) and shoot length ($p < 0.0005$) with the interaction between rootstock ages and grafting methods.

Table 2.4 Effect of rootstock age and grafting method on the survival rate of the grafting combination. Values represent the mean (±SD) of six replicates (each with 10 grafted plants) and different letters (within a column) represent significant differences between values ($p<0.05$), according to the LSD test.

Rootstock age	**Grafting techniques**			
	Slice		**Top wedge**	
	Survival rate (%)	**Shoot length (mm)**	**Survival rate (%)**	**Shoot length (mm)**
4 weeks	85.0 ± 5.0^{a}	86.2 ± 8.4^{a}	90.0 ± 6.7^{a}	52.3 ± 13.3^{a}
8 weeks	96.7 ± 4.4^{a}	70.8 ± 8.0^{b}	86.7 ± 6.7^{a}	15.0 ± 2.0^{b}
12 weeks	53.3 ± 8.9^{b}	0.8 ± 0.6^{c}	63.3 ± 7.8^{b}	6.6 ± 4.0^{b}

2.4 Discussion

More-efficient large-scale plant propagation is reality for Gac. The use of fresh seed is highlighted and optimal temperature conditions for seed germination have been defined. Further, larger seeds are not preferable to achieve better germinability unlike other cucurbit species (Nerson, 2007). The use of external plant hormone can be used to strike softwood cuttings to increase female plant numbers. Grafting is also an effective practice to graft female scions onto rootstock material.

In this study, the germination proportions are highest at 25-35 °C. This was in agreement with the optimum germination temperature of other seeds that belongs to the *Cucubitaceae* family such as *Momordica dioica* Roxb. (30°C) (Mohammad et al., 1991) and pumpkin (29-32 °C) (Motsa et al., 2015). Reduction of germination at high temperatures may be due to the change in structure of some proteins that are essential for germination (Hardegree, 2006, Kalemba and Pukacka, 2014). Other studies reported that the causes of reduced emergence of seed at excessively low or high temperatures were due to the high rate of respiration in seeds and failure or retarding of metabolic activity that was essential in seed germination (Motsa et al., 2015, Verma et al., 2010). Therefore, the traditional practice of cooking seed has no practical value and may even reduce seed germination with excessive temperatures.

In this study, seed age affected germination with the newest seed (6 months) having greater germination proportions compared with older seed (30 and 18 months). The germination proportion of the Gac seed declined more than 45% from 6 months to 18 months and those stored for over 2.5 years (30 months) had completely lost their viability. It appears that seed storage during this study (21 °C, 60 % RH) did not provide ideal conditions. One problem is that ideal conditions have not been determined for Gac seed. It is not known if Gac seed is desiccant-tolerant or desiccant-sensitive which will dictate

the conditions required. Most Cucurbits are desiccant-tolerant and require drying to a moisture content of about 5 % for long-term storage (Nerson, 2007). In this study, Gac seeds were dried to 10 % moisture content. If Gac seed has similar requirements to other melons including watermelon, then relative humidity conditions will need to be greater (about 75%) (Nerson, 2007) than that provided in this study (60%) for Gac.

This study showed that increased storage time dramatically reduced Gac seed viability and was also reflected by reduction in seed weight (Table 2.2). This may be related to the high oil content. Gac seed contains high amounts of fatty acids with about 60% as stearic acid and many other fatty acids (Ishida et al., 2004). A previous study indicated that peroxidation of oil content in red cedar seeds (*Thuja plicata* Donn ex D.Don) is also related to seed deterioration during storage (McDonald, 1999). The peroxidation of oil content in seed relates to the decline of antioxidant enzymes activity in soybean seed (Sharma et al., 2013), and in almonds to increased activity of lipoxygenase enzymes which are involved in degradation of the lipid in seed (Zacheo et al., 1998). Therefore, the determination the activity of these enzymes in Gac seed in further studies would permit a better understanding of the process of seed viability loss during storage.

An effective propagation system for Gac can be utilized by using softwood cuttings as an additional material to semi-hardwood and hardwood cuttings which do not require plant hormone but require a large biomass. The traditional practice of propagation by farmers is to use semi-hardwood or hardwood cuttings without hormone. Thus, the use of hormone with mature cuttings is unlikely to be beneficial. The most appropriate concentration of IBA for treatment of softwood was 3-5g/L. In a study on watermelon, shoot tip and one node cuttings dipped in IBA solution improved vegetative growth of

shoots, and produced an early yield compared with the seedling control (El-Eslamboly, 2014). In this study, two cuttings were kept and flowering and fruiting occurred for one of these plants. The benefits of using cuttings in terms of timing of flowering and yield need further investigation.

The survival of Gac grafted combination depends on rootstock age (Table 2.4). Younger rootstock increased survival rate of the grafts. The highest survival rate and the shoot length of grafting combinations were obtained at rootstock ages of 4 and 8 weeks. Generally, cucurbit grafts use rootstock material of a younger seedling stage (10- 20 days) (Traka-Mavrona et al., 2000, Amin and Mona, 2014). However, Gac rootstock needs more than 20 days from emergence to reach an appropriate stage for grafting; therefore older rootstocks were used in this study. It appears from this study and a previous study (Joseph and Bharathi, 2008) that the grafting techniques of insert, slice and wedge can be successfully used for Gac.

It has been reported that the shoot length of grafting combination on 12 weeks old root stocks showed poor performance compared that at rootstock age 4 and 8 weeks. This may be due to rootstock age has relationship to ability of regenerating of plant part which is found in younger rootstocks. In old rootstocks, the meristematic cells have a lower activity, leading to late formation of callus and slower healing of grafting combination (Hartmann et al., 1997). In addition, the limitation in shoot length may relates to time required for breaking bud of grafting union (Islam et al., 2003) and it needs to be investigated for Gac.

2.5 Conclusion

Propagation techniques have been investigated for Gac using three approaches. In this study, the most suitable temperature for Gac seed germination was 30 °C, with 20 °C and 40 °C significantly reducing it. However, seed viability depended on age and the oldest seeds were not viable which was related to reduce seed weight. The utilization of external plant hormone significantly improved the survival rate of the softwood cuttings from 53% to 77% but not of semi-hardwood cuttings. The two grafting methods (slice and wedge) with younger rootstock (4 and 8 weeks old) were successful techniques. These methods could be widely used to propagate Gac with the purposes of increasing the number of female plants.

CHAPTER 3

POLLINATION AND ASSISTED POLLINATION TECHNIQUES

Availability of pollen is a potential problem in Gac production and hand pollination is necessary for greenhouse production which excludes pollinating insects. This chapter includes developing an *in vitro* method to evaluate pollen viability using different medium recipes and a range of temperatures. The effect of pollen storage on pollen viability and on Gac fruit quality was also investigated.

3.1 Developing an *in vitro* method for assessing Gac pollen viability

3.1.1 Introduction

The viability of pollen can be assessed by testing pollen germination, pollen metabolism and pollen staining to indicate the presence of living cytoplasm. Germination tests can include *in vitro, in vivo* and semi-vivo tests (Judy et al., 1995). Stains used include acetic carmine, aniline blue, cotton blue, iodide potassium, trifeniltetrazolium chloride and red tetrazolium to identify functional pollen grains (Alexander, 1969). This method is quicker and simpler than pollen germination but it has the disadvantage of overestimating of viability (Gaaliche et al., 2013). The *in vitro* germination test only uses a small sample of pollen grains that are cultured in an agar medium and it is a better method to test the ability of pollen for its function of delivering the sperm cell to the embryo sac after compatible pollination (Shekari et al., 2016). The *in vivo* test places the pollen grains on the receptive stigma of the flower and then evaluating the developing pollen tube under the microscope. For the *semi-vivo* test, the styles are removed from flowers after pollinating by hand into germination media and the pollen tubes develop out of the styles into the medium and are observed (Judy et al., 1995).

A previous study has evaluated Gac pollen germination using acetocarmine staining on a 3% sucrose medium, although the percentage of pollen grains germinated were not

reported (Maharana and Sahoo, 1995). A study has also assessed *in vitro* pollen germination of 14 species of cucurbit using BK's medium (Brewbaker and Kwack, 1963) but the components of the culture medium has not been optimized (Rashed Zaman, 2009).

The *in vitro* germination of pollen in medium is a technique that simulates the conditions of the stigma. Each species requires a specific medium to obtain maximum germination proportion (Soares et al., 2013). In general, the basic BK medium contains carbohydrates and other substances that stimulate germination such as boric acid, calcium nitrate, potassium nitrate and magnesium sulfate (Brewbaker and Kwack, 1963). The medium components amounts depend on the requirements of each species (Pham et al., 2015). The aim of this study was to develop an optimum method for *in vitro* germination of Gac pollen for further pollen viability studies. This chapter evaluated for Gac the *in vitro* test conditions found suitable for the germination of pollen in avocado (Alcaraz et al., 2011) and longan (Pham et al., 2015). The parameters included 1% agar, 0.01% H_3BO_3, 0.01% KNO_3 and modification of sucrose, $MgSO_4.7H_2O$ and $Ca(NO_3)_2.4H_2O$. Following this, the effect of temperature on pollen germinability was investigated.

3.1.2 Materials and Methods

3.1.2.1 Pollen collection

Male flowers were collected at 9am and delivered to the laboratory immediately for assessment. The petals of the male flowers were removed and placed on aluminium trays in at room temperature (21 ± 1 ºC).

3.1.2.2 Modifying the in vitro medium to enhance pollen germination

The basic medium (BK medium) containing 1% agar, 0.01% H_3BO_3, 0.01% KNO_3 used by Brewbaker and Kwack (1963) was used in this study. Sucrose treatments were: 0, 5, 10, 15, 20 and 30 % weight. Pollen germination and pollen tube length were then evaluated at the optimal sucrose determined but varying concentrations of $MgSO_4.7H_2O$ (150, 200, 250 and 300 mg L^{-1}) and $Ca(NO_3)_2.4H_2O$ (200, 300, 500 and 700mg L^{-1}). The pH of the medium was adjusted to 6.0 before autoclaving. Pollen was cultured on 9cm Petri dishes containing 15mL of the medium. For each treatment, there were 5 Petri dishes were assessed and each Petri dish had 4 fields of view containing 50-200 pollen grains per field of view to prevent the negative effect of high pollen density on germination rate. The mean of 4 fields of view per petri dish was considered a replicate. These dishes were placed in the dark in an incubator at room temperature (22 ± 1°C) for 24 hours. Pollen viability was determined through observation of grains using a microscope with 40x ocular lenses. Pollen grains were considered germinated when the length of the pollen tube was at least the length of the diameter of the pollen grain. After placing an incubator for 24h, pollen tube length was measured using an ocular micrometer attached to microscope. A minimum of 20 pollen tubes were measured per field of view and the averages were calculated.

3.1.2.3 The effect of temperature on pollen germination

The optimum medium that was developed in section 3.1.2.2 was used to evaluate temperature effects on pollen germination and pollen tube length. Pollen was cultured on petri dishes containing optimum medium at five temperature regimes (15, 20, 25, 30, 35 and 40 °C). Three incubators were used for each temperature and 5 petri dishes used for each incubator. Other conditions, germination proportion and tube length of pollen were similarly described in section *3.1.2.2*.

3.1.2.4 Statistical analysis

The statistical analyses were conducted using SPSS software version 24.0. Analysis of variance (ANOVA) was used to test for sucrose and temperature effects. Two way ANOVA was used to analyse the combined effect of Ca^{2+} and Mg^{2+} concentrations on germination and tube length. The least significant difference (LSD) at a 5% level was used to compare the means of different sucrose concentrations and temperatures. Post hoc tests for all analyses were made with LSD ($p< 0.05$).

3.1.3 Results

3.1.3.1 Optimization of the in vitro pollen germination medium

3.1.3.1.1 Effect of sugar concentrations on pollen viability and pollen tube length

At room temperature (22 ± 1°C), a low sugar concentration (below 5%) and a high concentration (above 20%) slightly decreased pollen germination. Pollen germination was maximized of 15% sucrose and pollen tube length was greatest at 5-15 % (Fig 3.1).

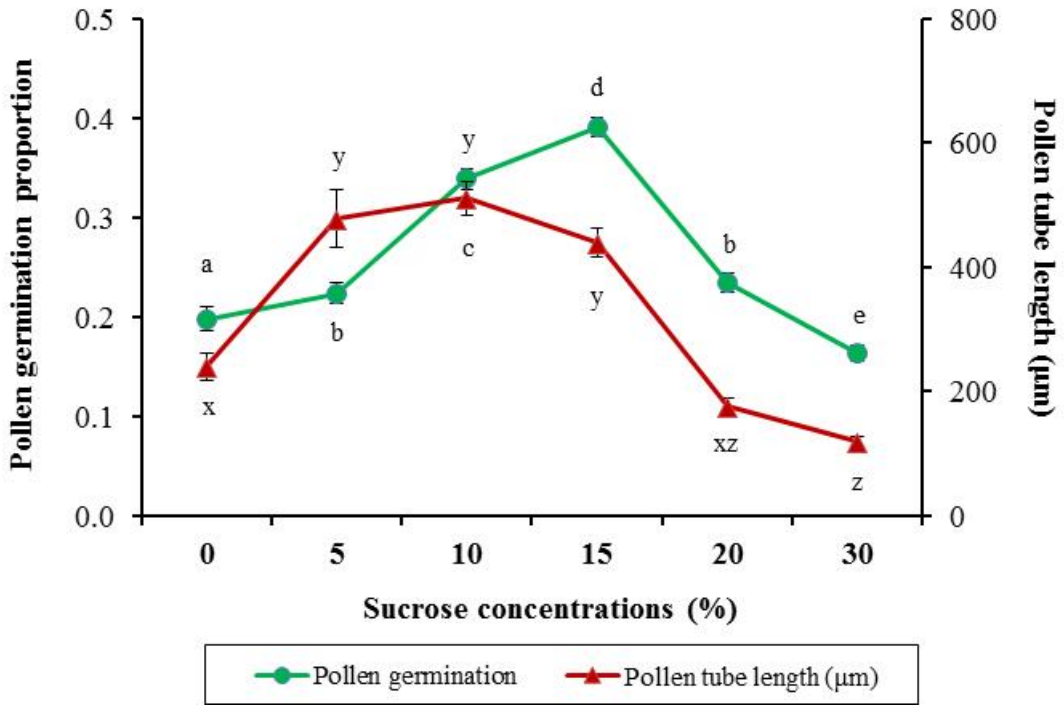


Fig 3.1 Effect of sucrose concentrations on mean pollen germination proportion and pollen tube length of Gac pollen. Mean with the same letter within germinated pollen or pollen tube length are not significantly different ($p<0.05$) ($n = 5$). Error bars represent SE of the mean.

3.1.3.1.2 Effects of Ca^{+2} and Mg^{+2} on pollen viability and pollen tube length

The effects of Ca^{+2} and Mg^{+2} concentration on pollen viability and pollen tube lenth are presented in Fig 3.2 and 3.3.

The two way ANOVA was conducted to examined the effect of Mg^{2+} and Ca^{2+} and interaction between Mg^{2+} and Ca^{2+} on germination proportion and tube length. The Ca^{2+} concentration had a significant impact on germination rate and tube length, $F(3, 304) = 13.823$ and 21.976, $p < 0.0005$, respectively while the Mg^{2+} concentration only had a significant effect on germination proportion, $F(3, 304) = 49.515$, $p < 0.0005$ and not on

tube length, F(3.304) = 1.829, p = 0.142. There was a statistically significant interaction between Mg^{2+} and Ca^{2+} concentrations on germination proportion and tube length, F(9, 304) = 16.600 and 19.609, p < 0.0005, respectively.

The concentrations of calcium and magnesium that maximized Gac pollen germination were obtained with 700 mgL^{-1} $Ca(NO_3).4H_2O$ and 250 mgL^{-1} $Mg(SO_4).7\ H_2O$ mainly based on the pollen germination proportion responses. Pollen tube length were more variable than pollen germination among treatments (Fig 3.3)

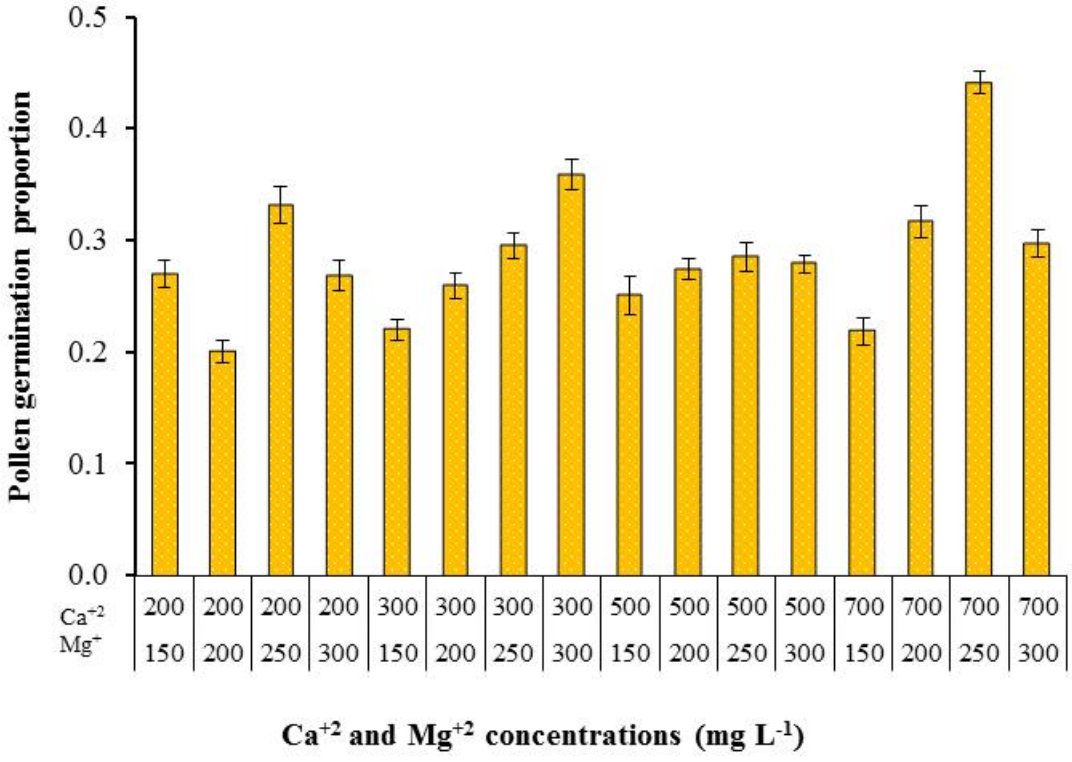


Fig 3.2 Effect of Ca^{+2} and Mg^{+2} concentrations on germination proportion of Gac pollen. Bars represent means ± SE (n =5).

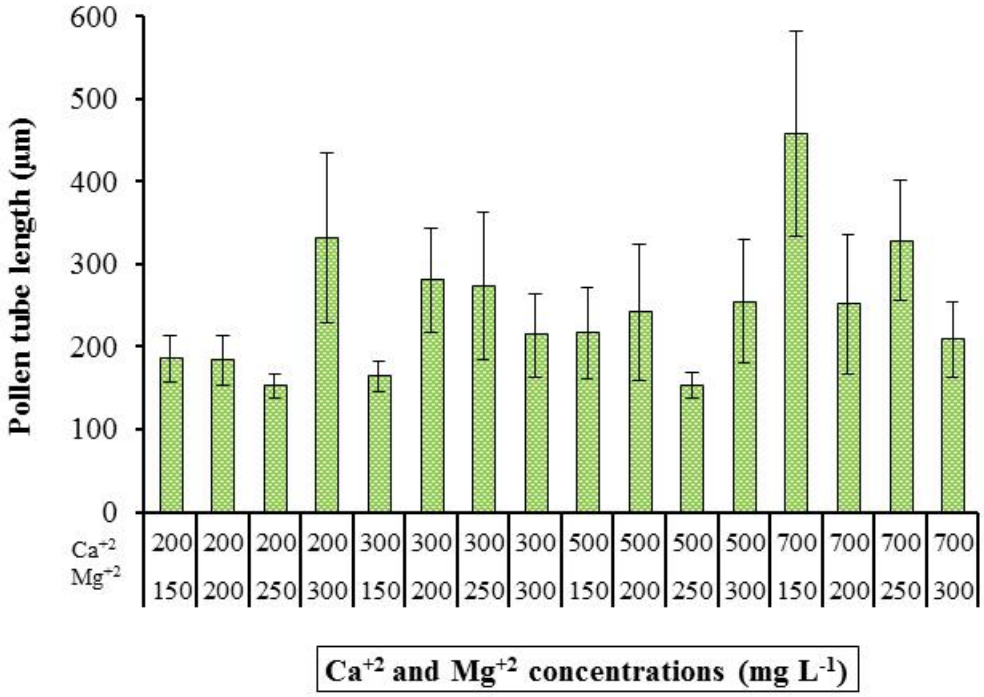


Fig 3.3 Effect of Ca^{+2} and Mg^{+2} concentrations on pollen tube length of Gac pollen. Bars represent means ± SE (n = 5).

3.1.3.2 Effects of temperature on pollen viability and pollen tube length

Effects of temperature on Gac pollen viability is presented in Fig 3.4 and 3.5. Fig 3.4 shows that the maximum germination proportion and tube length were obtained at 35ºC (0.6 ± 0.01 and 759.0 µm ± 41.5, respectively). Significant difference in pollen germination proportion occurred among temperatures, $F(5, 114) = 141.232$, $p < 0.0005$ but tube lengths were similar between 20 °C, 25 °C and 30 °C.

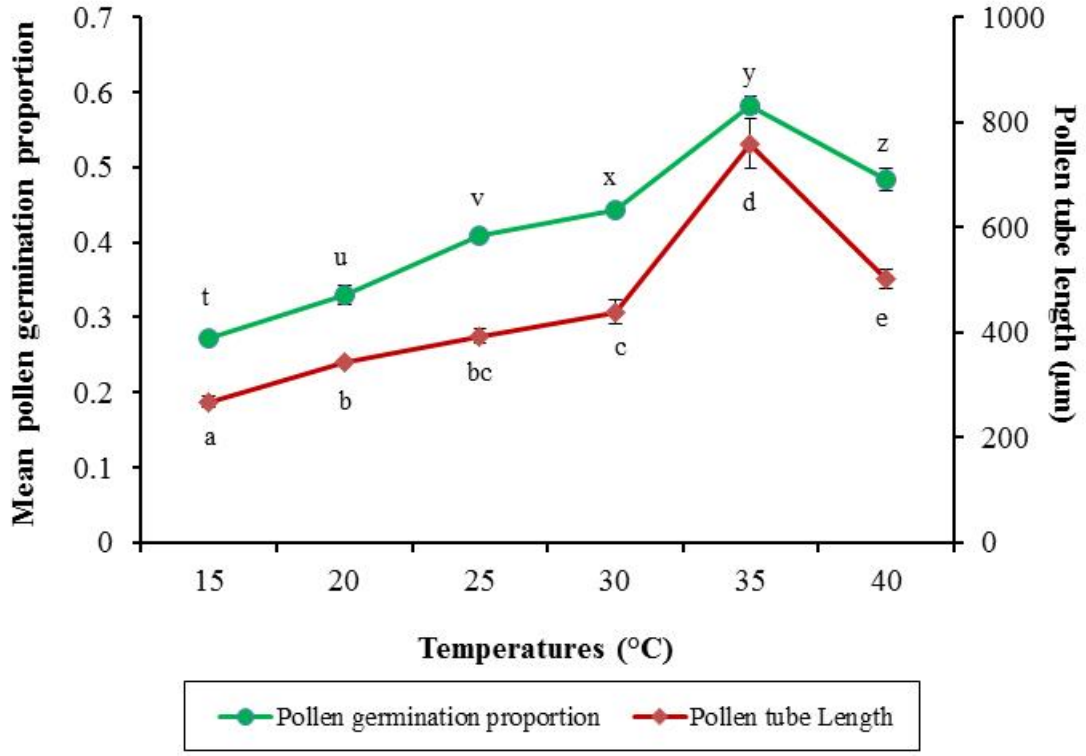
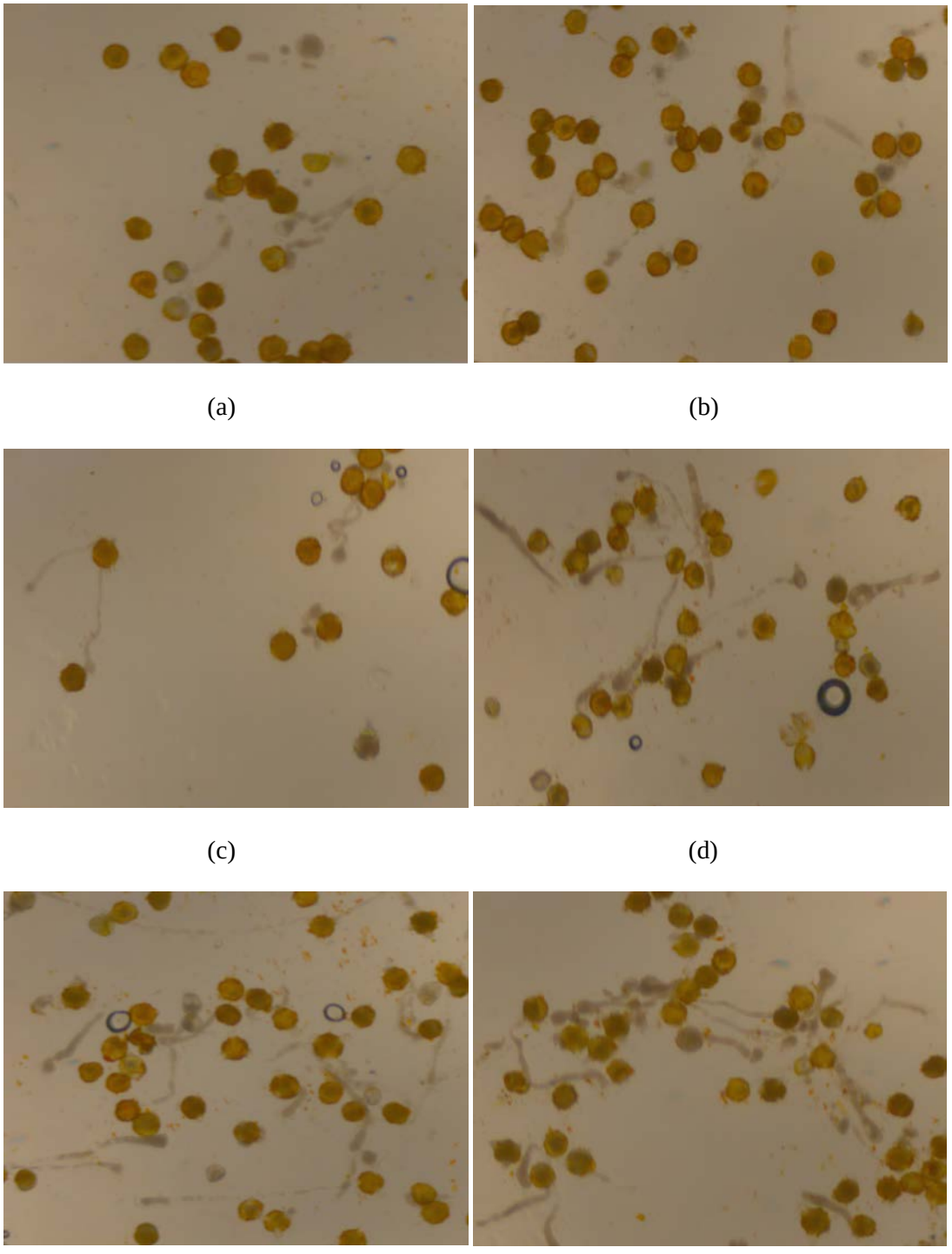


Fig 3.4 Effect of temperature on pollen germination proportion and pollen tube length. Means with different letters are significantly different ($p \leq 0.05$) by LSD test. Error bars represent standard errors (SE) of the mean (n = 5).



(a) (b)

(c) (d)

(e) (f)

Fig 3.5 Gac pollen viability at different temperatures: 15 °C (a), 20 °C (b), 25 °C (c), 30 °C (d), 35 °C (e) and 40 °C (f).

3.1.4 Discussion

In this study, an *in vitro* pollen germination medium for Gac has been optimized for the first time. Based on this study, the recommended medium for Gac pollen germination included 1% agar, 0.01% H_3BO_3, 0.01% KNO_3, 700 mgL^{-1} $Ca(NO_3).4H_2O$ and 250 mgL^{-1} $Mg(SO_4).7\ H_2O$.

Different sucrose concentrations added to BK medium (Brewbaker and Kwack, 1963) have been evaluated for several Curcubitace species. For example, in liquid BK medium, pumpkin pollen germinated well with sucrose 12.5 % (w/v) (Košmrlj et al., 2014) while the best sucrose concentration for germination of Teasle gourd pollen was 20% (Naik et al., 2016) and 6% for *Trichosanthes dioica* Roxb. pollen (Kumari et al., 2009). In a closely related species, the bitter gourd melon, the optimal germination medium contained 8.0%-8.8% sucrose (Hu et al., 2009). In this study, the highest germination proportion was obtained for Gac with 15 % sucrose. Sucrose improve pollen germination by the role of sucrose in culture medium as nutritious and osmotic compounds to improve pollen tube length development (Taylor and Hepler, 1997). In some other species, sucrose can be replaced with polyethyleneglycol (PEG) (Rihova et al., 1996), lactose (Trognitz, 1991) and mannitol (Rihova et al., 1996). These replacements may be investigated for Gac pollen to further improve germination proportion and tube length growth.

The evaluation of Gac pollen germination at different temperatures suggests that a temperature range of 30-35 °C may also be optimal for fertilization and fruit set for Gac in the field. Temperature is an important factor affecting the fertilization success and fruit set (Alcaraz et al., 2011, Nastari Nasrabadi and Neamati, 2015) and also influences pollen germination and tube growth (Kakani et al., 2005). The effect of temperature on

Gac pollen germination is similar to several some tropical and subtropical species including other cucurbits as described in Table 3.1.

Table 3.1 Optimal temperatures for pollen germination and tube length of some species.

Species	**Family**	**Temperature for optimal pollen germination**	**Temperature for optimal tube length**	**References**
Dimocarpus longan Lour.	Sapindaceae	30-35 °C	-	(Pham et al., 2015)
Litchi chinensis Sonn.	Sapindaceae	30 °C	-	(Stern and Gazit, 1998)
Cucumis melo L.	Cucurbitace	30 °C	35 °C	(Nastari Nasrabadi and Neamati, 2015)
Momordica cochinchinensis Spreng.	Cucurbitace	35 °C	35 °C	

3.1.5 Conclusion

An efficient *in vitro* method for pollen germination of Gac pollen has been developed as a result of this study. These conditions have been used in subsequent Gac pollen germination tests described in this thesis. The effect of temperature on pollen germination indicates that the optimal temperatures for successful pollination for Gac in *in vitro* and potentially the field are 30-35 °C.

3.2 Pollen storage and the effect of stored pollen on Gac fruit quality

3.2.1 Introduction

Traditionally, Gac is grown in the field and is pollinated by insects (Joseph and Bharathi, 2008). Ctenoplectra bees have been identified as the insect pollinator of Gac in India (Schafer, 2005). However, hand pollination for Gac has been recommended to maximise fruit set (90-100%) (Maharana and Sahoo, 1995), especially when bees are less active during overcast conditions. Hand pollination is also necessary for Gac crops produced in a greenhouse where insect pollinators are absent (Parks et al., 2013). Further, the male flowers tend to bloom before the female flowers and therefore pollen availability can be scarce at the time of anthesis of the female flowers (Parks et al., 2013). The use of Gac dried-frozen pollen to achieve fruit set has been demonstrated for three fruit (Parks et al., 2013) but otherwise has not been well described.

As the bioactive properties of Gac have dietary and commercial value, any effect of stored pollen on fruit quality requires investigation. The objective of this study was to determine the effect of pollen storage on fruit set and fruit quality. The viability of fresh and stored pollen was assessed *in vitro* by germinating it on an agar medium. The morphology of fresh and stored pollen was also observed using SEM techniques. Fresh pollen and pollen stored at 4 and -20 °C was used *in vivo* to pollinate flowers, with resulting fruit assessed for some physicochemical qualities.

3.2.2 Materials and Methods

3.2.2.1 Crop production

A Gac crop was produced in four climate-controlled greenhouses located at the NSW Department of Primary Industries Research Station, Ourimbah, NSW, Australia (151° 22'E, 33° 21'S) with temperature and the relative humidity maintained between 18 to 25°C and 60 to 80 %, respectively. A total of 44 plants (24 female and 20 male) were grown in

bags of coir substrate supplied hydroponically with water and nutrients through drippers (February, 2013). Gac pollen was collected from the male flowers for the storage studies. The female flowers were pollinated with fresh and stored pollen for the *in vivo* test.

3.2.2.2 Pollen collection and storage

Approximately 40-50 male flowers were collected and stored daily (7 days per week) for 3 months to supply the blooming female flowers with pollen at anthesis. The petals of the male flowers were removed and placed on aluminium trays in the laboratory at room temperature (21 ± 1 ºC, RH 60%). The fresh pollen was dried for 1 hour at room temperature and collected in vials, with each vial containing 1.0g pollen. For viability tests, these vials were used immediately or stored in the refrigerator compartment (4ºC, 60 % RH) or freezer compartment (-20 ºC, 70% RH) of three combined refrigerators/freezers until required. Pollen was also stored at room temperature for 9 days to observe moisture loss. The moisture content of pollen was approximately 13-14% prior to storage. Pollen was dried at 70°C in a vacuum oven (Vord-46OD, Australasian Scientific Marketing Group, Kotara, NSW, Australia) until a constant weight was obtained. Moisture content (%) = (FW-DW)/FW*100.

3.2.2.3 Scanning electron microscopy

Three samples of fresh pollen and pollen stored at 4 °C and -20 °C for 12 weeks were taken out of storage 2 hours and placed on stubs and examined using a FEI Quanta 200 SEM (Hillsboro, Oregon, USA) scanning electron microscope (SEM). Scanning electron micrograph images were taken at 600 ×, 1200 × and 2400 × magnifications (Fig 3.8 a-d). Measurements of the equatorial diameter and polar axis lengths were made of 10 pollen grains and are expressed in microns (µm). Means (±SD) are presented.

3.2.2.4 In vitro pollen viability assessments

Pollen viability was evaluated by germinating it in agar medium containing 15 % sucrose, 1% agar, 0.01% H_3BO_3, 0.01% KNO_3, 250 mgL^{-1} $MgSO_4$ $7H_2O$ and 700 mgL^{-1} $Ca(NO_3)_2$ $4H_2O$. The pH of the medium was adjusted to 6.0 before autoclaving. Pollen was cultured on 9cm Petri dishes containing 15mL of the medium. Each Petri dish had 4 fields of view containing 50-200 pollen grains per field of view to prevent the negative effect of high pollen density on germination rate. After the storage treatment, these dishes were placed in the dark in an incubator at a temperature of 35 °C for 24 hours. Pollen viability was determined through observation of grains using a microscope with 40x ocular lenses. Pollen grains were considered germinated when a pollen tube was observed, being at least the length of the diameter of the pollen grain. A minimum of 5 Petri dishes were assessed per treatment and the mean of 4 fields of view per petri dish was considered a replicate. After placing an incubator for 24h, pollen tube length was measured using an ocular micrometer attached to microscope. A minimum of 20 pollen tubes were measured per field of view and the averages were calculated.

3.2.2.5 Pollination of flowers with stored pollen

The experiments were designed with two factors (storage time and temperature). Gac pollen was stored at 4°C and -20°C for 2 weeks, 4 weeks, 8 weeks or 12 weeks of storage. The 8 treatments were randomly allocated to plots and pollinated in sequential order of female flower opening. Each treatment contained three replicates of ten female flowers. Pollination with fresh pollen was used as a control.

The female flowers were pollinated on the first day of opening at 9 am. Pollen was taken out from the refrigerator or freezer and left at room temperature for 1 hour before use.

Approximately 0.05 g pollen (the equivalent weight of pollen from one fresh male flower) from each treatment was rubbed on to the stigma of the female flower with a brush. The control sample was pollinated directly with fresh pollen. The pollination date was labelled on each pollinated flower and Gac fruits were harvested mature at 14 weeks after pollination. The number of fruit set was counted 4 weeks after pollinating when the fruitlets had started to develop. Gac fruit with a weight of over 1.0 kilogram were considered to be of a commercial size and calculated by total number of fruit > 1kg/total number of fruit) ×100.

3.2.2.6 Fruit physiological properties

Fruit weight was measured using an electronic balance (Kern & Sohn, Balingen, Germany, ± 0.01 g). Fruit length (polar axis-distance between the apex and stem) and fruit diameter (the maximum width from perpendicular to the polar axis) were measured using a vernier calliper (Mitutoyo, Kawasaki, Japan, ± 0.01 mm). Skin colour was measured using a Minolta Chroma Meter CR-400/410 (Minolta Corp, Osaka, Japan). Ten measurements (L^*, a^*, b^*) were measured along the equatorial axis of fruit with three fruits per treatment. The colour parameters included chroma ($C^* = (a^{*2}+b^{*2})^{1/2}$) and hue angle ($h° = \arctan(b^*/a^*)$) and were calculated for fruit skin colour as described by McLellan et al. (1995). The firmness of fruit was determined with a Penetrometer (Facchini, Alfonsine, Italy) with an 8mm flat plunger. The value of fruit firmness was an average of ten points per fruit with 3 replicates. The results were expressed by the load in kilograms force (kgf).

3.2.2.7 Chemical properties of the aril

3.2.2.7.1 Total soluble solid (TSS) of the red aril

The red aril was separated from the seed and blended in an electric food processer. The aril juice was then filtered using a cloth to determine total soluble solids (TSS). TSS was measured using a digital refractometer (Atago Co.Ltd, Japan).

3.2.2.7.2 Determination of total oil content and carotenoid content

The total oil content and carotenoid contents (β-carotene and lycopene) of Gac fruit aril were measured using Soxhlet extraction and high performance liquid chromatography (HPLC) as described by Kha (2013b).

3.2.2.8 Statistical analysis

Data were analysed using ANOVA. Statistical analyses were performed using SPSS 22 version software. Analysis of variance used a Generalised Linear Model procedure to predict the effect of storage temperature and time on germination proportion and pollen tube length. Means were compared using least significant differences (LSD) at 0.05 levels. The effect of stored pollen on fruit set and on the physicochemical properties of Gac was analysed using GenStat 18 with the treatment structure being a 4*2 factorial plus an added control. Graphs were generated using GraphPad Prism 7.

3.2.3 Results

3.2.3.1 The viability of pollen stored at 21^{o}C

The viability of fresh pollen declined with time as indicated by reductions in germination and pollen tube length (Fig 3.6). The analysis of variance showed significant differences in germination proportion ($p < 0.0005$) and tube length ($p < 0.0005$) among pollen ages. The linear regression was run to predict germination proportion and tube length from pollen age. These variables predicted germination proportion statistically significantly,

F(1, 198) = 2760.06, $p < 0.0005$, $R^2 = 0.933$ and tube length, F(1, 198) = 515.42, $p < 0.0005$, $R^2 = 0.722$. As the pollen moisture content (PMC) declined, this was associated with decreased germination.

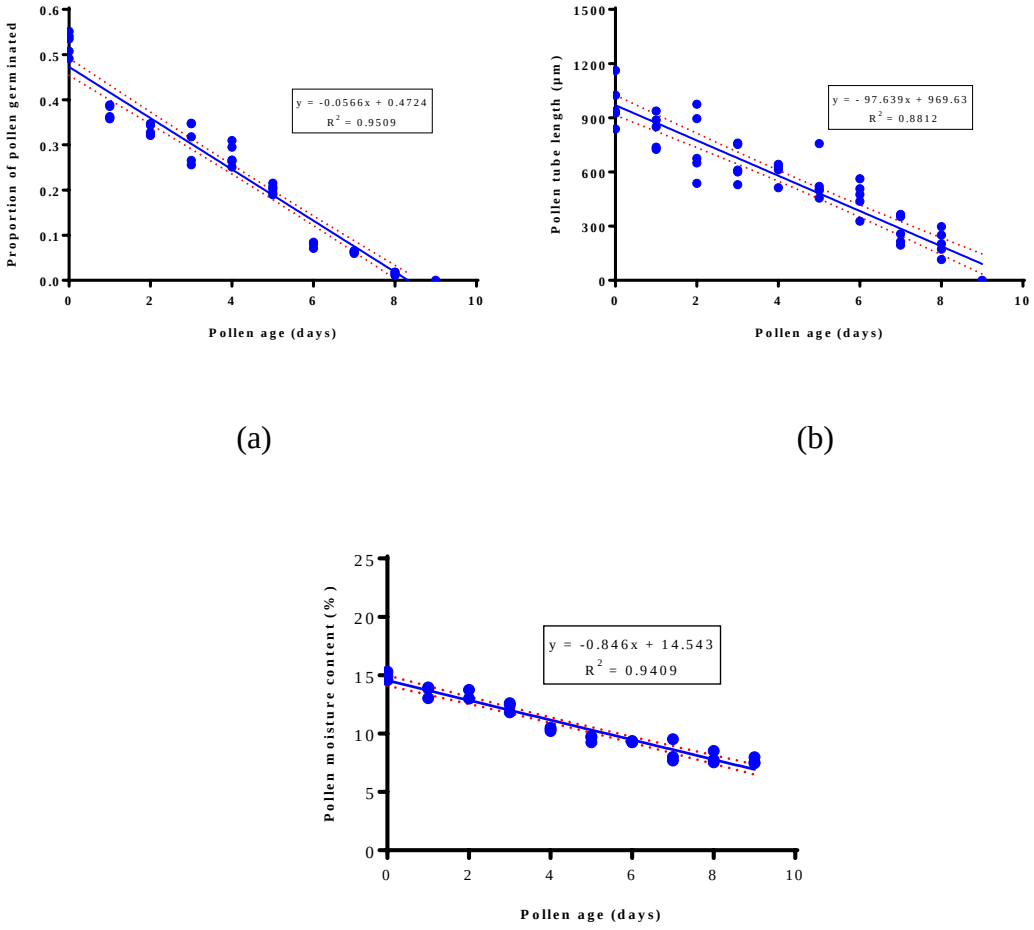


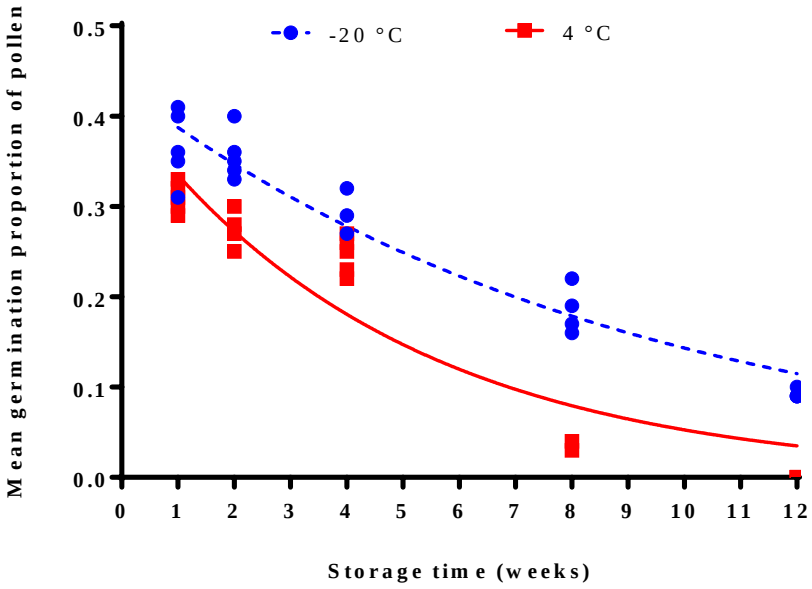
Fig 3.6 The predicted proportion of pollen germinated on an agar medium at 35 °C (a), their pollen tube lengths (b) and moisture content over time (c). Solid lines are linear regressions, with dotted lines indicating 95% confidence limits.

3.2.3.2 The viability of cold-stored pollen

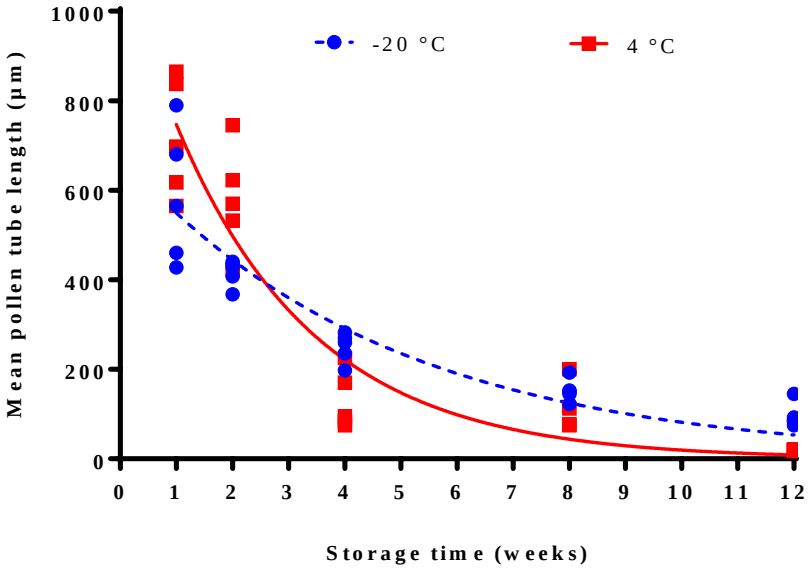
The viability of Gac pollen stored at 4 °C and -20 °C for up to 12 weeks is presented in Fig 3.7 (a and b).

Pollen germination and pollen tube length were significantly reduced after 4 weeks in storage and at 12 weeks, viability was minimal. Germinability was generally better for pollen stored at -20 °C but pollen tube lengths were similar for both storage temperatures. The analysis of variance indicated that storage time of pollen affected germination proportion ($p < 0.0005$) and tube length ($p < 0.0005$) while storage temperature only impacted on germination ($p < 0.0005$) and did not affect tube length ($p = 0.482$). The interaction between temperature and storage time on germination and tube length was significantly difference ($p < 0.0005$). The linear regression was run to predict germination proportion and tube length from storage temperature and time. These variables statistically significantly predicted germination, $F(2, 197) = 886.47$, $p < 0.0005$, $R^2 = 0.898$; tube length, $F(2, 197) = 149.13$, $p < 0.0005$, $R^2 = 0.602$.

The linear correlation between pollen germination proportion and storage time were observed at both temperatures, $y= -2.612 x + 40.20$, $r^2= 0.99$ for -20 °C and $y= -3.042 x + 33.81$, $r^2= 0.93$ for 4 °C. Similar, correlation between pollen tube length and storage time were $y= -39.66 x + 513.76$, $r^2= 0.82$ for -20 °C and $y= -58.77 x + 627.95$, $r^2= 0.73$ for 4 °C.



(a)

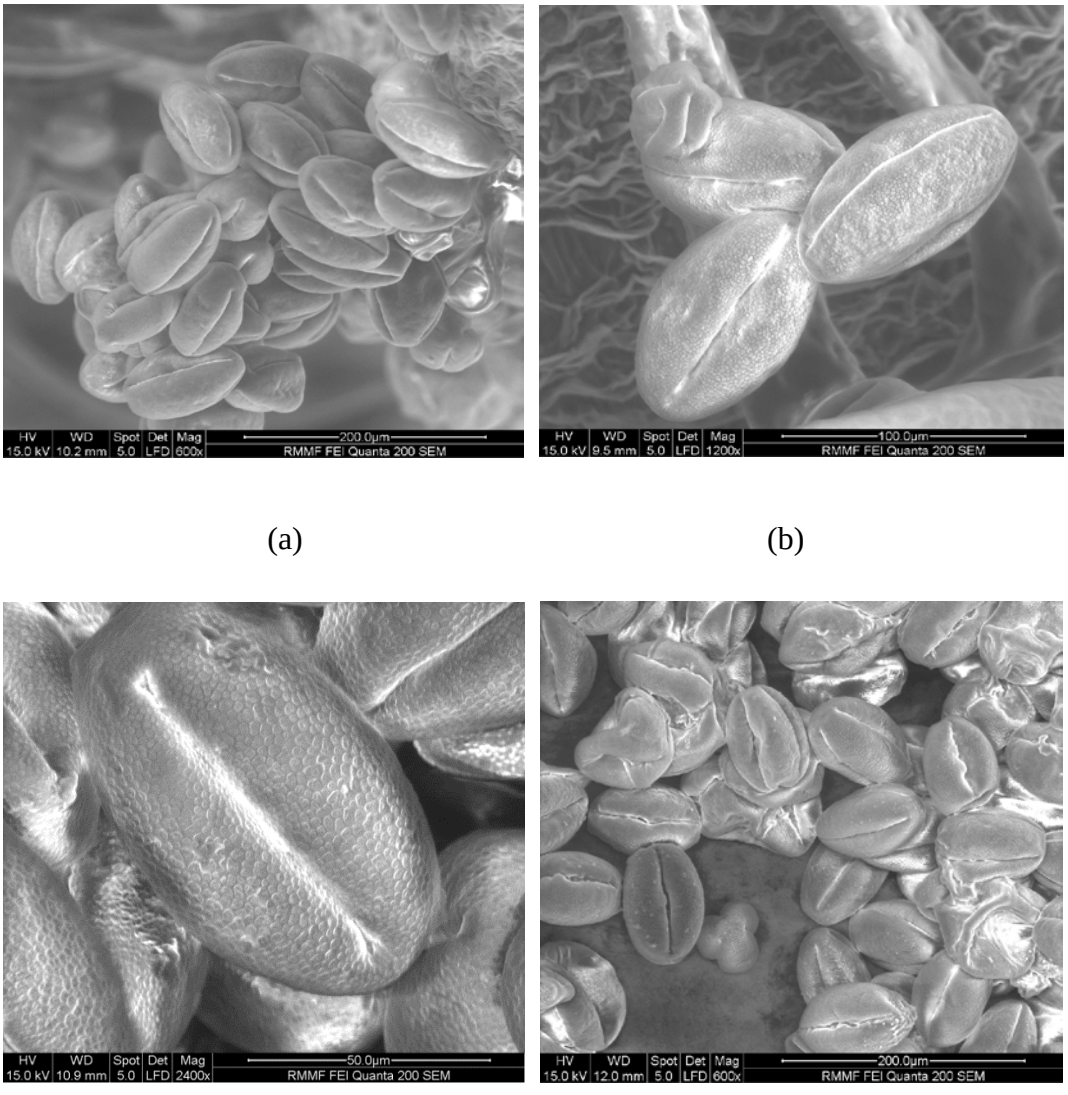


(b)

Fig 3.7 The proportion of germination (a) and the tube length (b) of Gac pollen in storage at 4 °C (solid line) and -20 °C (dashed line) over time (n=5). The fitted regression line is fitted to data across the five storage times.

3.2.3.3 Scanning electron microscopy

The shape of Gac pollen is shown to be elliptical in the equatorial view with three colpi (three furrows containing pores) (Fig 3.8), prolate (longer than wide), has long sunken colpus (apertures) and shows reticulate (net-like) ornamentation on the surface (Fig 3.8c). The length (vertical distance) of pollen was measured at 109 ± 4 µm. The stored pollen appeared to be deformed compared with the fresh pollen, particularly those stored at -20 °C.



(a) (b)

(c) (d)

Fig 3.8 Images of Gac pollen under a SEM microscope: fresh pollen (a and b), pollen stored at 4 °C (c), pollen stored at -20 °C (d).

3.2.3.4 The effect of stored pollen on fruit set and number of commercial fruit

The fruit set and the amount of commercial fruit produced from stored pollen is shown in Table 3.2.

Table 3.2 The effect of storing pollen on total fruit set and commercial fruit set.

Storage temperatures (°C)	**Storage time of pollen (weeks)**	**Fruit set (%)**	**Commercial fruit (%)**
C	0	96.7 ± 3.3^{a}	83.0 ± 3.0^{a}
	2	76.7 ± 3.3^{c}	61.3 ± 6.2^{ab}
	4	80.0 ± 0.0^{b}	66.7 ± 4.2^{ab}
	8	40.0 ± 0.0^{d}	33.3 ± 8.3^{b}
	12	10.0 ± 0.0^{e}	0.0 ± 0.0^{c}
	2	86.7 ± 3.3^{b}	73.2 ± 3.3^{ab}
	4	83.3 ± 3.3^{bc}	60.0 ± 5.0^{ab}
	8	73.3 ± 3.3^{c}	45.2 ± 2.4^{b}
	12	13.3 ± 3.3^{e}	50.0 ± 28.8^{b}

The values followed by different letters (a, b, and c) in a column indicate significant differences ($p<0.05$) using the least significant difference (LSD) test. Mean ± S.E presented, n=3 (10 flowers in each replicate).

A high fruit set and high numbers of commercial fruit were obtained when using fresh pollen for pollination. Fruit set significantly declined with longer storage times for pollen but was nonetheless suitable when cold-stored for 4 weeks with fruit set of about 80%, with over 60% fruit being of a commercial quality. Resonable fruit set of commercial fruit was not obtained using pollen that was cold stored for 8 weeks (<50%).

3.2.3.5 Physiological properties of fruits pollinated with stored pollen

3.2.3.5.1 Morphological and physical properties

Increasing the storage time of cold-stored pollen resulted in reduced fruit weight, fruit size (length and diameter) and a reduced proportion of aril (Table 3.3). The lowest weight (827 ± 24 grams) was obtained with fruits which pollinated by stored pollen at 4 °C for 12 weeks, only 60% weight of fruit pollinated by fresh pollen. The total seed number and number of developed seeds declined for pollen stored at 8 and 12 weeks (4 °C) and at 12 weeks (-20 °C) whilst the number of undeveloped seed considerable increased in fruits pollinated by stored pollen at 4 °C for 8 weeks and -20 °C for 12 weeks. Firmness was not affected by pollen storage treatment and no clear trends were evident for the skin colour characteristic.

Table 3.3 Physiological properties of Gac fruit pollinated by stored pollen.

Fruit attributes	**Storage temperature and time**								
	Fresh pollen	**4 °C**				**-20 °C**			
	Control	**2w**	**4w**	**8w**	**12w**	**2w**	**4w**	**8w**	**12w**
Fruit weight (g)	1403 ± 19^{a}	1173 ± 11^{bc}	1193 ± 9^{bc}	921 ± 8^{d}	827 ± 24^{d}	1288 ± 6^{ab}	1252 ± 5^{ab}	1102 ±1^{c}	909 ± 149^{d}
Fruit length (cm)	22.8 ± 0.2^{ab}	22.1 ± 0.5^{bc}	22.0 ± 0.1^{c}	20.0 ± 0.3^{d}	20.5 ± 0.3^{d}	23.1 ± 0.1^{a}	22.3 ± 0.0^{bc}	21.6 ± 0.3^{c}	20.6 ± 0.3^{d}
Fruit diameter (cm)	46.0 ± 0.2^{a}	44.1 ± 0.2^{a}	44.0 ± 0.2^{a}	41.2 ± 0.4^{bc}	41.0 ± 0.5^{c}	45.1 ± 0.1^{a}	44.5 ± 0.2^{a}	43.6 ± 0.1^{ab}	40.8 ± 2.4^{c}
Firmness (kgf)	2.4 ± 0.0^{a}	2.3 ± 0.0^{a}	2.4 ± 0.1^{a}	2.5 ± 0.2^{a}	2.4 ± 0.2^{a}	2.3 ± 0.03^{a}	2.4 ± 0.0^{a}	2.4 ± 0.1^{a}	2.6 ± 0.2^{a}
Total seed number	31.0 ± 0.7^{a}	30.2 ± 0.9^{ab}	28.5 ± 0.2^{ab}	28.6 ± 0.6^{ab}	16.0 ± 2.0^{c}	28.6 ± 1.8^{ab}	29.4 ± 1.5^{ab}	27.8 ± 2.7^{ab}	21.2 ± 7.9^{bc}
Developed seeds	27.4 ±0.9^{a}	25.7 ± 0.7^{a}	24.0 ± 0.6^{a}	16.3 ± 1.6^{b}	10.0 ± 0.6^{c}	22.9 ± 1.3^{ab}	25.3 ± 1.5^{ab}	21.9 ± 0.9^{ab}	6.0 ± 3.5^{c}
Undeveloped seeds	3.6 ± 0.4^{b}	4.3 ± 0.3^{b}	4.5 ± 0.6^{b}	12.3 ± 1.0^{a}	6.0 ± 2.5^{b}	5.7 ± 1.2^{b}	4.1 ± 0.7^{b}	5.9 ± 2.5^{b}	15.2 ± 4.5^{a}
Proportion of aril (%)	26.4 ± 1.0^{a}	23.2 ± 0.9^{ab}	23.4 ± 0.7^{ab}	22.5 ± 0.8^{ab}	19.1 ± 0.1^{bc}	23.7 ± 0.7^{ab}	22.7 ± 0.7^{ab}	23.1 ± 0.8^{ab}	15.8 ± 5.8^{c}
Colour									
*C**	60.4 ± 1.9^{a}	60.3 ± 0.7^{a}	62.3 ± 0.9^{a}	62.0 ± 1.3^{a}	56.0 ± 0.5^{b}	59.5 ± 0.4^{a}	62.0 ± 1.0^{a}	55.9 ± 0.7^{b}	55.0 ± 1.8^{b}
h°	49.1 ± 0.4^{b}	50.7 ± 1.5^{bc}	51.8 ± 0.6^{bc}	55.3 ± 1.4^{a}	46.2 ± 1.5^{d}	49.3 ± 1.5^{bcd}	52.5 ± 0.7^{ab}	51.2 ± 0.5^{bc}	50.7 ± 1.1^{bc}
L	39.7 ± 0.9^{ab}	38.7 ± 0.9^{b}	39.6 ± 0.1^{ab}	41.2 ± 1.3^{ab}	33.5 ± 1.4^{c}	37.8 ± 1.1^{b}	39.1 ± 0.3^{ab}	40.7 ± 0.6^{ab}	42.0 ± 1.1^{a}

Values followed by different letters (a, b, and c) in a row indicate significant differences ($p<0.05$) using the least significant difference (LSD) test. Mean ± S.E presented (n=3).

3.2.3.5.2 Chemical properties of Gac fruit produced by stored pollen

Total soluble solids (TSS), oil content, the lycopene and β-carotene content were measured in Gac fruit pollinated by fresh and stored pollen and are presented in Table 3.4.

Table 3.4 Some chemical properties of Gac fruit pollinated by fresh and stored pollen.

Storage temp (°C)	**Storage time (weeks)**	**Fruit attributes**			
		TSS (°Brix)	**Oil content** (g g^{-1} DW)	**Lycopene** (mg g^{-1} FW)	**β-carotene** (mg g^{-1} FW)
C	**0**	16.2 ± 0.8^{a}	0.29 ± 0.08^{a}	0.37 ± 0.07^{a}	0.24 ± 0.05^{ab}
	2	12.6 ± 0.1^{b}	0.18 ± 0.03^{b}	0.32 ± 0.03^{a}	0.22 ± 0.00^{ab}
	4	13.2 ± 0.7^{b}	0.13 ± 0.00^{b}	0.27 ± 0.01^{a}	0.19 ± 0.05^{ab}
	8	12.8 ± 0.2^{b}	0.13 ± 0.00^{b}	0.38 ± 0.04^{a}	0.30 ± 0.03^{a}
	12	12.2 ± 0.2^{b}	0.12 ± 0.01^{b}	0.28 ± 0.11^{a}	0.13 ± 0.01^{b}
	2	13.6 ± 0.1^{b}	0.17 ± 0.01^{b}	0.34 ± 0.02^{a}	0.22 ± 0.05^{ab}
	4	12.9 ± 0.6^{b}	0.21 ± 0.00^{ab}	0.34 ± 0.04^{a}	0.26 ± 0.10^{ab}
	8	14.4 ± 0.8^{ab}	0.15 ± 0.01^{b}	0.37 ± 0.05^{a}	0.29 ± 0.04^{a}
	12	12.3 ± 0.2^{b}	0.19 ± 0.02^{b}	0.23 ± 0.03^{b}	0.16 ± 0.03^{b}

Means (± SE) with different letters with columns are significant different ($p<0.05$) using the least significant difference (LSD) test, n=3.

Cold storage of pollen reduced the TSS and oil content of Gac aril. TSS related well to the oil content but less so to the lycopene and β-carotene contents which appeared un-affected by pollen storage time (<12 weeks) or storage temperature.

3.2.4 Discussion

Despite storage causing some loss of pollen viability, pollen stored at 4 °C for 4 weeks and at and -20 °C for 8 weeks still provided a high level of fruit set (>70 %) with only limited effects on fruit quality. In another cucurbit, *Citrullus lanatus* Thunb., pollen stored at 4 and -10°C for four weeks showed a similar decline to Gac pollen in germination rates (Kwon et al., 2005). This extends the use of pollen well beyond that previously shown (30 hrs) for pollen stored at ambient temperature (Maharana and Sahoo, 1995). This research has shown that storage of Gac pollen in a domestic fridge or freezer permits sufficient time for growers to collect pollen and consolidate stores prior to the flush of female flowers. Further, the numbers of male plants required for the fruit crop could be reduced, increasing the efficiency of production.

The subject of Gac pollen and its desiccation sensitivity or desiccation tolerance remains unresolved. It is possible that Gac pollen is desiccation-sensitive or recalcitrant demonstrated for another cucurbit, *Cucurbita pepo* L. (Gay et al., 1987). Understanding the nature of desiccation sensitivity is important for the development of protocols for long-term storage of pollen, particularly for breeding and conservation purposes. The significant loss of viability associated with reduced pollen moisture in Gac pollen may indicate desiccation-sensitivity but further studies are required using pollen with controlled moisture contents to ascertain moisture requirements under storage. In contrast, the presence of furrows on Gac pollen described in this study is typical of

desiccation-tolerant pollens (Franchi et al., 2011). Yet, the flowering behaviour of corolla closure at the end of stigmatic receptivity in Gac (Maharana and Sahoo, 1995) is characteristic of species with recalcitrant pollen (Franchi et al., 2011). If recalcitrant, protocols will need to be developed that accommodate the presence of freezeable water in the pollen specimens under cold storage (Ishikawa et al., 2005). Viable pollen has been demonstrated for *Momordica doica* and *M. sahyadrica* after cryopreservation for 48 hours but the content and control of pollen moisture were not discussed (Rajasekharan, 2010).

Classification of pollen can be determined by image processing (Pozo-Baños et al., 2012). The SEM images of Gac pollen show grain morphology as being tricolporate (with three furrows containing pores) without spines. This feature corresponds with the pollen structure of one *Momordica* species *Momordica balsamina* (Van Rensburg et al., 1985) and of the *Momordica charantia* type as one of three types in the Cucurbitaceae as described by Perveen and Qaiser (2008). In particular, it is prolate (longer than wide), has long sunken colpus (apertures) and shows reticulate (net-like) ornamentation on the surface (Fig 3.8c). The Gac pollen size was classified as large (> 100 µm) and much larger than that recorded for Gac in a previous study (Maharana and Sahoo, 1995). However, the exine sculpture is also a feature used for phylogenetic classification (Gilani et al., 2010, Arzani et al., 2005) and has not been described here for Gac. The exine feature is an important character used to identify the Cucurbitace family. Pollen structure of the Cucurbitace family is thinnest at the aperture when pollen grains mature (Van Rensburg et al., 1985). Therefore, further observations of Gac pollen apertures and exine features would more confidently classify this pollen species.

In this study, the storage of pollen directly influenced fruit quality of Gac. Gac fruit weight, size, seed number and proportion of aril associated declined with stored pollen at both temperatures (4 and -20 °C) and generally declined as storage time increased. Storage of pollen for more than 4 weeks at 4°C and at -20°C for more than 8 weeks results in unacceptable production of marketable fruit (less than one third of fruit set). Reduced fruit weight, size and seed number was also observed in "Lessard Thai" sugar apple (*Annona squamosa*) with pollen stored in the refrigerator after 24 hours and for Gefner atemoya (*A. cherimola* × *A. squamosa*) with pollen stored in the refrigerator after 24 and 48 hours (Pereira et al., 2014). In Gac, seed number of fruits produced using stored pollen was not affected when pollen storage was less than 8 weeks at 4°C or 12 weeks at -20°C. Reduced seed numbers is associated with reduced fruit weight in apple (Buccheri and Di Vaio, 2004) and capsicum (Marcelis and Baan Hofman-Eijer, 1997). Since oil concentration was also reduced with stored pollen, some fruit qualities are clearly compromised by the practice of pollen storage. Although the concentrations of carotenoids did not decline, one would expect to extract less from smaller fruits with a reduced proportion of aril. Despite this, marketable fruits were produced using stored pollen which appears to be a suitable practice when pollen is otherwise limited or if pollination is affected by other environmental factors.

3.2.5 Conclusion

This is the first report on storage of Gac pollen at low temperatures. Although pollen germination declined with storage time, hand pollination with stored pollen (for up to four weeks at 4 °C and eight weeks at -20 °C) showed a high fruit set (>73%) and no differences in fruit quality (lycopene, β-carotene) compared with fresh pollen. Pollen

stored for 4 weeks at 4 °C, or for up to 8 weeks at -20 °C can be used to produce marketable fruit (>1kg). Further information on pollen morphology may be useful in the further classification of this diverse species. Understanding the desiccation sensitivity of Gac pollen will help develop the method for appropriate storage conditions

CHAPTER 4

FRUIT LOAD EFFECTS ON FRUIT QUALITY

4.1 Introduction

Fruit production is strongly affected by biomass allocation and it is regulated by source and sink strength (Marcelis, 1993). The source strength (assimilate supply) can be defined as the carbon assimilates that are produced by the plant (Marcelis, 1996). A high yield is desirable for fruit crops and a high biomass allocation to fruits is crucial. However, since fruit growth is associated with leaf area formation, allocating too much biomass to fruit can adversely affect the crop (Heuvelink, 1997). Gac is a perennial climber with high dry matter production and the plant height can reach up to 25 meters (Joseph and Bharathi, 2008). It is possible that fruit load (the number fruit per plant) and fruit position on the plant impacts on fruit size and carotenoid content in Gac since in a previous study fruit weight declined as the season progressed (Parks et al., 2013).

The crop load and fruit position has been shown to affect fruit size and fruit quality in a number of species. For example, in olive, a high crop load was shown to reduce fruit oil concentration (Gucci et al., 2007, Trentacoste et al., 2010). A lower leaf-fruit ratio in persimmon showed a high yield but fruit weight, soluble solid and fruit colour declined (Choi et al., 2010). In cucumber, increasing the number of fruits per plant reduced fruit weight due to decreasing dry-matter assimilation per fruit (Marcelis, 1994). Similar results were observed in tomato (Heuvelink, 1997). Further, the fruit position also impacts on fruit size. In cherimoya, heavier fruits were produced at basal nodes than at the shoot apex (González and Cuevas, 2008). Information on the effect of fruit load and fruit

position on fruit weight and fruit quality could be used to manage the number of fruit per plant to achieve the highest quality and yield.

Leaf area index (LAI) is a useful measurement for estimating plant growth (Ma et al., 1992, Bonan, 1993, Labbafi et al., 2017). It is defined as the ratio of the leaf area of the plant to the ground area it occupies (Labbafi et al., 2017). Previous studies indicated that the close relationship between leaf area index (LAI) and leaf dry weight or leaf number per plant can be used to estimate LAI of a plant when no LAI meter is available (Ma et al., 1992, Labbafi et al., 2017). The use of the LAI has not been evaluated for Gac. It is possible that the relationship between LAI and leaf measurement of Gac plant may be used to predict this index. If so, these will provide tools for estimating the growth of Gac.

The objective of this chapter is to gain a better understanding of the effect of fruit load and fruit-set order on fruit weight and quality of Gac and to explore resource allocation among leaves and fruits. Since the quality in Gac in terms of oil, lycopene and β-carotene concentrations is strongly related to the total soluble solids (TSS) of the aril (Tran et al., 2016), TSS was used as a quality indicator in this study.

4.2 Materials and methods

4.2.1 Experimental site

The experiment was carried out during September 2014- July 2015 season at Ourimbah research station, NSW, Australia (151° 22'E, 33° 21'S). 16 female plants (2 years old) were grown in the greenhouse with 2 × 2 m spacing. Each Gac plant was trained from the main stem to make a small canopy (100 × 80 cm) with 2m above ground level. The

environmental parameters and growing conditions that were used are described in Chapter 3.

4.2.2 Treatment and experimental design

Four treatments of 1, 5, 9 or unrestricted fruit numbers per plant were established in a randomised complete block design with four replications. The unrestricted fruit numbers obtained were 9, 9, 10 and 9. The first female flower was pollinated at the 20th leaf position and successive flowers were pollinated to obtain the number of fruit as required for each treatment. Surplus female flowers were removed. Fruits were harvested at 14 weeks after pollination date.

4.2.3 Canopy measurements

4.2.3.1 Leaf area index

Leaf area index (LAI) was measured at 2 months after planting using an LAI-2200C Plant canopy analyser, (Licor, Lincoln, NE, USA) with one reading above and nine readings below the canopy at a distance of 40 cm.

4.2.3.2 Leaf number

The leaf number, separated into small, medium and large size of each plant was counted prior to harvesting the plants.

4.2.3.3 Leaf area

The leaf area of each leaf size (small, medium, large) was measured using an area meter (LI-COR, model 3100A, Nebraska, USA). The total leaf area of each plant was calculated using the mean leaf area of 3 leaves of each size × total leaf number of each size.

4.2.3.4 Dry weight of plant components

The fresh plant parts (leaves, fruits, branches, roots and aril) were dried at the end of the experiment in an oven at 105 °C for 15 minutes, then at 80 °C and weighted until their mass was constant. A balance (Kern & Sohn, Balingen, Germany, ± 0.01 g) was used to measure dry weight.

4.2.4 Calculations

Average leaf number per fruit was calculated by total leaf number of plant per total fruit number of that plant. Relationships were explored between: fruit number × aril weight and aril proportion, fruit number × leaf number, fruit weight × leaf number, LAI × leaf area, LAI × leaf dry weight and LAI × total leaf area.

4.2.5 Fruit quality measurements

4.2.5.1 Fruit physical measurements

Fruit weights, including aril weights at harvest were measured using an electronic balance (Kern & Sohn, Balingen, Germany, ± 0.01 g). The proportion of fruit as aril was calculated for each fruit (aril weight/fruit weight). The seeds were also counted.

Fruit length (polar axis-distance between the apex and stem) and fruit diameter (the maximum width from perpendicular to the polar axis) were measured using a vernier calliper (Mitutoyo, Kawasaki, Japan, ± 0.01 mm).

4.2.5.2 Total soluble solids (TSS) of the aril

The red aril was separated from the seed and the aril juice was then filtered using a cloth to determine total soluble solids (TSS). TSS was measured using a digital refractometer

(Atago Co.Ltd, Japan) and expressed as °Brix (°Bx). TSS values were measured for 3 samples in every fruit.

4.2.6 Statistical analysis

Analysis of variance (ANOVA) was used to test for fruit load and fruit position effects on seed number, TSS and biomass allocation. Differences between the treatment means were detected by least significant differences (LSD) test ($p<0.05$). The linear and multiple regressions were used to analyse the data using SPSS version 22.0. The linear regression or polynomial regression models were fitted to describe the relationship between fruit load with mean aril weight per fruit, mean fruit dry weight per plant, average leaf number per plant, fruit position with aril fresh weight and fruit dry weight, fruit weight with aril proportion and average leaf number per fruit, LAI and leaf dry weight per plant.

4.3 Results

4.3.1 Effects of fruit load and fruit position on the fresh weight of Gac fruit and the relationship between fruit weight and aril proportion

Increasing the number of fruit per plant and increasing the order of fruit position considerably diminished the fruit weight and fruit size (Table 4.1). The Gac fruit obtained its largest average weight and size with 1 fruit per plant and were least with the unrestricted fruit load. The plants with 5 fruits per plant produced equitable weight and size (1.1kg to 1.5kg, diameter 44-50cm). The fruit weight and fruit size declined with increasing fruit position. The fruit at the first position on plants obtained largest average weight (1.5kg) and declined considerably by the tenth position (373g). Multiple regressions were run to predict fruit weight and fruit size (length and diameter) from fruit

load, fruit position and the interaction of two these factors. These variables statistically significantly predicted on fruit weight, $F(3,93) = 82.558$, $p < 0.0005$, $R^2 = 0.727$, fruit length $F(3,93) = 66.11$, $p < 0.0005$, $R^2 = 0.681$ and fruit diameter $F(3,93) = 75.294$, $p < 0.0005$, $R^2 = 0.708$. All three variables added significantly to the prediction, $p<0.05$.

Table 4.1 The effect of fruit load and fruit-set order on fruit fresh weight and fruit size. Data are means of four replicate plants ± SE (n = 4).

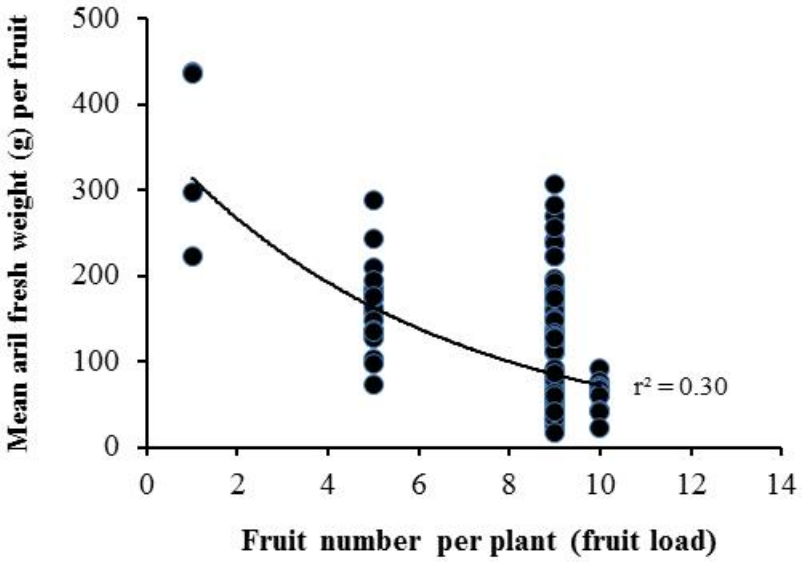
No. of fruit per plant	Node position	Fruit-set order	Fruit weight (g)	Fruit length (cm)	Fruit diameter (cm)

1	20	1	2073 ± 297	27.5 ± 1.3	54.5 ± 1.8
5	20	1	1584 ± 193	25.0 ± 1.8	49.8 ± 2.8
5	21	2	1244 ± 136	22.9 ± 1.6	45.0 ± 0.8
5	22	3	1214 ± 87	22.8 ± 0.6	45.3 ± 1.3
5	23	4	1134 ± 149	22.8 ± 1.0	43.8 ± 1.9
5	24	5	1134 ± 92	22.5 ± 0.8	44.3 ± 0.9
9	20	1	1496 ± 261	25.0 ± 1.5	48.6 ± 3.1
9	21	2	1129 ± 203	22.0 ± 1.5	43.4 ± 2.4
9	22	3	1059 ± 225	21.9 ± 2.1	43.1 ± 2.6
9	23	4	1013 ± 206	21.1 ± 2.4	42.6 ± 2.9
9	24	5	952± 189	20.9 ± 1.1	42.1 ± 2.4
9	25	6	873 ± 163	20.4 ± 0.9	40.8 ± 2.8
9	26	7	815 ± 98	20.4 ± 0.4	40.8 ± 1.3
9	27	8	659 ± 114	18.8 ± 0.8	38.3 ± 2.0
9	28	9	440± 111	17.9 ± 0.6	32.8 ± 2.6
Unrestricted	20	1	1130 ± 305	22.8 ± 2.0	44.6 ± 3.6
Unrestricted	21	2	1123 ± 259	22.3 ± 1.4	43.9 ± 3.4
Unrestricted	22	3	1053 ± 282	21.6 ± 1.1	43.6 ± 3.4
Unrestricted	23	4	961 ± 218	21.0 ± 1.3	45.0 ± 3.5
Unrestricted	24	5	732 ± 51	19.8 ± 0.8	39.8 ± 0.6
Unrestricted	25	6	654 ± 43	19.0 ± 1.0	38.0 ± 1.0
Unrestricted	26	7	621 ± 72	18.6 ± 0.6	37.4 ± 1.6
Unrestricted	27	8	554 ± 14	19.1 ± 0.9	35.8 ± 1.0
Unrestricted	28	9	374 ± 58	17.0 ± 1.0	33.6 ± 0.7
Unrestricted	29	10	413 ± 0	15.0 ± 0.0	32.5 ± 0.0

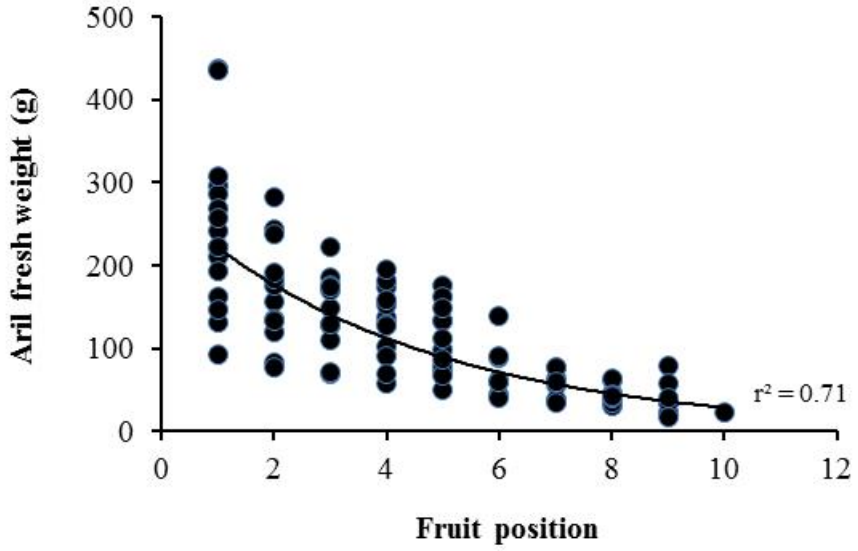
The fruit load and fruit position strongly diminished the aril weight (Fig 4.1 a and b). The aril weight of the plant with one fruit per plant was largest (220g to 430g) while this fruit component was under 100g with unrestricted fruit load plants (Fig 4.1a). The first set fruit on plants obtained an average aril weight (220g) higher than that of the fruits at other positions (Fig 4.1b). Multiple regressions were run to predict fresh aril weight and dry

aril weight from fruit load, fruit position and the interaction of two these factors. These variables statistically significantly predicted on aril weight, $F(3,93) = 66.079$, $p < 0.0005$, $R^2 = 0.681$ and dry aril weight $F(3,93) = 34.845$, $p < 0.0005$, $R^2 = 0.529$. Two variables (fruit position and interaction between fruit load and fruit position) added significantly to the prediction, $p<0.05$.

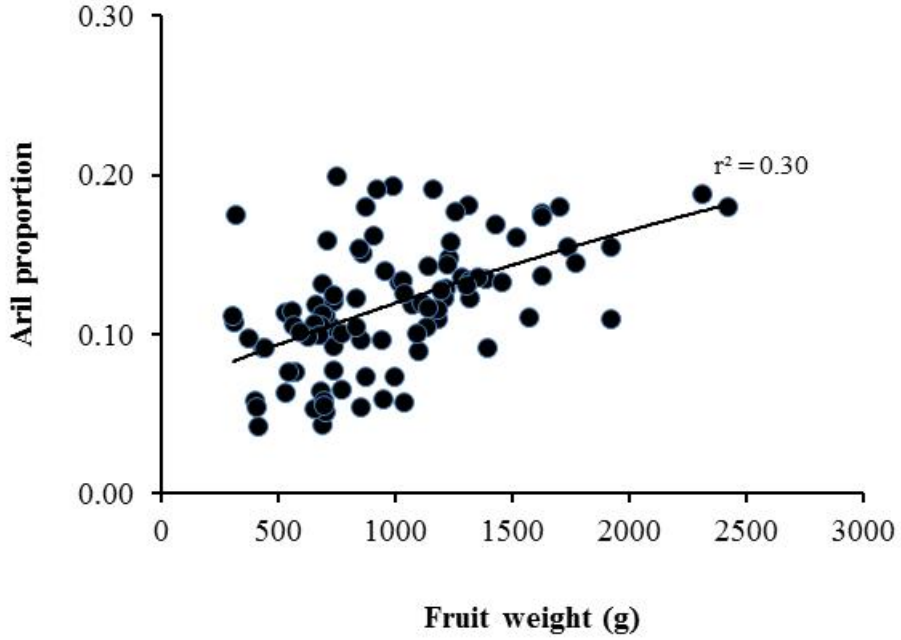
Small fruits had relatively less aril than bigger fruits. The fitted relation between aril proportion to fruit weight accounted for 30% of variability ($y = -5E\text{-}09x^2 + 6E\text{-}05x + 0.0646$). (Fig 4.1c).



(a)



(b)

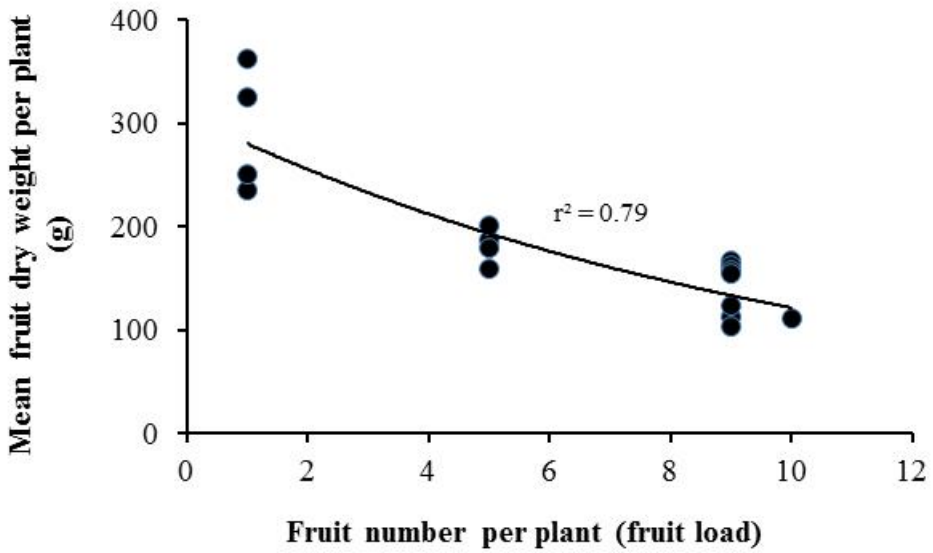


(c)

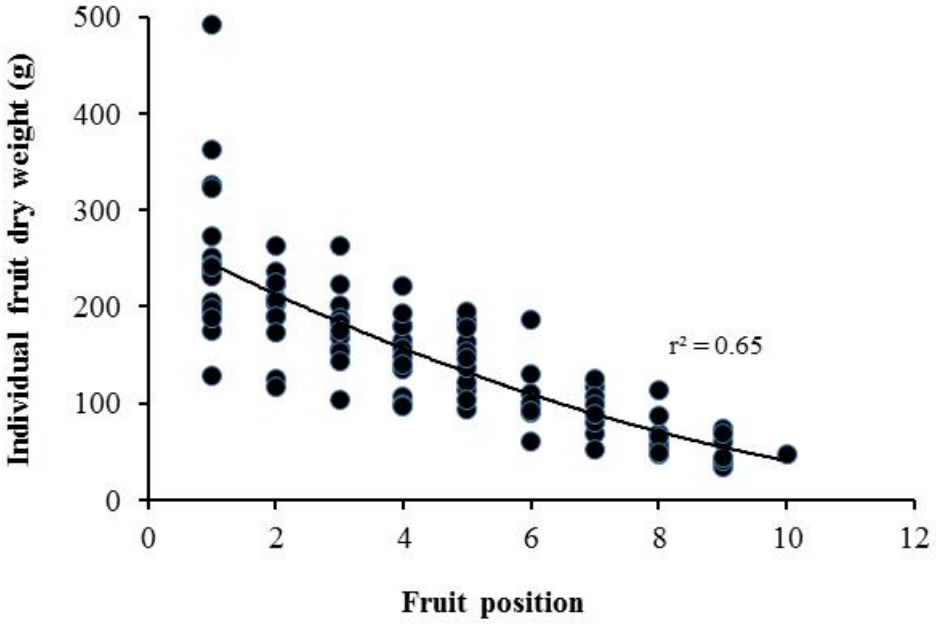
Fig 4.1 Effect of fruit load and fruit position on the aril weight of Gac fruit. The single model (solid line) fitted the data across four fruit loads (a) and ten fruit position (b). The relationship between fruit weight and aril proportion (c).

4.3.2 Effects of fruit load and fruit position on dry weight of Gac fruit

Increasing fruit load levels and more distant fruit positions resulted in declining in mean fruit dry weight per plant similar to fresh weights (Fig 4.2 a and b). The increasing of fruit number per plant from one to nine double declined the mean fruit dry weight per plant (Fig 4.2a). A positive correlation between fruit load levels and mean fruit dry weight per plant was observed ($y = 307.75e^{-0.093x}$, $R^2 = 0.79$). The average dry fruit weight at the first position was 500g more than the average dry fruit formed at the tenth position. The relationship between fruit position and dry fruit weight was negative ($y = 1.0877x^2 - 34.594x + 277.65$, $R^2 = 0.65$) (Fig 4.2b). The multiple regression was performed to ascertain the effects of fruit load, fruit position and the interaction of fruit load and fruit position on dry weight of fruit. These variables statistically significantly predicted dry fruit weight, $F(3,93) = 58.484$, $p < 0.0005$, $R^2 = 0.654$. Only two variables (fruit position and the interaction of fruit load and fruit position) added significantly to the prediction, $p<0.05$.



(a)



(b)

Fig 4.2 Effect of fruit load (a) and fruit position (b) on dry weight of Gac fruit. A single model (solid line) fitted the data across ten fruit-set order position.

4.3.3 Effects of fruit load and fruit position on biomass allocation of Gac plant

In general, the dry matter distribution to fruits increased with increasing fruit number per plant (Fig 4.3). Increasing the number of fruit per plant resulted in a declining average leaf number per fruit and smaller fruits. A positive relationship was detected between fruit load levels and average leaf number per fruit ($y = 138.7e^{-0.244x}$, $R^2 = 0.88$ and $p<0.001$) (Fig 4.4a). The fruit weight increased as average leaf number per fruit increased ($y = 0.0894x - 63.631$, $R^2 = 0.84$) (Fig 4.4b).

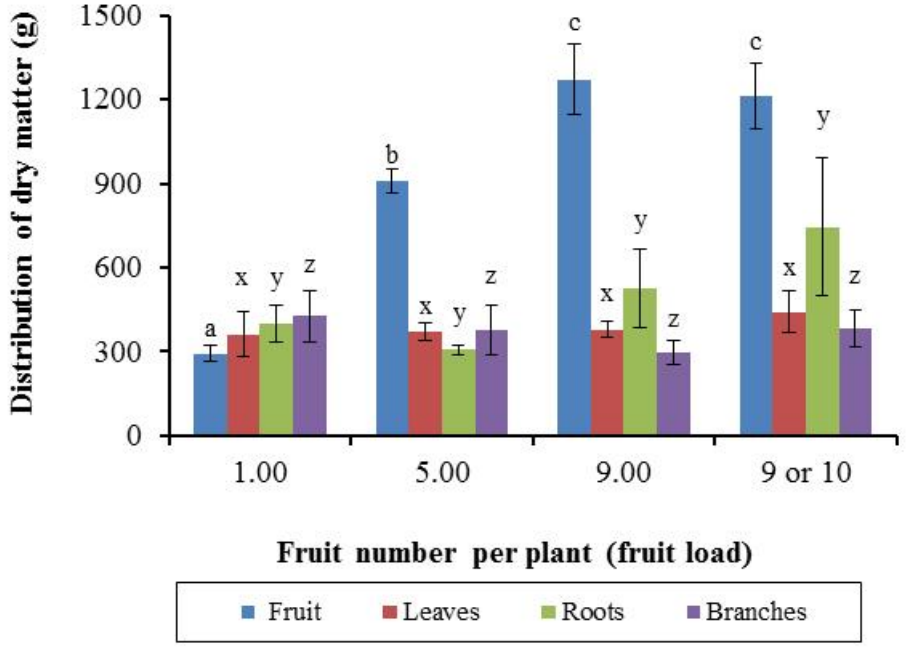
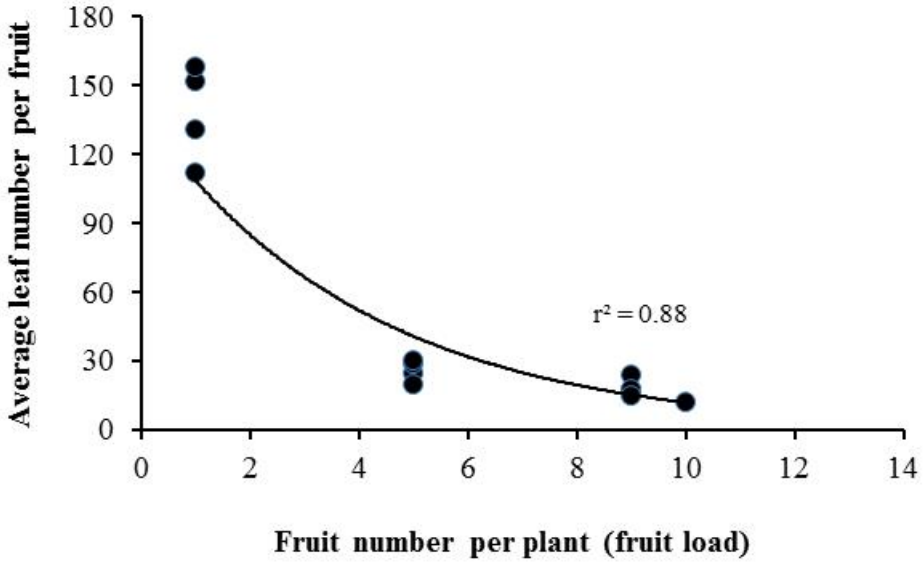
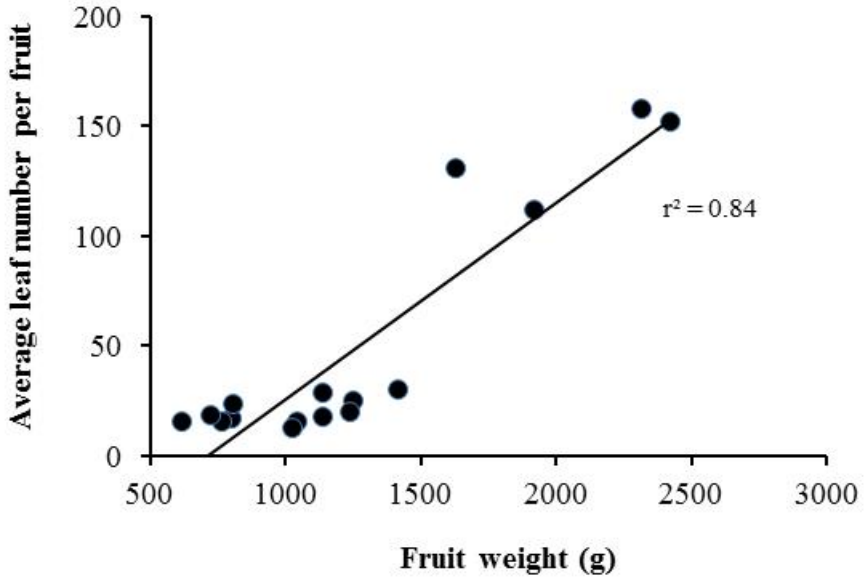


Fig 4.3 Dry matter distribution for four fruit loads. Data are means ± SE (n=4). For each plant part, values sharing the same letter are not significantly different from each other by LSD test at $p<0.05$.



(a)

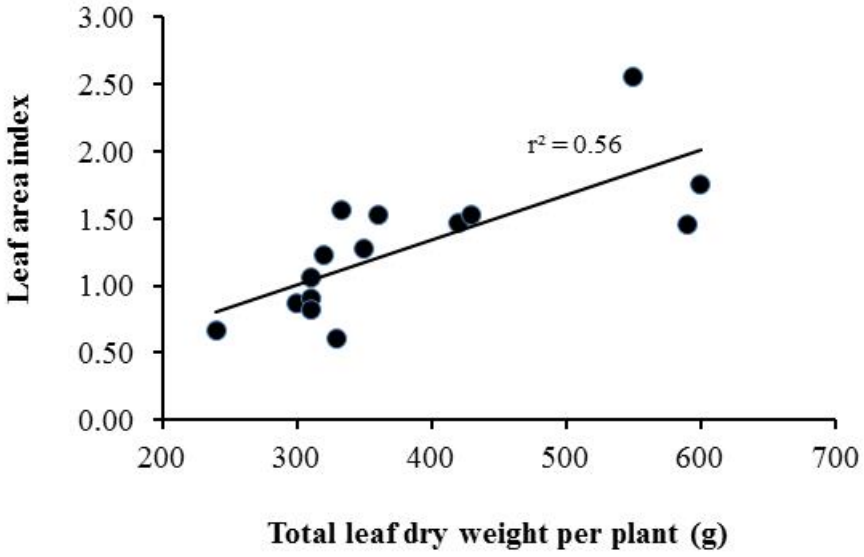


(b)

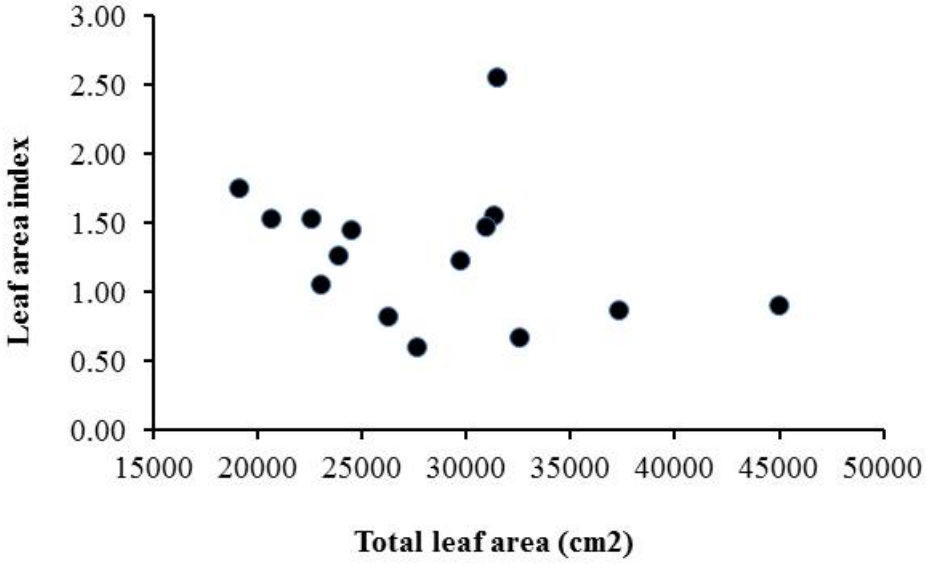
Fig 4.4 Relationship between fruit load (a) and fruit weight (b) with average leaf number per fruit. The single models (solid lines) are fitted to the data across four fruit load levels (a) and fruit weight (b).

4.3.4 Relationship between leaf area index (LAI) with total leaf dry weight per plant, total leaf area and leaf number per plant

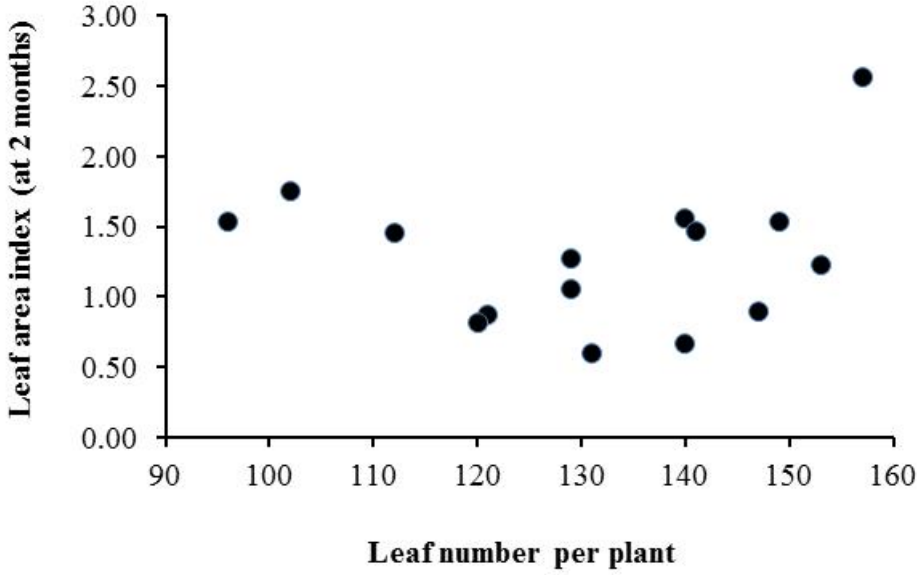
The relationship between LAI with total leaf dry weight per plant, total leaf area and leaf number per plant are presented in Fig 4.5 (a, b and c). The positive correlations were found between LAI and total leaf dry weight per plant ($y = 0.0033x + 0.0004$, $R^2 = 0.56$) (Fig 4.5 a). There was no obvious relationship between LAI with total leaf area and leaf number (Fig 4.5 b and c).


3.00
2.50
2.00
1.50
1.00
0.50
0.00
Leaf area index
r² = 0.56
200
300
400
500
600
700
Total leaf dry weight per plant (g)

(a)


3.00
2.50
2.00
1.50
1.00
0.50
0.00
Leaf area index
15000
20000
25000
30000
35000
40000
45000
50000
Total leaf area (cm2)

(b)



(c)

Fig 4.5 The relationship between leaf area index with total leaf dry weight per plant (a), total leag area (b) and leaf number per plant (c). The single model (solid line) is fitted to the data across total leaf dry weight.

4.3.5 Effects of fruit load and fruit position on fruit quality

A low fruit load (1 fruit per plant) obtained the highest proportion of aril. At other fruit load levels, there were no differences in aril proportion. TSS of the Gac aril decreased with increasing fruit load from 15.4 to 12.3 °Brix (Table 4.2). There was a significant fruit load effect between 1 and 5 fruits per plant and 9 and unrestricted fruits per plant on TSS and the number of developed seeds. The number of undeveloped seeds and the skin colour of Gac fruit were similar across the four fruit load levels (data not shown).

Table 4.2 Effect of fruit load on some quality characteristics of Gac fruit. Data are means ± SE. Values sharing the same letter in a column are not significantly different from each other by the LSD test at $p<0.05$.

Fruit number/plant	Aril proportion	TSS of aril (° Brix)	Developed seed number	Undeveloped seed number
1	0.16 ± 0.01^{a}	15.4 ± 0.6^{a}	45.0 ± 5.1^{a}	1.5 ± 0.7^{a}
5	0.13 ± 0.01^{b}	14.3 ± 0.3^{ab}	41.2 ± 2.8^{a}	2.6 ± 1.5^{a}
9	0.12 ± 0.01^{b}	12.8 ± 1.2^{b}	27.6 ± 3.2^{b}	1.8 ± 0.2^{a}
9-10	0.11 ± 0.01^{b}	12.3 ± 0.6^{b}	25.8 ± 4.9^{b}	1.2 ± 0.4^{a}

4.4 Discussion

These results highlight the commercial and nutritional value of fruits related to their size. The largest fruits contained the highest weights of aril, the most seeds and the best quality aril in terms of high TSS values (Table 4.2). In other fruit such as pomegranate, larger fruit sizes contain higher aril numbers (Wetzstein et al., 2011). The larger and heavier fruits also contained proportionally more aril compared with smaller fruits (Fig 4.1c) but the relationship was poorer than that found in a previous study (Parks et al., 2013). Thus, in the market place, this information can be used with a recommendation to select fruit based on largest size since it relates to the highest quality. Also, it is recommended that practices on farm to maximize fruit size on the plant need to be developed to optimize fruit quality at harvest.

In several species, increasing crop load has been shown to reduce fruit size and fruit components. A low crop load in cherimoya (*Annona cherimola* Mill.) produced heavier

fruits (González and Cuevas, 2008) and manual removal of cherries improved fruit size (Bennewitz et al., 2010). In Gac, an increasing distribution of dry matter to fruit (Fig 4.2) was associated with smaller fruit size, less aril and fewer seed numbers. Declining seed number with fruit-set order was also shown in a previous study on melon (*Cucumis melo* L.) (Nerson, 2004). The seed number per fruit also decreased with increasing fruit-set order position in cucumber grown in the field (Nerson, 2008). These data provide a better understanding of fruit production relationships between fruits on the same Gac plant. Earlier developing fruits appear to be a stronger sink compared with later- setting fruit as has been shown in cucumbers (Nerson, 2008).

Higher fruit load reduced aril quality in terms of declined TSS level. TSS reflects sugar content and similarly a high fruit load in Clementine plant showed a reduction in fruit size and a lower total sugar concentration compared with a low fruit load (Poiroux-Gonord et al., 2012). Similarly, in Redfree and Goldrush apples, TSS was higher in fruits from a low crop load compared with a high crop load (Katuuramu et al., 2012). TSS also increased as crop load was reduced in apricots (Stanley et al., 2015) and strawberry (Correia et al., 2011).

Estimating canopy area is a useful growth indicator for Gac and will be useful in future research on resource allocation in this species. The positive correlation of LAI with leaf dry weight suggests that LAI is a suitable indicator of growth. The lack of a relationship between LAI and leaf number or total leaf area requires further investigation. For total leaf area, the estimate was not accurate in this study as each individual leaf was not measured. Nonetheless, since for another cucurbit (*Cucurbita pepo* L.), LAI related well to leaf area, leaf dry weight, leaf fresh weight and leaf number (Labbafi et al., 2017), it

would be worth exploring these relationships further for Gac. LAI is easily measured non-destructively but the instrument is expensive. Therefore, determining other methods of growth estimates (for example, leaf number, node number, leaf fresh weight) would be a less expensive alternative.

4.5 Conclusion

Under a limited canopy, declining fruit weight is associated with declining aril TSS. Increasing fruit load and fruit-set order drives reduced fruit weight. This study also highlights that LAI provided a good non-destructive indicator of canopy area, having a positive relationship with leaf dry weight ($r^2 = 0.56$) and it may be suitable for use in future studies requiring canopy area estimates.

CHAPTER 5

FRUIT QUALITY, BIOACTIVE CONTENT IN GAC FRUIT AS AFFECTED BY STORAGE DURATION

5.1 Introduction

Traditionally, in Vietnam, Gac fruit are harvested by farmers according to the tactile feel of fruit firmness, and by how much of the skin has turned orange. The fruit are stored for between one and four weeks but the effect of storage on the quality of Gac fruit has not been well characterised. In one study limited to the measurement of aril carotenoid contents at one and two weeks of storage, carotenoid contents had significantly declined at two weeks (Nhung et al., 2010). The development of methods to estimate aril quality using readily-available instruments such as a penetrometer for fruit firmness or a colorimeter to detect skin colour changes would improve the management of fruit quality at harvest and during postharvest.

The effect of storage on aril qualities such as oil content has not been investigated but has health and commercial implications. For example, understanding how the oil content or volume extracted from aril is affected by storage is useful commercially since the oil is currently extracted and sold as a health supplement. Further, the oil has the potential to be used as a healthy alternative to saturated fatty acids in the diet (Kha et al., 2013a) and the extraction and use of Gac oil in poor households has been identified as an important measure to improve family health and nutrition in Vietnam (Vuong and King, 2003).

The nutritional quality of Gac fruit is potentially affected by intrinsic factors such as variety and stage of maturity, and by extrinsic influences like growing and storage conditions. The stage of fruit maturity has a strong effect on aril quality. For example,

Gac fruit harvested at a fully ripened stage were of a higher quality than less ripe fruits in terms of their carotenoid concentrations (Nhung et al., 2010, Kubola and Siriamornpun, 2011) and oil concentrations (Tran et al., 2016). This species also has high genetic diversity and is associated with morphologically diverse fruits (Wimalasiri et al., 2016). This may also translate to variations in fruit quality as has been shown for varieties of the closely related bitter melon (*Momordica charantia*) (Tan et al., 2014). Such potential intrinsic and extrinsic factors need consideration prior to conducting postharvest studies. In the current study, sourcing material from contrasting growing and storage conditions has provided data with a broader scope of inference compared with previous Gac studies which generally used material with a common history (Golding and Spohr, 2015).

Since the time between harvest and consumption of Gac can be weeks and large changes in aril carotenoid concentrations can occur during storage, more detail of these changes is required alongside the measurement of other aril qualities and postharvest traits that may also be affected. Thus, the purpose of this chapter was to evaluate the impact of postharvest storage on some physiochemical characteristics of mature Gac fruit including weight loss, skin colour and firmness of the entire fruit, and for the aril surrounding the seeds: TSS, and the concentrations of oil, lycopene and β-carotene. The relationships between the aril qualities of nutritional value (carotenoid and oil concentrations) and several traits commonly measured during postharvest (skin colour, TSS and firmness) were also explored to identify any potential indicators of aril quality for future work.

5.2 Materials and Methods

Three experiments on Gac fruit storage were conducted for this study, two in Vietnam and one in Australia with contrasting conditions (Table 5.1).

Table 5.1 Production and postharvest conditions for the three experiments.

Harvest month	Location	Growing system	Postharvest storage temperature	Days stored
December 2011	Vietnam	Soil / Outdoors	30 °C	7
December 2012	Vietnam	Soil / Outdoors	30 °C	7
March 2013	Australia	Substrate/ Greenhouses	21 °C	21

5.2.1 Crop production

In Vietnam, a Gac crop was grown from seed in the field in the rural area of Trang Bang, Tay Ninh Province (11° 2'N, 106° 22'E) and harvested at two and three years old in December 2011 and 2012. The plants were grown according to typical practices for Gac production. The plants were fertilised using an organic fertiliser and composted straw. In Australia, a Gac crop was grown from two-year-old root stock in May, 2013 in a climate-controlled greenhouse at the NSW Department of Primary Industries Research Station in Ourimbah, NSW, (151° 22'E, 33° 21'S) and was harvested in March, 2014, 13 weeks after pollination. The crop was grown in bags of soilless coir and irrigated and fed using hydroponic nutrient solution. Practices were as described for greenhouse production of Gac in Chapter 3.

5.2.2 Harvest, sorting and storage of fruit

In Vietnam, the fruit were harvested and transported to the laboratory of the Faculty of

Food Science and Technology, Nong Lam University, Ho Chi Minh City for storage and analysis. In Australia, the Gac fruits were harvested and stored and analysed at the postharvest facilities on-site. For Australian crop, fruits were harvested at a vine-ripened stage, this stage of maturity was between 3 and 4 of the Gac maturity scale with orange patches to fully covered orange skin and red aril (Tran et al., 2016). Vietnamese Gac fruits were picked at fully ripen stage with orange skin, red aril and were considered ready-to-eat at picking.

The Gac fruits were stored in plastic containers with dimension of 50 cm long, 35 cm wide and 30 cm high (five fruits per container) under ambient conditions until they appeared unacceptable to consumers (7 days in Vietnam and 21 days in Australia). In Australia, the temperature and the relative humidity (RH) of the preservation room were maintained at 21±1°C and 60-70%, respectively. In Vietnam, the average temperature of the preservation room was 30±1^{o}C (RH not available). For each experiment, the physicochemical properties were determined on three randomly selected fruit, daily for 7 days in the two Vietnamese experiments, and on every third day for 21 days in the Australian experiment.

5.2.3 Measurements on whole fruit

The physiological loss in weight of Gac fruit during storage was calculated as the percentage difference between the initial weight and the final weight of the individual fruit. The colour (L*, a*, b*) of fruit skin was measured using a Minolta Chroma Meter CR-400/410 (Minolta Corp, Osaka, Japan). Calculations were made of two colour parameters, based on the equations for chroma [$C^* = (a^{*2} + b^{*2})^{0.5}$] and hue angle [H° =

arctan(b*/a*)] (McLellan et al., 1995). The equatorial axis of the fruit was selected to take the measurements, 10 per fruit.

The firmness of each Gac fruit was determined using a Penetrometer (Facchini, Alfonsine, Italy) with an 8 mm flat plunger under constant force to penetrate into the fruit. Means of 10 values per fruit were calculated and expressed as kilograms force (kgf).

5.2.4 Measurements on the aril

The total soluble solids (TSS-°Brix) of the filtered aril juice were measured using a digital refractometer (Atago Co. Ltd, Japan).

The Soxhlet extraction method was used to obtain the total oil content in aril samples (Kha et al. (2013b). These were expressed as g g^{-1} of dry weight. In brief, hexane was used as the solvent and the oil extract was dried at 70^{o}C to constant weight.

High performance liquid chromatography (HPLC) was used to obtain the contents of β-carotene and lycopene in aril (Kha et al. (2013b) and these were expressed as mg g^{-1} of dry weight. Briefly, an ethanol: hexane (4:3, v:v) solvent was used for the extraction. The Agilent 1200 and Shimadzu LC-10AD HPLC systems were used with coupled Luna C18 and Jupiter C18 columns and the 20 µl volumes were injected at a flow rate of 1.0 mL min^{-1} and detected at 450 nm.

5.2.5 Statistical analyses

Data were assessed by analysis of variance (ANOVA) and the least significant difference (LSD) post-hoc test (95% confidence interval) was employed to separate means using the SPSS-PASW GradPack 22.0 for Mac (IBM Corp., Armonk, NY, USA). Correlation coefficients (r) were determined by Pearson's correlation matrix method also using SPSS software.

5.3 Results

The Gac fruit in this study from both Vietnam and Australia were similar in their whole fruit and aril weights. The proportion of aril in the fruit did not relate to fruit weight in any of the experiments (data not shown).

5.3.1 Whole-fruit quality changes during storage

During storage, weight loss increased and this was associated with a significant decline in firmness for all three experiments (Tables 5.2, 5.3 and 5.4). At the beginning of storage, the Vietnamese fruit were generally firmer than the Australian fruit but all fruit were similar in firmness at one week of storage.

Skin colour as indicated by the degree of lightness or darkness (L), vividness (chroma), and the tint of the colour (hue angle) did not reveal any general trends during storage of gac fruit (Tables 5.2, 5.3 and 5.4). However, the Australian fruit were generally more vivid in colour, indicated by a higher range of chroma values. Also, the Vietnamese fruit from 2012 declined in hue and chroma values, signifying that the skin became redder, and duller with storage time (Table 5.3).

Table 5.2 Some properties of stored mature Gac on whole fruits (weight loss, firmness, skin colour) and concentrations of oil, β-carotene and lycopene grown in Vietnam in 2011 (30±1°C).

Storage time (day)	Weight loss (%)	Firmness (kgf)	TSS (°Brix)	Fruit skin colour			Oil (g g^{-1}DW)	β-carotene (mg g^{-1}DW)	Lycopene (mg g^{-1}DW)
				L	C	H°			
0	0.00±0.00^{a}	6.86±0.25^{f}	9.78±0.08^{a}	41.60±0.90^{b}	55.74±0.63^{c}	54.67±0.85^{a}	0.16±0.01^{a}	0.49±0.06^{a}	0.68±0.03^{a}
1	0.23±0.09^{a}	6.17±0.15^{e}	10.64±0.29^{ac}	43.05±0.90^{bc}	55.20±0.31^{bc}	53.00±1.49^{a}	0.19±0.01^{ab}	0.82±0.21^{a}	1.40±0.14^{b}
2	0.30±0.06^{a}	5.68±0.23^{e}	11.53±0.18^{ab}	41.95±0.70^{bc}	49.92±1.04^{ab}	48.67±0.90^{a}	0.22±0.01^{b}	1.19±0.09^{b}	1.76±0.17^{b}
3	0.73±0.19^{b}	4.91±0.26^{d}	14.07±1.07^{bc}	39.65±1.31^{ab}	49.09±1.95^{a}	48.87±2.90^{a}	0.33±0.01^{c}	0.80±0.07^{a}	1.75±0.11^{b}
4	1.40±0.12^{b}	4.19±0.15^{c}	13.25±0.94^{abc}	36.87±2.30^{a}	46.57±2.19^{a}	43.05±3.69^{a}	0.40±0.03^{d}	1.19±0.06^{b}	2.23±0.10^{c}
5	3.43±0.64^{c}	3.41±0.09^{b}	15.53±1.44^{cde}	41.26±0.33^{b}	50.78±1.92^{ac}	47.42±1.79^{a}	0.40±0.03^{d}	1.17±0.06^{b}	2.46±0.27^{c}
6	4.10±0.40^{cd}	2.89±0.15^{b}	15.60±2.64^{cde}	42.79±2.11^{bc}	50.03±3.17^{ab}	48.11±3.42^{a}	0.33±0.01^{c}	1.34±0.15^{b}	2.80±0.20^{c}
7	4.43±0.47^{d}	2.03±0.26^{a}	18.13±0.93^{ef}	45.90±1.01^{c}	54.66±1.06^{bc}	50.39±2.08^{a}	0.34±0.02^{c}	1.49±0.13^{b}	3.50±0.20^{d}

The values are means ± SE (n=3) and those sharing the same superscript letters in a column are not significantly different ($p<0.05$) as determined by ANOVA and the LSD post-hoc test.

Table 5.3 Some properties of stored mature Gac on whole fruits (weight loss, firmness, skin colour) and concentrations of oil, β-carotene and lycopene grown in Vietnam in 2012 (30±1°C).

Storage time (day)	Weight loss (%)	Firmness (kgf)	TSS (°Brix)	Fruit skin colour			Oil (g g^{-1}DW)	β-carotene (mg g^{-1}DW)	Lycopene (mg g^{-1}DW)
				L	C	H°			
0	0.00±0.00^{a}	7.79±0.17^{e}	9.57±0.28^{a}	41.76±0.82^{a}	55.63±1.27^{d}	55.50±1.09^{b}	0.16±0.01^{a}	0.37±0.07^{a}	0.18±0.04^{a}
1	0.70±0.21^{a}	6.75±0.51^{d}	10.07±0.11^{a}	41.45±2.43^{a}	53.96±0.97^{cd}	50.59±0.38^{c}	0.21±0.02^{b}	0.48±0.07^{ab}	0.99±0.34^{b}
2	0.63±0.09^{a}	5.46±0.21^{c}	11.34±0.22^{abc}	40.55±1.42^{a}	53.65±1.96^{cd}	46.79±0.85^{ac}	0.25±0.01^{b}	0.62±0.08^{bc}	1.86±0.34^{c}
3	1.40±0.29^{b}	4.77±0.08^{c}	13.99±1.00^{bc}	36.08±2.41^{a}	51.41±1.91^{cd}	48.08±0.82^{c}	0.41±0.02^{c}	0.75±0.12^{c}	1.64±0.08^{c}
4	1.80±0.15^{b}	3.70±0.54^{b}	13.20±0.90^{cd}	37.72±1.73^{a}	48.09±1.70^{ac}	44.51±2.93^{a}	0.40±0.02^{c}	1.04±0.06^{d}	2.27±0.25^{c}
5	3.23±0.28^{c}	3.55±0.18^{b}	15.72±1.28^{cde}	39.20±0.53^{a}	51.39±1.51^{cd}	45.00±1.04^{a}	0.37±0.02^{c}	1.07±0.05^{d}	3.26±0.06^{d}
6	4.00±0.26^{d}	2.59±0.10^{a}	17.63±2.05^{e}	41.38±2.42^{a}	49.73±2.15^{abc}	43.42±2.32^{a}	0.36±0.01^{c}	1.14±0.05^{d}	3.76±0.19^{d}
7	4.70±0.44^{d}	1.93±0.18^{a}	17.86±0.92^{e}	45.19±2.43^{a}	46.08±2.05^{a}	44.38±0.63^{a}	0.36±0.01^{c}	1.26±0.02^{e}	4.33±0.09^{e}

The values are means ± SE (n=3) and those sharing the same superscript letters in a column are not significantly different ($p<0.05$) as determined by ANOVA and the LSD post-hoc test.

Table 5.4 Some properties of stored mature Gac on whole fruits (weight loss, firmness, skin colour) and concentrations of oil, β-carotene and lycopene grown in Australia in 2013 (21 ± 1°C).

Storage time (days)	Weight loss (%)	Firmness (kgf)	TSS (°Brix)	Fruit skin colour			Oil (g g^{-1}DW)	β-carotene (mg g^{-1}DW)	Lycopene (mg g^{-1}DW)
				L	C	Hº			
0	0.00 ± 0.00^{a}	2.73 ± 0.08^{e}	12.10 ± 0.98^{a}	35.76 ± 2.27^{a}	56.84 ± 1.69^{a}	44.60 ± 2.38^{ab}	0.23±0.01^{b}	1.35±0.29^{a}	1.85±0.06^{a}
3	3.47± 0.33^{b}	2.36 ± 0.10^{d}	13.07 ± 0.53^{a}	40.95 ± 1.54^{b}	57.75 ± 5.42^{a}	48.53 ± 3.22^{ab}	0.21±0.01^{b}	1.10±0.15^{ab}	1.96±0.25^{a}
6	3.76 ± 0.51^{bc}	2.20 ± 0.06^{cd}	13.32 ± 1.61^{ab}	37.43 ± 1.72^{ab}	60.11± 2.31^{a}	50.01 ± 2.06^{bc}	0.21±0.00^{b}	1.31±0.14^{a}	1.85±0.21^{a}
9	5.45 ± 0.49^{cd}	2.05 ± 0.06^{c}	13.65 ± 0.94^{ab}	38.39 ± 2.72^{ab}	52.55 ± 1.25^{a}	45.33 ± 3.33^{ab}	0.25±0.01^{a}	1.16±0.11^{ab}	2.32±0.21^{a}
12	6.59 ± 0.68^{de}	1.78 ± 0.14^{bc}	14.30 ± 0.98^{bc}	33.43 ± 1.56^{a}	56.26 ± 0.07^{a}	43.28 ± 1.83^{ac}	0.24±0.01^{a}	1.05±0.09^{ab}	2.45±0.20^{a}
15	8.25 ± 0.68^{e}	1.78 ± 0.08^{ab}	14.64 ± 0.53^{bc}	38.05 ± 0.60^{ab}	56.88 ± 2.86^{a}	42.29 ± 3.79^{ac}	0.21±0.01^{b}	1.12±0.17^{ab}	1.28±0.25^{a}
18	14.00 ± 1.09^{f}	1.60 ± 0.04^{a}	15.81 ± 0.59^{cd}	41.49 ± 1.43^{b}	56.26 ± 2.28^{a}	52.01 ± 2.36^{b}	0.19±0.01^{c}	0.65±0.06^{b}	0.70±0.06^{b}
21	11.56 ± 2.15^{f}	1.65 ± 0.14^{a}	16.75 ± 0.28^{d}	36.60 ± 1.30^{ab}	56.17 ± 1.48^{a}	41.83 ± 0.46^{a}	0.19±0.01^{c}	0.66±0.06^{b}	0.70±0.12^{b}

The values are means ± SE (n=3) and those not sharing the same letters superscript in a column are significant differences ($p<0.05$) as determined using the ANOVA and LSD post-hoc test.

5.3.2 Aril quality changes during storage

The TSS of the aril appeared slightly higher for the Australian fruit compared with the Vietnamese fruit at the beginning of storage but all aril significantly increased in TSS with storage time to similar levels. In general, the contents of oil, β-carotene and lycopene in the aril of Gac fruit either increased or remained stable with at least 7 days storage. In the Australian fruit, which was stored for longer and at a lower temperature than the Vietnamese fruit, the decline in these qualities was marked after 12 days of storage (Table 5.4). Although the two Vietnamese fruit samples from 2011 and 2012 were very similar in most qualities, they were dissimilar in their range of values for β-carotene and lycopene contents (Table 5.2 and 5.3).

5.3.3 Interactions of whole fruit and aril characteristics

With storage up to 7 days (2011, 2012), TSS, lycopene, β-carotene and oil increased in aril, and fruit firmness decreased. These variables were related (Tables 5.5 and 6.6). However, storage for longer than 12 days (2013) was associated with a reduction in lycopene, β-carotene and oil and these values did not relate well to the associated increases in TSS or to the reduction in firmness (Table 5.7). The TSS of aril was positively correlated with lycopene, β-carotene and oil concentration in 2011and 2012 (Tables 5.5 and 5.6). In 2013, TSS was negatively correlated with lycopene, β-carotene and oil content (Table 5.7).

Fruit firmness was negatively correlated to lycopene, β-carotene and oil concentration in aril in 2011 and 2012 (Tables 5.5 and 5.6). In 2013, firmness was positively correlated with lycopene and β-carotene but negatively correlated with oil concentration (Table 5.7). Fruit firmness was negatively correlated to aril TSS in 2011 ($r = -0.764$), 2012 ($r = -0.805$) and 2013 ($r = -0.675$). The colourmetric measurements of the skin, chroma and hue angle

were negatively correlated with lycopene and β-carotene in aril, but only in 2012 (Table 5.6).

Table 5.5 Pearson correlations coefficients (r) among the quality properties of stored Gac fruits in Vietnam in 2011 (30±1°C).

Parameters	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
1. Firmness	1							
2. TSS	-0.803**	1						
3. Lightness	-0.200	0.347	1					
4. Chroma	0.227	-0.079	0.684**	1				
5. Hue angle	0.334	0.000	0.640**	0.726**	1			
6. Oil content	-0.764**	0.672**	-0.169	-0.424*	-0.516**	1		
7. Carotene	-0.754**	0.664**	0.350	-0.182	-0.244	0.519**	1	
8. Lycopene	-0.923**	0.747**	0.290	-0.237	-0.321	0.713**	0.786**	1

* $p < 0.05$ (2- tailed).

** $p < 0.01$ (2-tailed).

Table 5.6 Pearson correlations coefficients (r) among the quality properties of stored Gac fruits in Vietnam in 2012 (30±1ºC).

Parameters	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
1. Firmness	1							
2. TSS	-0.834**	1						
3. Lightness	-0.022	0.116	1					
4. Chroma	0.702**	-0.529**	-0.092	1				
5. Hue angle	0.823**	-0.558**	0.097	0.675**	1			
6. Oil content	-0.805**	0.649**	-0.347	-0.653**	-0.708**	1		
7. Carotene	-0.919**	0.749**	0.125	-0.766**	-0.822**	0.775**	1	
8. Lycopene	-0.935**	0.842**	0.202	-0.608**	-0.756**	0.630**	0.889**	1

* $p < 0.05$ (2- tailed).

** $p < 0.01$ (2-tailed).

Table 5.7 Pearson correlations coefficients (r) among the quality properties of stored Gac fruits in Australia in 2013 (21±1°C).

Parameters	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
1. Firmness	1							
2. TSS	-0.675**	1						
3. Lightness	-0.114	0.311	1					
4. Chroma	0.171	0.059	0.182	1				
5. Hue angle	0.122	0.068	0.610**	0.442*	1			
6. Oil content	-0.408*	-0.443*	-0.222	-0.324	-0.088	1		
7. Carotene	0.491*	-0.460*	-0.232	0.223	-0.078	0.320	1	
8. Lycopene	0.439*	-0.468*	-0.242	-0.204	-0.240	0.634**	0.542**	1

* $p < 0.05$ (2- tailed).

** $p < 0.01$ (2-tailed).

5.4 Discussion

This study raises the possibility of postharvest ripening and prolonged storage of semi-ripened fruit, without having to compromise on nutritional value. However, the development of objective quality measures will be required to assist in managing fruit quality during postharvest and some are proposed here.

The traits with high nutritional value, being the oil, lycopene and β-carotene contents in aril, increased during storage. These remained stable at maximum levels during storage for up to about 12 days at 21ºC (Table 5.3). Their maximum levels were comparable to fully vine-ripened fruits grown from the same Australian plants in a previous study (Tran et al., 2016). These findings corroborate the previous observation that under-ripe Gac fruit have increased lycopene contents in aril at one week of storage, similar to levels obtained in stored medium and fully ripened fruits (Nhung et al., 2010). Ripening in Gac requires further investigation in order to estimate stage of ripeness and for managing ripening under controlled conditions. For instance, the effect of temperature needs consideration since in bitter melon (*Momordica charantia*), postharvest ripening of fruits at 35ºC generally inhibited lycopene synthesis in the aril in contrast to its steady accumulation at 25ºC (Tran and Raymundo, 1999).

During the storage of Gac fruit, significant weight loss, softening and increases in TSS occurred, characteristic of ripening in a number of fruits such as mango (Baloch and Bibi, 2012) and pomegranate (Olaniyi and Umezuruike, 2013). Fruit firmness and TSS have some promise as postharvest quality indicators in Gac. TSS may be particularly useful since it can easily be measured in the aril juice using a hand-held refractometer. Fruit firmness and TSS were well correlated with the carotenoids and oil contents in both the

Vietnamese fruit sets. These were not well related in the Australian fruit, probably because the oil content declined after about 9 days of storage. Although colourmetric measures of skin can distinguish between different stages of Gac fruit maturity (Tran et al., 2016), in this study, the skin colour changes that occurred during storage occurred in and were related to carotenoid contents in only one of the three experiments. The uneven development of the orange colour on the skin as the Gac fruit ripens may render colourmetric measurements too impractical for application.

Firmer fruit from the Vietnamese experiments compared with those from the Australian experiment were potentially less mature, despite efforts to harvest all fruit at a similar stage of maturity. This was perhaps reflected by their slightly lower TSS, lycopene and β-carotene contents in the aril, characteristic of Gac fruit harvested before they are fully mature (Tran et al., 2016). However, other reasons for the firmer fruit cannot be discounted. In some species, fruit firmness is affected by variety, such as in kiwifruit (Islam et al., 2012). As Gac is high in genetic variability (Wimalasiri et al., 2016), it is possible that some physiochemical differences between the Vietnamese and Australian fruit may have a genetic basis. Further, differences in crop production may also be important. For example, in the field, water capacity can be lower than in hydroponic and this may contribute to difference in firmness of fruit. This information has been demonstrated in previous study with the increasing of firmness in tomato resulted in rising of soil moisture deficit (Patanè and Cosentino, 2010). In addition, the Australian crop was grown without the inclusion of silicon (Si) which is typical for hydroponic crops. Silicon application has been shown to increase the firmness of tomatoes (Weerahewa and David, 2015) and the absence of Si in the Australian fruit may have contributed to generally softer fruit at harvest.

Silicon is absorbed by roots as monosilicic acid and polymerized into polysilicic acid and then transformed to amorphous silica, which forms a thickened membrane of Si cellulose. This double-cuticular layer protects and mechanically strengthens at different parts of plants such as leaves, fruits. Silicon also might form complexes with organic compounds in the cell walls of epidermal cells, thus increasing their resistance to enzymes activities in cells. In addition, Si can accumulate beneath the cuticle of leaves and fruits to form a cuticle-Si double layer, preventing penetration of pathogens in leaves or hardening in fruit peel (Datnoff et al., 2007).

This study highlights the potential impact of sample origin and provenance on the quality and health value of Gac fruits. Despite the common features of fruit maturity, location and storage regime, the two Vietnamese fruit samples, picked on different occasions, differed in their range of values for carotenoid contents. In this case, obvious sources of variation may have included climate and plant age. In another example, in this study the proportion of aril in the fruit was not related to fruit weight, in contrast to a previous crop from the same genetic stock and growing system (Parks et al., 2013). Further studies on physiochemical measures of Gac fruit need to consider several distinct sources of fruit, preferably with known provenance, to avoid data with limited inference.

5.5 Conclusion

It has been established that Gac fruit harvested prior to full maturity can increase their nutritional qualities, in terms of oil, lycopene and β-carotene contents in aril, postharvest. With further work, it may be possible to use a simple measurement of TSS in aril juice or whole-fruit firmness to indicate fruit quality during ripening. Investigations are required to determine the appropriate storage conditions for maximising and maintaining fruit

quality. Ensuring that sources of fruit used in Gac research are not limited in their inference is important for the application of new postharvest techniques that are developed for use in a commercial context.

CHAPTER 6

THE QUALITY CHARACTERISTICS OF GAC FRUITS AS A FUNCTION OF MATURITY

6.1 Introduction

In general, indices of fruit maturity can be used to decide when a fruit is ready to pick or eat (Pinillos et al., 2011, Wanitchang et al., 2010). Changes during the developmental stages of a fruit result in changes in its quality and shelf-life (Zong et al., 1995) and the maturity of a fruit at harvest is an important factor determining its marketability (Bhutia et al., 2011). Maturity indices have not yet been developed for Gac fruit. In addition, these indices closely related to fruit classification which indicates Gac is climacteric or non-climacteric fruit. The climacteric fruit characterized by an increasing sharply in respiration during the ripening time, however, this information has not been investigated for Gac.

External subjective measures of fruit quality such as skin colour, firmness and fruit size influence consumer acceptance of some fruits, but many consumers are also interested in nutritional quality (Iglesias et al., 2012). For Gac fruit, the major consumer quality attributes include fruit size and weight, skin colour, firmness, aril thickness and colour. Traditionally, Vietnam consumers generally prefer Gac fruits to be about 1.2-2 kg with good firmness and to have red skin with thick dark-red aril around the seeds. Fruits that are very soft are considered 'too old' and of a poorer quality which attract lower prices. Understanding the relationships between the external measures of fruit quality such as skin colour and firmness and the nutritional qualities of the aril inside the fruit will lead to simple and quantifiable indicators of fruit quality.

The quality of Gac fruit is strongly affected by its maturity at harvest. Fruit harvested at full maturity has been shown to contain the highest concentration of carotenoids (lycopene and β-carotene content) in the aril compared with green and semi- ripe fruits (Kubola and Siriamornpun, 2011, Nhung et al., 2010). The fruit size can affect the proportion of aril which increases with increasing fruit size (Chapter 4). However, these previous studies are limited as they only considered up to three stages of fruit maturity, which were poorly defined and did not explore the relationships between other important physical characteristics such as fruit weight or skin colour and the nutritional quality of the aril. Using well defined maturity stages, such as the number of weeks after the time of pollination (WAP), provide a clear definition of maturity and allow a clear comparison between multiple maturity stages.

In this study, the relationships between a number of physical properties (fruit weight, size, skin colour and flesh firmness) and chemical properties (concentration of carotenoids, total soluble solid (TSS), pH, titratable acidity (TA) and oil content) of Gac fruit harvested at five maturity stages were made. The aim of this chapter was to determine if any of these properties could be used as an indirect measure of aril quality. Indirect measures of quality would be of great benefit to growers, consumers and processors to make informed decisions about when to harvest, sell and consume Gac fruits with an optimum nutritional content.

6.2 Materials and method

6.2.1 Crop production

A Gac crop was produced in greenhouses at the NSW Department of Primary Industries at Ourimbah, NSW, Australia (151° 22'E, 33° 21'S). The 18 plants (12 female and 6 male

plants) were grown from seed in February 2013. The seed was sourced from fruit grown locally in Sydney, Australia. The environmental parameters and growing conditions for the Gac crop are described by Parks et al. (2013). The greenhouse temperature and the relative humidity were maintained between 18 to 25 °C and 60 to 80 %, respectively. A hydroponic system provided water and fertilisers with a complete nutrient solution with a target electrical conductivity (EC) of 1.2 dS m^{-1}, and a pH of 5-6.5. At the flowering stage, the target EC of the nutrient solution was increased to 2.4 dS m^{-1}. The female flowers were hand pollinated using fresh pollen collected from the male flowers.

6.2.2 Fruit harvest

Gac fruits were harvested, between 12 November 2013 (first fruit) and 16 March 2014 (last fruit) from 12 female plants at five stages of maturity: 8, 10, 12, 14 and 16 weeks after the day of pollination (WAP) and each stage included eight fruits. The fruit characteristics at the five stages of maturity are described in Table 6.1.

Table 6.1 Description of Gac fruit at five stages of maturity.

Stages	WAP*	Description of maturity stages	Pictures
M1	8	Fully green skin, white pulp, light-yellow aril	
M2	10	Green skin, spines turning yellow at the top of fruit, white pulp, yellow or pink aril	
M3	12	Semi-ripe, skin starting to yellow or orange in patches, light yellow pulp, red-aril	
M4	14	Ripened, fully orange or red skin, yellow pulp, red aril	
M5	16	Fully ripe, dark-red skin, dark yellow pulp, dark-red aril	

*WAP: week after pollination

Two to four fruits were harvested per plant and every fruit from the same plant was assigned to a different stage of maturity. The eight fruit for each maturity stage were sampled on different dates with three fruits used for quality measurements (weight, size, colour, firmness, TSS, pH, oil content, lycopene and β-carotene content) and five fruits used for respiration and ethylene production measurements at 20°C for up to 20 days after harvest. The Gac fruits were all harvested in the morning and delivered to the laboratory within 30 minutes for analysis.

6.2.3 Fruit morphology and moisture content

The weight of the whole fruit and its components (skin, pulp, aril and seed (mature black seed and immature white seed) were determined using an electronic balance. Fruit length (polar axis-distance between the apex and the stem) and fruit diameter (the maximum width perpendicular to the polar axis) were measured. Skin colour was measured using a Minolta Chroma Meter CR-400/410 (Minolta Corp, Osaka, Japan) where ten measurements (L^*, a^*, b^*) were taken along the equatorial axis of each fruit for three fruits per maturity stage. The colour parameters chroma ($C^* = (a^{*2}+b^{*2})^{1/2}$) and hue angle ($h° = \arctan(b^*/a^*)$) were calculated for fruit skin colour as described by McLellan et al. (1995). The flesh firmness was determined using a drill-mounted penetrometer (Facchini, Alfonsine, Italy) and measuring the force to manually lower a flat penetrometer tip (8mm diameter) under constant force to penetrate into the fruit to a depth of 8mm, corresponding to a mark inscribed on the shaft of the probe (Harker et al., 1996). The value of fruit firmness was an average of ten points per fruit for three replicate fruits. The results were expressed as load in kilograms force (kgf). The moisture content of the fruit components was measured by drying at 70°C in a vacuum oven (Vord-46OD, Australasian Scientific Marketing Group, Kotara, NSW, Australia) until a constant weight was obtained.

6.2.4 Respiration rate and ethylene measurements

Approximately 1h after harvest, the respiration rate and the ethylene production of the fruit were determined by measuring the concentration of CO_2 and ethylene accumulated from each Gac fruit placed in a 4 L sealed container previously equilibrated for 24 hours in a controlled room at 20 °C and 80-85% RH for at least 30 minutes. Five replicate fruits were used for this experiment. The respiration rate and the ethylene production rates were determined daily for each fruit for 20 days after harvest at 20°C.

Respiration rate

A gas sample (5 mL) of the headspace was withdrawn from each sealed container, containing a single Gac fruit, the level of CO_2 within the sample were measured with ICA gas analyser (ICA 40 system, Tonbridge, Kent. UK) and the respiration rate were calculated and expressed as ml CO_2 $kg^{-1}h^{-1}$.

Ethylene production

A gas sample (1mL) of the headspace was withdrawn from each sealed containers, containing a single Gac fruit, and the concentration of ethylene determined with Gas Chromatograph (Gow-Mac-580 Instrument Co., Bethlehem, PA, USA) equipped with a stainless steel column (190mm × 216 mm) packed with activated alumina (80-100 mesh, Alltech, Sydney, NSW, Australia) and a flame ionisation detector. The operation temperatures were 70, 90 and 100°C for the injector, column and detector, respectively. The gas flow rates were 20, 30 and 300mL/minute for nitrogen, hydrogen and air, respectively.

Ethylene production rate for each fruit were determined daily for 20 days after harvest at 20°C and expressed as µl C_2H_4 $kg^{-1}h^{-1}$.

6.2.5 The aril quality of the Gac fruit

Total soluble solids (TSS), pH and titratable acidity (TA) of the aril

The red aril was separated from the seed then blended and filtered with cheese cloth to determine TSS, pH and TA of juice. The TSS was measured using a digital refractometer (Atago Co.Ltd, Tokyo, Japan) and the pH was determined at room temperature using a pH meter (Hanna Instruments Inc., Woonsocket, RI, USA). The TA was measured using

an automatic titrator (Mettler Toledo T50, Schwerzenbach, Switzerland) against 0.1N NaOH to an end-point of pH 8.2 and the results were expressed as g citric acid (CA) per 100mL.

6.2.6 HPLC analysis of β-carotene and lycopene content

The content of β-carotene and lycopene in Gac aril was evaluated at each maturity stage using high performance liquid chromatography (HPLC) as described by Kha et al. (2013b). Two grams of fresh aril were crushed and extracted with 60mL of ethanol: hexane (4:3 v/v) at room temperature until there was no colour left in the aril material. The extracted solution was filtered using a 0.45µm syringe filter (Grace Davison, Rowville, VIC, Australia) prior to injection onto the Agilent 1200 HPLC system and Shimadzu LC-10AD HPLC system equipped with a Luna C18 (100 × 46 mm) coupled to a Jupiter C18 (250 × 46 mm) column (Phenomenex, Lane Cove, NSW, Australia). The mobile phase consisted of acetonitrile (ACN), dichloromethane (DCM) and methanol (MeOH) (5:4:1, v/v/v). The injection volume was 20 µl, the flow rate was 1.0mL.min^{-1}, and detection was at 450 nm. The β-carotene and lycopene were quantified based on the retention times and standard curves of authentic standards (Sigma-Aldrich, Castle Hill, NSW, Australia) and their content was expressed as mg g^{-1} of fresh aril weight (FW).

6.2.7 Determination of total oil content

The total oil content of the Gac fruit aril was measured using the Soxhlet extraction method as described by Kha et al. (2013b). First, 200 grams of the fresh aril was dried using microwave oven (Model R42BST, Sharp Corporation, Artarmon, Australia) and three grams of the dried-aril was placed in a cellulose thimble, which was inserted into the Soxhlet apparatus and extracted with 300mL of boiling hexane until the sample was

colourless. The extracted Gac sample was left at room temperature for 15 minutes in a fumehood to evaporate any remaining hexane and then dried at 70°C in a vacuum oven (Vord-46OD, Australasian Scientific Marketing Group, Kotara, NSW, Australia) until a constant weight was obtained. The oil content was then calculated by difference and expressed as weight percentage (g g^{-1} of dry weight, DW).

6.2.8 Statistical analyses

All chemical characteristic measurements were done in triplicate. The values were expressed as means ± standard deviations (S.D) and statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) software version 22 (IBM Corp. Armonk, NY, USA). Analysis of variance (ANOVA), followed by the least significant difference (LSD), post-hoc test, was performed to determine statistical significance between the five stages of maturity (M1 to M5). Pearson's correlation test was used to determine whether there were any significant associations between the mean values for the variables. Statistical significance was given at $p < 0.05$.

6.3 Results

6.3.1 Fruit morphology

As expected, as the Gac fruits grew on the plant, the fruit from each maturity stage were larger and heavier. The results presented in Table 6.2 show that the fruit harvested at stages M4 and M5 were larger (length and diameter) and heavier compared to fruit harvested at stage M1. Gac fruit weight increased during the developmental stages, increasing 66% from stage M1 to stage M5. Although the fruit length and diameter increased as the fruit matured, the ratio of length/diameter remained similar during the five stages at 0.5 (Table 6.2).

Table 6.2 Physical properties of Gac fruit at five maturity stages.

Fruit Attributes	Fruit maturity stages				
	M1	**M2**	**M3**	**M4**	**M5**
Weight (g)	1040 ± 294^{a}	1277 ± 269^{ab}	1587 ± 174^{ab}	1666 ± 185^{b}	1730 ± 227^{b}
Length (cm)	21.3 ± 1.2^{a}	22.7 ± 1.4^{ab}	24.5 ± 1.7^{ab}	25.3 ± 1.4^{b}	26.3 ± 1.6^{b}
Diameter (cm)	42.5 ± 3.0^{a}	44.7 ± 2.8^{a}	48.7 ± 1.4^{b}	49.2 ± 1.8^{b}	50.8 ± 2.2^{b}
Length/diameter	0.5 ± 0.0^{a}	0.5 ± 0.0^{a}	0.5 ± 0.0^{a}	0.5 ± 0.0^{a}	0.5 ± 0.0^{a}
Total seeds	32.7 ± 9.1^{a}	33.0 ± 2.7^{a}	36.0 ± 4.7^{a}	37.7 ± 1.6^{a}	34.0 ± 8.7^{a}
Immature seeds	32.0 ± 9.3^{b}	4.7 ± 6.2^{a}	3.0 ± 1.3^{a}	4.7 ± 2.4^{a}	2.3 ± 1.6^{a}
Mature seeds	0.7 ± 0.8^{a}	28.3 ± 4.9^{b}	33.0 ± 4.7^{b}	33.0 ± 2.0^{b}	31.7 ± 9.8^{b}
Colour					
*a**	-18.3 ± 3.7^{a}	-8.1 ± 6.8^{ab}	-4.8 ± 6.1^{b}	40.1 ± 3.6^{c}	44.1 ± 6.1^{c}
*b**	39.9 ± 5.1^{a}	40.3 ± 4.5^{a}	49.0 ± 5.7^{ab}	51.6 ± 11.2^{ab}	58.7 ± 6.1^{b}
*L**	37.4 ± 1.3^{a}	38.5 ± 3.1^{a}	46.1 ± 3.2^{a}	42.2 ± 3.9^{a}	43.1 ± 5.6^{a}
h°	114.4 ± 2.9^{c}	101.0 ± 9.4^{bc}	96.4 ± 7.2^{b}	51.5 ± 3.2^{a}	53.0 ± 5.6^{a}
Firmness (kgf)	> 15	> 15	9.7 ± 3.3^{b}	2.9 ± 0.1^{a}	2.3 ± 0.1^{a}

The values are means ± S.D (n = 3) and those not sharing the same letter superscripts in a row are significantly different ($p<0.05$) as determined using the ANOVA and the LSD post-hoc test. The different stages of fruit maturity (M1 to M5) are defined in Table 6.1.

The development of the seeds within the Gac fruit was affected by the harvest maturity stage, where there was a significant difference in the number of immature white seeds relative to fully mature black seeds between stage M1 and the other stages (Table 6.2). At stage M1, the seeds were almost all white (98%) and they were not completely developed, whereas at the other stages they were mostly black (86-93%) and well formed.

The external colour of the Gac fruit skin as measured with the Minolta colour meter showed a distinct progression through the five distinct stages described in Table 6.1. Skin colour at M1 was dark green but changed as the fruit matured to green with some

yellowing (M2), to yellow and orange (M3), to orange and red (M4) and finally dark red (M5). The changes of skin colour were clearly reflected by the Chroma meter a^* value (which indicates 'greenness' when it is negative and 'redness' when it is positive), increasing progressively from being negative at stage M1, M2 and M3 to being highly positive at stage M4 with no further increase at the last stage (M5) (Table 6.2). Where there were significant increases between stage M1, M3 and stage M4, fruit skin changed from fully green at M1 to semi-ripe at M3 and fully ripen at M4. The L^* value (which indicates the lightness or darkness of fruit), remained stable at the five maturity stages and the positive values indicated that the Gac fruit remained fairly light in colour throughout. Conversely, the hue angle (h° value, which integrates both a* and b* values) decreased significantly between stage M3 (in the yellow range) and M4 (in the red range). There was no hue angle colour differences between M1 to M2 (in the green range) and between M4 to M5 (in the red range).

Flesh firmness could not be determined with the M1 and M2 stages as the fruits were too hard (>15 kgf), the maximum value on the penetrometer. However, during the growth and maturity, flesh firmness declined by ≥ 70% between the M3 stage and the M4 and M5 stages (Table 6.2), where by the final maturity stage (M5), the fruit were very soft (2 kgf).

The relative weights of the four main fruit components (skin, yellow pulp, aril and seeds), as a percentage of the whole fruit weight, and their moisture content at the five different maturity stages are presented in Table 6.3. The results show that the pulp made up the highest component of the fruit (36.2 - 43.9%) and the seeds comprised the lowest component (9.3- 12.2%). The aril is the component of most interest, accounting for approximately 25-30% of the total fruit weight whist the skin contributed 14- 19% to the

total weight of the fruit. The results showed that although the total weight increased during growth (Table 6.2), the relative amounts of the fruit components were not affected by the maturity stages (Table 6.3).

Table 6.3 The components of Gac fruit and moisture contents at five maturity stages (n=3).

Parameters	**Maturity stages**				
(%)	**M1**	**M2**	**M3**	**M4**	**M5**
Percentage of fruit Skin	19.1 ± 5.4^{e}	16.9 ± 4.8^{e}	15.7 ± 2.5^{e}	13.9 ± 2.3^{e}	15.2 ± 0.2^{e}
Pulp	38.0 ± 2.1^{f}	37.0 ± 3.4^{f}	36.2 ± 1.2^{f}	43.9 ± 5.0^{f}	42.7 ± 3.9^{f}
Aril	24.9 ± 1.6^{g}	26.6 ± 2.4^{g}	29.2 ± 2.2^{g}	30.0 ± 3.8^{g}	29.5 ± 4.4^{g}
Seed	12.2 ± 1.9^{h}	9.8 ± 1.7^{h}	10.2 ± 1.5^{h}	9.3 ± 0.7^{h}	9.8 ± 1.7^{h}
Moisture content					
Skin	86.9 ± 0.3^{c}	85.3 ± 0.9^{c}	78.1 ± 1.8^{a}	84.0 ± 0.3^{c}	79.1 ± 1.9^{b}
Pulp	94.0 ± 0.1^{d}	91.2 ± 0.2^{c}	89.6 ± 0.7^{b}	91.4 ± 0.2^{c}	88.7 ± 0.3^{a}
Aril	89.7 ± 0.4^{e}	76.3 ± 0.5^{b}	78.9 ± 0.8^{c}	82.3 ± 0.3^{d}	72.5 ± 1.6^{a}
Seed	64.8 ± 1.2^{d}	30.7 ± 0.7^{b}	37.9 ± 2.1^{c}	30.4 ± 0.6^{b}	25.9 ± 0.9^{a}

The values are means ± S.D. (n = 3) and those not sharing the same letter superscripts for the proportion of fruit or for the moisture content are significantly different ($p<0.05$) as determined using the ANOVA and the LSD post-hoc test. The different stages of fruit maturity (M1 to M5) are defined in Table 6.1.

The moisture content of the five components was influenced by the harvest maturity stage. A significant difference in the moisture content of the aril was observed at all five maturity stages, where the moisture content of the four components were higher at stage M1 and decreased through the other stages to be 9% lower for the skin, 5.6% lower for the pulp, 19% lower for the aril and 60% lower for the seeds at stage M5.

6.3.2 Physiological properties

Respiration rate

The results of the respiration rates of the Gac fruit from the different harvest maturity stages are presented in Fig 6.1 and show that the respiration rate of fruit at the first day after harvest depended on the stage of maturity. The results showed that fruit from the early harvest maturities had higher respiration rates than those fruit at the more mature stages (Fig 6.1). The maximum respiration rate was obtained on the first day after harvest for the M1 stage (71 mL CO_2 $kg^{-1}h^{-1}$) and it was lower for the M2 (46 mL CO_2 $kg^{-1}h^{-1}$), M3 (51 mL CO_2 $kg^{-1}h^{-1}$), M4 (36 mL CO_2 $kg^{-1}h^{-1}$) and M5 stages (10 mL CO_2 $kg^{-1}h^{-1}$).

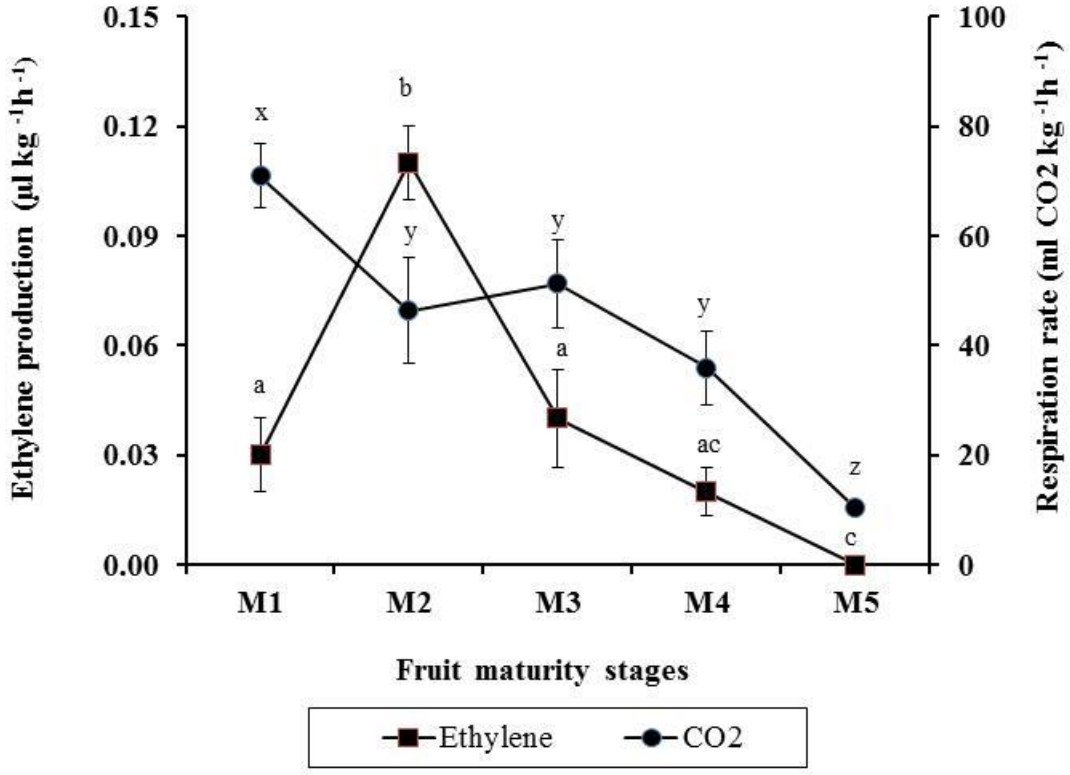


Fig 6.1 The respiration rate and ethylene production of Gac fruits harvested at five stages of maturity. Measurements were taken one day following harvest. The values are means ± S.D. (n = 5) and those not sharing a letter (a-d for ethylene, x-z for respiration) are significantly different ($p<0.05$).

The respiration rates over time after fruit harvest from the different harvest maturities are presented in Fig 6.2 and show that the production of carbon dioxide generally declined during postharvest storage at 20°C.

Ethylene production

The levels of ethylene production on the first day after harvest in fruit at the M2 stage were higher than all other maturity stages (Fig 6.1). Conversely the ethylene production rates of fruit harvested at the final maturity stage, M5 was the lowest ethylene production rate (Fig 6.1).

During the 20 days of storage at 20°C, a peak of ethylene production was observed on different days for different maturity stages (Fig 6.2). At stage M1, the highest level of ethylene (0.59 µl C_2H_4 $kg^{-1}h^{-1}$) was obtained 17 days after harvesting. Similarly, the peak ethylene production rate for the M2 stage (0.26 µl C_2H_4 $kg^{-1}h^{-1}$) was observed 17 days after harvest (Fig 6.2). Unfortunately, skin colour was not monitored during the postharvest storage life at 20°C but it was observed that the colour of fruit's skin had completely changed from green to yellow at day 17 at the M1 stage.

In contrast, the highest concentration of ethylene for the M3 stage was 0.52 µl C_2H_4 $kg^{-1}h^{-1}$ 12 days after harvest (Fig 6.2). Very low levels were observed for the M4 stage, with a peak of 0.06 µl C_2H_4 $kg^{-1}h^{-1}$ two days after harvesting and no ethylene production was detected for the M5 stage (Fig 6.2).

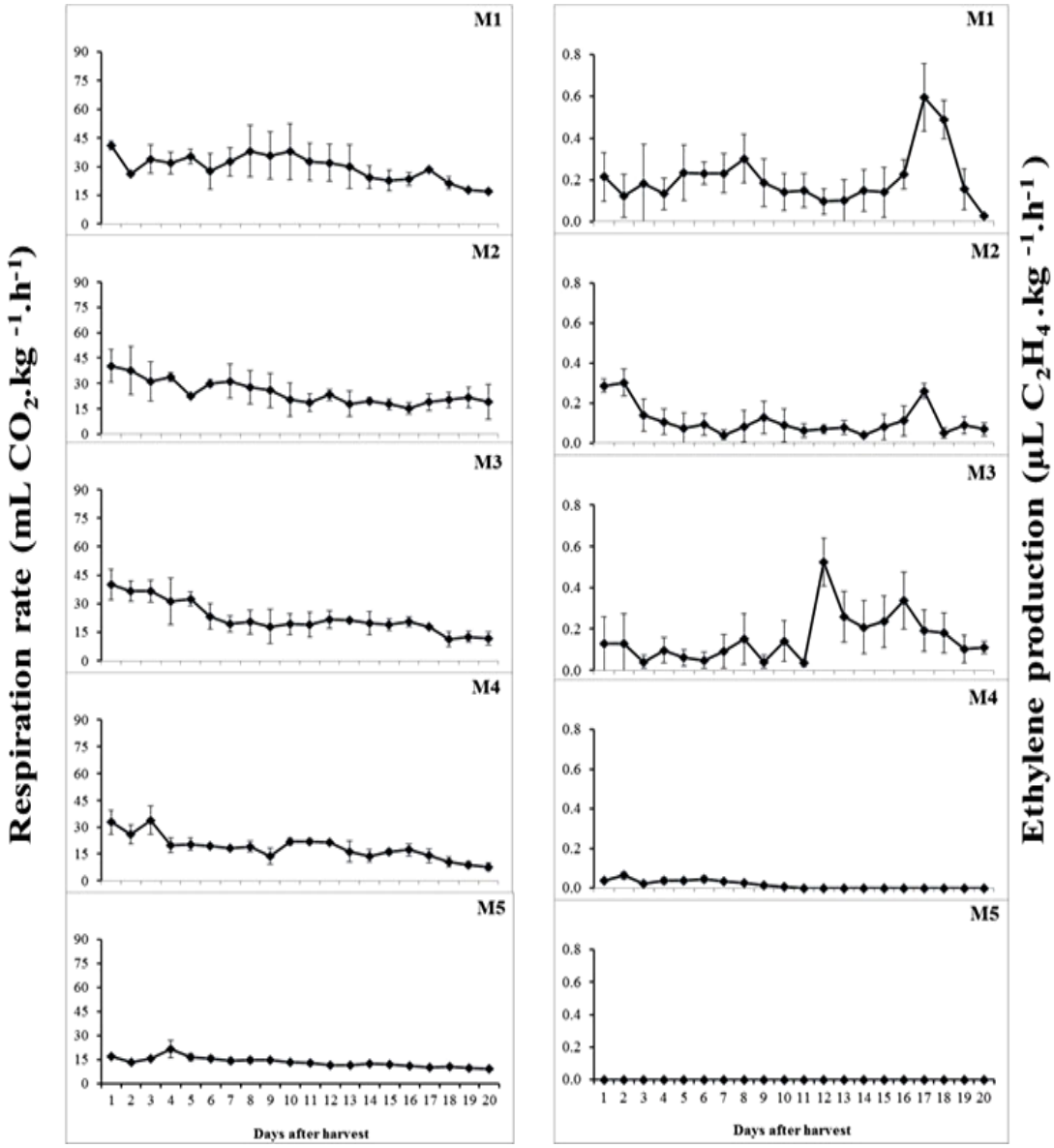


Fig 6.2 The respiration rate and ethylene production of Gac fruit during 20 days after harvest at the five stages of maturity: (a) respiration rate; (b) ethylene production. The values are means ± S.D. (n = 5).

6.3.3 Fruit quality

Total soluble solids (TSS) and pH of the aril

A close relationship between harvest maturity and TSS in the aril of the Gac fruit was observed. The results in Table 6.4 show that the TSS of the Gac aril was significantly

different at each of the five maturity stages with the highest levels of TSS measured at the M4 stage. There was an increase in the TSS of the Gac aril from the M1 stage (4.9%) to the M4 stage (15.8%) but these levels declined to 13.9% at the final maturity stage (M5 stage) (Table 6.4).

As the TSS of the Gac aril increased during maturation, the pH of the aril remained stable (Table 6.4). Titratable acidity (TA) were generally low across all the maturity stages, but were lowest at M2 stages then followed by an increase at semi-mature stage (M3). A gradual decrease in TA was observed with advancing fruit maturity (M3 to M5). The TSS/TA ratio increased from 20.9 at the M1 stage to 66.0 at the M5 stage.

Table 6.4 Chemical properties of Gac aril juice at different maturity stages.

Attributes	**Fruit maturity stages**				
	M1	**M2**	**M3**	**M4**	**M5**
Total soluble solid, TSS (°Brix)	4.9 ± 0.4^{a}	6.3 ± 0.1^{b}	12.4 ± 0.4^{c}	15.8 ± 0.1^{d}	13.9 ± 0.9^{e}
pH	6.5 ± 0.1^{a}	6.7 ± 0.1^{a}	6.6 ± 0.2^{a}	6.7 ± 0.1^{a}	6.5 ± 0.1^{a}
TA (g CA per 100mL)	0.2 ± 0.0^{bc}	0.2 ± 0.0^{a}	0.3 ± 0.1^{c}	0.3 ± 0.0^{bc}	0.2 ± 0.0^{ab}
TSS:TA	20.9 ± 1.7^{a}	36.1 ± 2.0^{b}	44.6 ± 6.7^{b}	59.7 ± 1.0^{c}	66.0 ± 2.9^{c}

The values are means ± S.D. (n = 3) and those not sharing the same letter superscripts in a row are significantly different ($p<0.05$) as determined using the ANOVA and the LSD post-hoc test

Oil, lycopene and β-carotene contents in the aril

The harvest maturity stages strongly influenced the oil content of Gac aril (Table 6.5). The oil content increased significantly from 0.03 g g^{-1} DW at M1 stage to a maximum level of 0.27 g g^{-1} DW at M4; the M5, the oil content was 0.25 g g^{-1} DW and was not different from the values measured at M3 and M4 (Table 6.5).

The content of lycopene and β-carotene in the aril increased with fruit maturity, similar to the increase for oil content (Table 6.5). At the fully green stage, the lycopene and β-carotene concentrations were at their lowest values (0.12 and 0.11 mg g^{-1} DW, respectively). The lycopene concentration was highest at the M4 stage (2.27 mg g^{-1} DW) before it decreased to at the M5 harvest maturity stage (1.42 mg g^{-1} DW). Similarly, the β-carotene concentration increased at the M4 stage (1.63 mg g^{-1} DW) but decreased at the M5 stage (1.02 mg g^{-1} DW). Whilst, the lycopene concentration in the aril was not different at the three stages M2, M3 and M5 and the β-carotene content at the M5 stage was not different from its contents at the M3 and M4 stages. The total carotenoids content (total of lycopene and β-carotene content) was highest at M4 stage (3.90 mg g^{-1} DW) where there were no differences between the M2, M3 and M5 maturity stages.

Table 6.5 The oil, lycopene and β-carotene concentrations of Gac fruit at the five maturity stages.

Attributes	**Fruit maturity stages**				
	M1	**M2**	**M3**	**M4**	**M5**
Oil content (g g^{-1} DW)	0.03 ± 0.01^{a}	0.12 ± 0.01^{b}	0.23 ± 0.02^{c}	0.27 ± 0.02^{c}	0.25 ± 0.01^{c}
Lycopene (mg g^{-1} DW)	0.12 ± 0.01^{a}	1.16 ± 0.20^{b}	1.57 ± 0.22^{b}	2.27 ± 0.49^{c}	1.42 ± 0.02^{b}
β-carotene (mg g^{-1} DW)	0.11 ± 0.03^{a}	0.65 ± 0.23^{b}	1.09 ± 0.31^{cd}	1.63 ± 0.25^{d}	1.02 ± 0.10^{bc}
Total carotenoids (mg g^{-1} DW)	0.23 ± 0.04^{a}	1.81 ± 0.31^{b}	2.66 ± 0.28^{b}	3.90 ± 0.62^{c}	2.44 ± 0.10^{b}

The values are means ± S.D. (n = 3) and those not sharing the same letter superscripts in a row are significantly different ($p<0.05$) as determined using the ANOVA and the LSD post-hoc test; DW: dried aril weight.

6.3.4 Multivariate analysis

The relationships between the morphological properties and the quality parameters of the Gac fruit during fruit development were investigated using the Pearson correlation and the results are presented in Table 6.6. The results showed that there were positive correlations ($r > 0.65$, $p < 0.01$) between the fruit size parameters (weight, length and diameter) and the aril TSS and oil content. Fruit diameter possessed the highest correlations ($r > 0.70$, $p<0.01$). Fruit length and diameter also had strong inverse correlations with the respiration rate after harvest ($r = -0.68$ and $r = -0.67$, $p<0.01$, respectively) but there was no correlation with ethylene production after harvest.

There was a positive correlation between the colour of the skin fruit (a* value) and the quality indices (TSS, oil content, lycopene and β-carotene, $r > 0.575$, $p<0.05$) while the inverse correlation between a* value and firmness ($r = -0.884$, $p<0.01$) and respiration rate ($r = -0.939$, $p<0.01$) were observed. Significant ($p<0.01$) negative correlation was observed between TSS and respiration rate ($r = -0.776$, $p<0.01$).

Strong relationships ($p<0.01$) between the TSS of the aril and the content of oil, lycopene and β-carotene in the aril were observed (oil: $r = 0.941$, lycopene: $r = 0.810$, β-carotene: $r = 0.835$). Although significant ($p<0.05$), the correlations between the fruit size parameters (weight, length and diameter) and the lycopene and β-carotene content of the aril were less strong ($r < 0.62$) than for the aril TSS and oil content. There was an inverse correlation between the hue angle and the TSS, the oil, lycopene and β-carotene content of the aril (TSS: $r = -0.84$, oil: $r = -0.78$, lycopene: $r = -0.71$, β-carotene: $r = -0.74$, $p<0.01$) and this showed that the TSS, oil, lycopene and β-carotene content of the aril increased as the fruit matured.

Table 6.6 Correlations between the morphological properties and the quality parameter of the Gac during fruit development.

Parameters	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1. Weight	1													
2. Length	0.952**	1												
3. Diameter	0.983**	0.948**	1											
4. L^*	0.553*	0.618*	0.611*	1										
5. a^*	0.569*	0.637*	0.614*	0.334	1									
6. Hue angle	-0.535*	-0.580*	-0.573*	-0.303	-0.985**	1								
7. Firmness	-0.043	-0.043	-0.152	0.261	-0.884**	0.893*	1							
8. CO_2	-0.617*	-0.676**	-0.671**	-0.292	-0.939**	0.912**	0.832**	1						
9. C_2H_4	-0.333	-0.335	-0.410	-0.129	-0.569*	0.529*	0.892**	0.553*	1					
10. TSS	0.678**	0.671**	0.709**	0.413	0.817**	-0.841**	-0.539	-0.776**	-0.568*	1				
11. TA	0.057	-0.049	0.093	0.159	0.047	-0.083	0.278	0.105	-0.442	0.423	1			
12. Oil	0.686**	0.657**	0.710**	0.421	0.736**	-0.780**	-0.240	-0.719**	-0.363	0.941**	0.352	1		
13. Lycopene	0.618*	0.575*	0.599*	0.456	0.638*	-0.705**	-0.032	-0.565*	-0.103	0.810**	0.175	0.862**	1	
14.β-carotene	0.553*	0.477*	0.578*	0.385	0.575*	-0.740**	-0.422	-0.655**	-0.318	0.835**	0.281	0.858**	0.855**	1

* $p < 0.05$ (2- tailed).

** $p < 0.01$ (2-tailed).

6.4 Discussion

This study clearly demonstrated some of the important changes in several physical and chemical properties in Gac fruit harvested at increasing stages of maturity and provides the first comprehensive account of maturation and ripening in Gac fruit. Ripening of Gac fruit was defined by the changes of skin colour (green to orange and red), aril colour (yellow to red), pulp colour (white to yellow), seed colour (white to black) and firmness of fruit (firm to soft). Ripening commenced on the vine in the fruit harvested at a mature stage (M3) and following harvest in less mature fruit (M2). The appearance of yellowing on the fruit skin spines (M2) was a sign that the maturation process had commenced. Fruit harvested at this stage of maturity had a prominent level of ethylene production relative to the other fruit maturities (Fig 6.1). For fruits harvested green (M1), a peak of ethylene production occurred 17 days following harvest (Fig 6.2). This production peak is evidence of ethylene climacteric behaviour but this needs further investigation as respiration rates did not peak and were not characteristic of climacteric fruit. Nonetheless, respiration declined during storage at all maturity stages. Fruit rots did not occur at any stage of the experiment and thus did not exacerbate ethylene or respiration observations. Fruit ripening was characterised by fruit softening and increases in TSS, oil and carotenoid contents.

The proportion of aril did not change as a function of maturity or fruit size, the larger the fruit size, the greater the internal aril volume. The aril, the most utilised part of the fruit, and the other components declined in moisture content with maturity (Table 6.3). The total number of seeds was not related to fruit quality (Table 6.2). Mature and immature seed number did not relate to fruit quality for fruit between M2 to M5 stages and thus do not provide a good index of quality. A significant increase of mature seeds from M1 to

M2 and corresponding decrease in immature seeds reflects the maturation of these seeds between these stages of fruit maturity. Further work is required to investigate the potential effect of the mature seed number on the storage life of Gac.

Between M3 and M4 stages, the firmness of Gac fruit significantly declined (>70%) and this suggests that this characteristic may be a good indicator of quality as M4 fruit was of the best quality. As fruit firmness could not be measured in M1 and M2, data was limited and more research is required to evaluate this hypothesis.

Fruit maturity at harvest had a strong effect on the carotenoid contents in the aril where increasing carotenoids were measured in the later maturity stages. The highest lycopene (2.27 mg g^{-1} DW) and β-carotene (1.63 mg g^{-1} DW) contents were obtained in M4 fruit. The most advanced stage of ripeness (completely dark red skin, M5 stage) had lower levels of carotenoids than completely orange fruit (M4 stage) indicating that the fruit were over-ripening (Table 6.5). Kha et al. (2013a) reviewed the carotenoid contents in Gac fruit reported the contents were variable. The carotenoid contents obtained in this study are within the range reported by others (Aoki et al., 2002, Vuong et al., 2006, Ishida et al., 2004, Nhung et al., 2010) but clearly showed differences in the stage of fruit maturity at harvest. However, other variables such as variety, techniques used for analysis and variable production conditions will also have an effect on the quality and carotenoid contents and should be further evaluated.

The skin colour of Gac fruit showed a positive correlation with quality indices (TSS, carotenoid and oil contents) (Table 6.6). Growers and consumers could use this simple parameter to identify fully mature fruits with high quality. Although objective parameters of colour (Minolta) and firmness (penetrometer) did not significantly separate the two most mature categories, the difference in colour was apparent to the eye highlighting the

large variability in the Minolta colour measurements. Thus skin colour, a parameter already used by consumers, when choosing fruits for purchase or when deciding if a fruit is ready to eat (Opara et al., 2009) is appropriate for Gac fruit.

As more mature Gac fruits were harvested, the TSS of the Gac aril considerably increased from 4.9% Brix at M1 to 15.8% Brix at M4. Such increases in the TSS level are common with fruit maturation and it has been observed in many fruit types (Fawole and Opara, 2013, Salvador et al., 2007, Wanitchang et al., 2010). In addition, is likely that TSS also increases in Gac fruit as it ripens after harvest. It is observed that TSS in the Gac aril increase during storage for Gac fruit harvested at a stage between M3 and M4 (Chapter 5). As TSS is strongly correlated to the oil and carotenoid contents ($r > 0.81$; $p<0.01$) (Table 6.6), the simple measurement of TSS with a refractometer may be a practical and simple tool to use as an indicator of fruit quality. However more research is required to verify this relationship in different varieties and growing situations.

The increase in the ratio TSS/TA of Gac aril juice during fruit maturation was similar to other fruits (Fawole and Opara, 2013, Pinillos et al., 2011) and is also a reliable indicator of quality (Table 6.4). The TSS represents the total soluble solids such as sugars, acids and other components (Bailen et al., 2006). The relatively high level of TSS in Gac aril (15.8% Brix at M4) is interesting, as the informal assessment of taste of Gac fruit is not sweet like other fruits with similar levels. Although taste is a complex interaction of many characteristics, this may reflect that sugar levels may be a small proportion of the TSS but this needs further investigation.

6.5 Conclusions

This chapter has identified several measures that provide an index of maturity which relates to Gac fruit quality in terms of high contents of oil and carotenoids. The objective

indices useful for identifying high quality fruit are skin colour, TSS, the TSS: TA ratio and most likely firmness. Those measures identified as not being suitable are total seed number and TA. Gac fruits harvested at the M4 stage were ripe and soft, had completely orange or red skin with a yellow pulp and red aril. These were of the highest quality which could be considered best for consumption. However, we propose that Gac fruits can be picked at the M3 stage for transportation and distribution to the consumer. The fruit described at the M3 stage were semi-ripe and firm with skin starting to yellow or orange in patches and with light yellow pulp and red aril. Whether or not fruits harvested at the M2 and M3 stage can be ripened in postharvest to obtain acceptable levels of carotenoids and oil contents needs to be clarified. In addition, confirming the climacteric nature of Gac fruit needs further investigation.

CHAPTER 7

GENERAL DISCUSSION

7.1 Maximising production efficiency and Gac fruit quality

The focus of the work undertaken in this study on the Gac plant was to develop production and postharvest practices that would increase yield and maximize fruit quality. Until recently, this crop was underutilised and usually only home-grown. Large gaps in knowledge of its production potential have existed until the present. Specifically, methods of propagation, pollination, canopy management and postharvest practices have been improved as a result of this project. These improved methods have been condensed into guidelines for this discussion and are summarized in Tables 7.1 to 7.5. The guidelines may also be useful for a range of applications in the production of the crop, postharvest management of the fruit, and in future research on Gac.

More-efficient, large-scale plant production is a reality for Gac. To grow a Gac crop from seed, we need to use seed of no more than 6 months old (if stored at room temperature of 21 °C) and to germinate it at 25-35 °C (Table 7.1). The use of hormone dips will be useful to strike large numbers of female softwood cuttings and female scion can be grafted onto seedling rootstock of unknown sex to ensure the supply of fruiting plants (Table 7.1).

Table 7.1 Suitable conditions for Gac propagation as observed in this study.

Method	Requirements	Outcomes
Seed germination	Temperature range: 25-35°C Seed age: < 6 months (stored at 21 °C, 60-70% RH)	Time to > 90% germination: 7-8 days Time to planting: 35 days
Cuttings		
Semi-hardwood Softwood	No hormone required IBA hormone concentration: 3-5gL^{-1}	Survival rate: > 71% for both types of cuttings Time to planting: 50 days
Grafting		
Top-wedge Slice	Rootstock age: 4 to 8 weeks old Rootstock age: 4 to 8 weeks old	Survival rate: > 85% for both grafting methods Time to planting: 45 days

The use of stored pollen, with techniques developed in this study, potentially may reduce the number of male plant needed and allow more female plants to be grown, increasing yield capacity (Table 7.2).

Table 7.2 Conditions that limit viability loss of Gac pollen during storage. Pollen was dried for 1 hour before storage to obtain 13-14% pollen moisture content.

Storage temperature and RH	Storage time	Outcome
21 °C, 60 % RH	1 hour	Maximum pollen germination: 53 % Fruit set: 96 % *Commercial fruit set: 83% Time to harvest: 14 WAP
-20 °C, 70% RH	Up to 8 weeks	Maximum pollen germination: 37 % Fruit set: 86 % Commercial fruit set: 73 % Time to harvest: 14 WAP
4°C, 60% RH	Up to 4 weeks	Maximum pollen germination: 30 % Maximum fruit set: 76 % Commercial fruit set: 61 % Time to harvest: 14 WAP

*Commercial fruit is defined as > 1 kg fruit weight

The pollination studies highlight the importance of high quality pollen to obtaining high quality fruits. Limiting pollination with poor quality pollen or limited amount of pollen will reduce the size and nutritional qualities of Gac fruit. Further, this study has developed a method to assess Gac pollen germinability, so that pollen studies can continue to improve efficiencies in this practice (Table 7.3).

Table 7.3 The optimal *in vitro* medium and incubation temperature developed in this study for Gac pollen germination. Pollen germination and pollen tube length are assessed after 24 hours.

Conditions	Amounts
Culture medium:	
Agar	1 %
KNO_3	0.01 %
H_3BO_3	0.01%
Sucrose	15 %
$Ca(NO_3).4H_2O$	700 mgL^{-1}
$Mg(SO_4).7\ H_2O$	250 mgL^{-1}
Incubator temperature	35 °C

Investigations of fruit maturity and storage of fruit have illustrated the impact of a number of factors on fruit quality. In terms of managing fruit quality, firmness and the TSS of aril are potential indicators of the lycopene and β-carotene concentrations in aril (Table 7.4 and 7.5). Commercially, these bioactive compounds are highly valued with processed Gac products made from the aril such as powers and capsules already available (Kha et al., 2013a). Estimating the quality of the aril at harvest and at processing using simple tools (penetrometer for firmness, refractometer for brix and colorimeter for colour) will be a useful application for growers and processors to monitor fruit quality. These tools are already being used in other fruits. For example, in European plums (*Prunus domestica* L.), firmness is being used as an indicator of maturity (Usenik et al., 2014), skin colour is being used as a quality indicator for 'Ligol' and 'Jonagored' apples (Łysiak et al., 2014) and in two commercial mango cultivars 'Sindhri' and 'Samar Bahisht (S.B.) Chaunsa', TSS of the flesh is used as an indicator of harvest maturity (Amin et al., 2013). A recent study citing the current work (Tran et al., 2016), has further shown that if the stage of fruit maturity is known, the lycopene and β-carotene concentrations in aril can be reliably

predicted (Bhumsaidon and Chamchong, 2016). At the very least, the described stages of Gac fruit maturity, developed in Chapter 6 (Table 6.1) as colour posters, for example, provide a suitable guide for pickers, consumers and retailers to assess the potential quality of the fruit using external appearance.

Table 7.4 High quality fruit and their associated characteristics. Values are means of 3 replicates ± SE (Chapter 6).

Maturity stages	**Maturity Stages**	**WAP***	**Potential quality indices**		**Bioactives**		
			Firmness (kgf)	**Aril TSS (°Brix)**	**Oil content (g g^{-1} DW)**	**Lycopene (mgg^{-1} DW)**	**β-Carotene (mgg^{-1} DW)**
Just-ripened	M4	14	2.96 ± 0.10	15.8 ± 0.13	0.27 ± 0.02	2.27 ± 0.49	1.63 ± 0.25
Fully ripe	M5	16	2.28 ± 0.09	13.9 ± 0.87	0.25 ± 0.01	1.42 ± 0.02	1.02 ± 0.10

*WAP: weeks after pollination

Table 7.5 The conditions at harvest and during storage associated with high quality Gac fruit. Values are means of 3 replicates ± SE (Chapter 5).

Study location	**Growing system**	**Storage temperature**	**Time to maximum quality in storage**	**Potential quality indices**		**Bioactives**		
				Firmness (kgf)	**Aril TSS (°Brix)**	**Oil content (g g^{-1}DW)**	**Lycopene (mg g^{-1}DW)**	**β-carotene (mg g^{-1}DW)**
Vietnam	Field Tropical	30 °C	7 days	1.93 ± 0.18	17.86 ± 0.92	0.36 ± 0.01	3.50 ± 0.20	1.26 ± 0.02
Australia	Greenhouse Temperate	21 °C	12 days	1.78 ± 0.14	14.3 ± 0.98	0.24 ± 0.01	2.45 ± 0.20	1.05 ± 0.09

7.2 Towards improvements in plant production of Gac

Vigour tests are a potential practice to assist in determining seed storage requirements, and to improve Gac seed quality control, since Gac seed is sensitive to viability loss as shown in this study. Vigour encompasses the ability to rapidly germinate within negative environments (Milošević et al., 2010). Vigour testing is useful because vigour declines before germinability is lost (Shaban, 2013). Using physical characteristics do not have much promise for Gac as indicators of viability since seed mass does not relate well to the timing of radicle emergence (Chapter 2). Other vigour tests which may be suitable for future work on Gac include germiniating the seed under cold conditions, or exposing the seed to a short period of high temperature and high humidity conditions known as accelerated aging (Milošević et al., 2010).

Grafted plants in this study performed better than plants produced from seed in terms of earlier fruiting. This observation was anecdotal since yield data was not collected from these experiments. However, this warrants further investigation. Initially, the benefits of grafting on timing of flowering would need to be demonstrated for Gac. Interestingly, the rootstock of grafted plants were male raising the question of the genetic influence of the rootstock on the timing of flowering and fruiting. Potentially, the female root system may have a role in inhibiting flowering in this species. Investigating the role of rootstock in flower formation, as it has been previously investigated for cucumber (Satoh, 1996), would be of benefit for Gac. In another cucurbit species, Galia melon (*Cucumis melo* cv Arava) grafted with a hybrid squash rootstock (*Cucurbita maxima* × *Cucurbita moschate*) delayed the timing of anthesis of female flowers but the timing of the yield was not affected (Guan et al., 2015).

The benefit of grating for Gac has wider potential. For example, grafted cucurbit plants can improve tolerance of vegetables to thermal, water or organic pollutant stresses (Schwarz et al., 2010). This would allow Gac to be grown in areas where it is not normally grown, for example in more temperate or dry conditions. In any case, this study has demonstrated that grafting is a suitable method that growers can use to make redundant male plants into productive plants by grafting female scions onto male rootstock or seedling of unknown sex.

In other propagation methods, the earliness in flowering and fruiting of cuttings is worth investigating for Gac since in another melon (*Citrullus lanatus*) plants produced from cuttings flowered and fruited earlier than seed produced plants (El-Eslamboly, 2014). Also, micro propagation using tissue culture methods appear to be suitable for Gac. They are used in cucurbit production, for example in *Cucumis trigonus* Roxb. (Mali and Chavan, 2016) and preliminary Vietnamese work on Gac in micro propagation is promising (Hoa et al., 2009).

7.3 Determination of Gac as a climacteric fruit

This study points to Gac fruit as having climacteric characteristics. The continued ripening and peak of ethylene production in fruits harvested at maturity stage M2, provided evidence for this. Ethylene production and respiration (measured by CO_2 production) is closely associated with fruit ripening in climacteric fruit (Wills et al., 2007). In this study, there was limited information on carbon dioxide production during respiration of Gac fruit (Tran et al., 2016) and this requires further investigation. In addition, other measures of ripening need to be investigated including flesh softening, texture and colour changes and the accumulation of organic compounds (Wills et al., 2007). A better understanding of the ripening characteristics of Gac fruit during

postharvest will lead to the development of guidelines for temperature and humidity during storage. Where storage conditions cannot be controlled, predicting the longevity of fruits will also be possible. Ultimately better information on ripening will allow growers and fruit sellers to control Gac fruit quality.

7.4 Continued conservation of Gac

This study has improved the conservation of Gac through better production practices for this species (as outlined in 7.1 and 7.2). Further classification of this species will also be of benefit to its conservation. It is already known that Gac has high genetic diversity based on morphological and molecular characteristics of leaves, fruit and seed (Wimalasiri et al., 2016). However, it is apparent from the current study that the pollen of Gac has not been well classified (Chapter 3). Developments in pollen classification for Gac will further assist in identifying genetic diversity for this species. Also long-term storage strategies for pollen and seed will assist the conservation of this species. In particular, cryopreservation, a technique used for a range of species may be useful for Gac. This involves storage in liquid nitrogen at a temperature of -80 °C. In order to achieve this, the conditions required controlling the moisture content of seed and pollen will need to be developed in future research.

In conclusion, the agronomic practices including propagation, pollination, canopy management methods and postharvest practices have been improved as a result of this study. These practices can be used as a base for further developments in the commercialization and conservation of this species.

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