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Aspects of mango (*Mangifera indica* L.) fruit rind morphology and chemistry and their implication for postharvest quality

by

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DECLARATION

I, the undersigned, declare that the thesis, which I hereby submit for the degree of Doctor of Philosophy at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Wilma du Plooy

Signed in Pretoria on 10 March 2006

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To Bert, Dimari and Phillip

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List of Abbreviations

Abbreviation / Symbol	
ν_x	Wavenumber
CPD	Critical point drying
EDS	Electron dispersive spectroscopy
FE-SEM	Field emission scanning electron microscopy
FTIR	Fourier transform infrared
MCT	Mercury-Cadmium-Telurite
RP-HPLC	Reverse phase high performance liquid chromatography
r_t	Retention time
SEM	Scanning electron microscopy
TEM	Transmission electron microscopy
TFA	Trifluoroacetic acid
XRD	X-ray diffraction
XRF	X-ray fluorescence
λ	Wavelength

The way to do research is to attack the facts at the point of greatest astonishment.

Celia Green (1935 -)

British philosopher, psychologist and physicist

Chapter 1

GENERAL INTRODUCTION

Few fruit embody summer lusciousness more vividly than the tropical sensuality of a mango (*Mangifera indica* L. (Anacardiaceae)) fruit. After pineapples (45.2 %), it is the second most important tropical fruit crop (25.2 %) (Food and Agriculture Organisation, 2002; Saúco, 2004).

Considered indigenous to eastern Asia, Myanmar and North Eastern India, mangoes have been cultivated for at least the past 4000 years. Tree crop farmers in South Africa experimented with its cultivation since the turn of the 1900th century and in 1920 the first commercial production began in the vicinity of Tzaneen (S 23º49' E 30º20') (South African Mango Growers Association, 2004). Mango trees are evergreen, andromonoecious and capable of growing up to 250 years old, annually bearing abundantly flowering thyrses (Watson & Dallwitz, 1992; Judd *et al*., 2002). However, the plant culls the majority of its flowers, eventually bearing only a few fleshy drupes.

Variations in sensorial characteristics are cultivar dependant (Knight, 1997). As a globally popular commodity that also has cultural importance, a wide array of varieties in terms of colour, shape, smell and taste have been developed. The characteristic smells and tastes result from the organic acid, terpenoid and free sugar content of the fruit, varying from almost bland to distinctly turpentine, from sweet to acidic (Singh *et al*., 2004). Cultivar development and selection depends on these chemical profiles, along with other genetically determined traits such as fibrousness, colour and shape (Lavi *et al*., 1997).

Not all cultivars are suitable for cultivation in all production areas, since physical variables related to weather and geographic location affect fruit physiology, tree architecture and innate resistance to pathogens (Bompard, 1993; Human & Rheeder, 2004). Even if initially successful, commercial development of a cultivar includes consideration of other critical factors that may only become apparent after several seasons of cultivation, marketing and processing (López *et al*., 1995; Ponce de León *et al*., 2000). One of the reasons for this is

that physiologically, commercially important cultivars have to deal with strenuous export conditions and extended export periods. Inland production areas are up to 2000 km away from Cape Town, the major export harbour, and fruit is transported in refrigerated units until eventual transfer to specialised shipping facilities. Turn around time can be up to 28 days before the product reaches the European port terminals, some 10 000 km away from Cape Town. During this period, the fruit must withstand desiccation and might be exposed to chilling injury. As an example, 'Heidi' was initially developed for the export market, but was unable to withstand prolonged cooling under commercial conditions. This only became evident after all other selection criteria were successfully addressed and commercial growers invested in the cultivar. Irregular bearing exacerbates the cultivar's problems. However, this is not the only example of cultivar failure (Finnemore, 2000). Many mango difficulties reflected by specific cultivars are related to horticultural and farming practices.

Apart from physical parameters that influence production within a geographical area, ethnic and cultural preferences have a remarkable impact on horticultural decisions around the globe. Although preferences of the local markets within a production area will influence cultivar selection to satisfy these requirements, consideration of cultivar popularity within targeted export markets are more important (Hofman *et al*., 1997). South African producers abide by the same principles; cultivars once popular locally (e.g. 'Zill') are sacrificed in favour of cultivars with good export demand, such as 'Keitt' and 'Kent'. These cultivars do well in the major production areas of South Africa, nevertheless the potential horticultural success is not fully developed, due to seasonal constraints. Southern hemisphere summers dictate that the local production and marketing period is from late November to early April. This coincides with a number of competitors from South American and Indo-Asian countries who are targeting the same markets as South African producers (Segré, 1998). In addition, these competitors have the added advantage of a shorter export period (typically only 7 to 15 days from harvest). Furthermore, the volumes produced by these countries (Fig. 1) create an unassailable competitive advantage. This situation will become even more difficult in future, since cultivar development is steadily diminishing the seasonal limitations of all mango-producing countries (Saúco, 2004). Considering the increasing global popularity of mangoes, the seasonal niche, local production capacity and quality of South African cultivars are encouraging aspects for development of the local industry (Finnemore, 2000; Human & Rheeder, 2004). It is therefore prudent to make an effort to ensure the industry reaches its full potential.

In the South African context, the mango industry plays a significant socio-economic role within the production regions. In the 2002 season, 45.3 % of the mango harvest came from operational units in former resettlement areas, contributing 41.4 % of their annual income. According to Shabalala *et al*. (2004), some 106 000 farming operations produce mangoes in South Africa. However, in terms of all the fruit produced in South Africa, the quantities harvested and total annual income derived is less than the average for similar tree crops (Table 1). Moreover, South African mango production is also a small industry when local production of 67 metric tons (MT) and export figures (17 MT) are compared with figures for global production (25 754 509 MT) and global export (21 946 MT) (Food and Agriculture Organisation, 2002; South African Mango Growers' Association, 2004). Globally, however, industry size, potential and economic importance is not correlated. From Figure 1 it can be seen that Guatemala contributes only 0.73 % to world production, yet the high yield of 350.20 kg/Ha (Fig. 2) indicates an intensive industry. On the contrary, India produces the highest volume of mangoes (44.26 %, Fig. 1), but at a yield of only 99.49 kg/Ha (Fig. 2). Commercial mango production in South Africa averages yields of 193.59 kg/Ha that, similar to Guatemala, indicates high-density commercial operations (Food and Agriculture Organisation, 2002). Any increase in the productivity of such intense operational units will significantly increase the respective country's contribution to global production and export figures. Although mangoes are an important food source in many rural areas, increasing marketable volumes are imperative to develop prosperity.

Non-commercial cultivation for households sees large portions of harvests consumed by producers themselves, reducing their return on investment (Saúco, 2004). In 2002, it was estimated that up to 22 % of the crop produced by emerging South African farmers was consumed in this way (Shabalala *et al*., 2004). Self-consumption of marketable produce is only one of the reasons for low export volumes. Other factors that contribute to poor return on investment include important pre- and postharvest problems such as disease development, sunburn, insect damage and physiological disorders. Under preharvest conditions, these difficulties are often approached by prioritising postharvest outcomes concerned with fruit physiology, nutrition and pest control (Combrink *et al*., 1994). Crop management take the form of manipulation of both nutritional and water status, disease control by biological and chemical means, physical alteration through horticultural practices, and genetically predetermined breeding strategies (Lavi *et al*., 1997; Human & Rheeder, 2004; Saúco, 2004). Morphology, and to a lesser extend, ontogeny, is studied as part of horticultural searches to cultivar improvement (Núñez-Elisea & Davenport, 1995; Ponce de León *et al*., 2000). However, no studies considering the impact of mechanical or chemical interference on the epicuticular membrane on fruit physiology

were found. As the interface with its environment, this membrane is crucial to the development of the fruit up to and after harvest (Jeffree, 1996; Kolattukudy, 1996).

Postharvest problems in general arise because fruit respiration and metabolism continues after harvest, making physiological deterioration inevitable (Yamaki, 1995). This is a natural process since fruit, once physiologically mature, contains a viable embryo that needs to be dispersed (Esau, 1977). Postharvest control measures against this deterioration involve managing disease onset, fruit physiology and fruit appearance. Applying a commercial wax to the fruit surface is an important postharvest procedure. It is intended to enhance fruit appearance, to control weight loss through reduction of moisture loss and to manipulate physiological changes (López *et al*., 1995; Manzano *et al*., 1997). To attain this level of functionality demands a complex formulation that can combine with the natural wax without negatively affecting respiration and fruit tissue (Yamaki, 1995; Manzano *et al*., 1997). Commercial wax formulations are often the first suspected point of deviance in cases of postharvest failures, as was the case with lenticel discolouration, one of the most serious problems for South African producers. The random nature of the development of the condition, however, makes it clear that such a view is over-simplistic.

It is also not clear why certain cultivars seem to be more prone to developing the condition than others, making it difficult at this stage to breed cultivars resistant against lenticel discolouration. Several cultivars are affected with varying degrees of severity, annually causing up to 20 % loss in potential export volumes (Le Lagadec, 2003). Although innocuous, the discolouration causes superficial blemishes that render fruit cosmetically unacceptable on the export markets. Preliminary chemical and morphological investigations of this condition indicate that it is a physiological reaction caused by stress and not a pathogen-induced condition (Du Plooy *et al*., 2003). Discolouration development is often delayed, affecting cultivar performance during export, which has financial as well as reputational consequences. Consignments leaving South African shores with flawless fruit may reach the importing destination with afflicted fruit, causing a downgrade of goods and resulting in financial and customer confidence losses. Understanding and alleviating this problem would make it possible for local producers to become more competitive.

Like lenticel discolouration, sunburn and insect pests diminish the export value of crop substantially. Recent developments in the control of these problems lead to the preharvest application of kaolin on pome fruit in America. (Glenn *et al*., 2002). European growers soon followed suit and recently local commercial growers showed interest in utilising this alternative approach, already applied successfully on vegetable crops (De Night, 2004).

Kaolin has characteristics which makes its application seemingly advantageous, such as high reflectivity and high dispersibility. Reportedly effective in sunburn prevention, control of insect pests in more than one life stage and accepted as an alternative organic preharvest pest control measure it is an important new option in crop management (Glenn *et al*., 1999). Very little information on pre- and postharvest effects of both commercial forms of kaolin on mangoes is available (Le Lagadec, 2003; Du Plooy *et al*., 2004), with some studies on the possible insect control (Joubert *et al*., 2002). There are no studies on interference of kaolin with commercial wax application on any of the tree crops, or on the physiological impact that this specialised clay may have, particularly on the developing epicuticular membrane.

The epicuticular membrane is a common denominator in both pre- and postharvest management of mango fruit crops. No studies of cultivar dependent variation of this structure and its components have been conducted, making it difficult to determine any structural relation between cultivar susceptibility and physiological stress. Amplification of physiological stress conditions or induction of postharvest problems through the application of preharvest pest control measures is reported to be dependent on the inherent robustness of individual apple cultivars (Knight, 1997).

Fruit cultivar development seeks out and combines desirable traits from parent generations that can lead to improved horticultural qualities, but importantly, also to enhanced physical appearance, texture, smell and taste. Cultivar differences in terms of the sensorial characteristics are well documented (Singh *et al*., 2004). However, catering for consumer preference, breeding programmes and monoculture has diminished the effectiveness of many self-defence strategies employed by the plant. One such self-defence strategy often neglected is the epicuticular wax and other surface structures of the fruit (Eigenbrode, 1996).

Providing innate protection against excessive radiation, pathogen attacks and environmental interference, plant surfaces are expensive structural barriers in terms of energy investment (Kolattukudy, 1996). Fruit, as a vital survival mechanism, is therefore initiated and matured with chemical and structural efficiency (Esau, 1977; Yamaki, 1995). During cultivar development, differences in ability for these efficiencies may be augmented and could account for some of the variances in resistance against environmentally and physiologically induced disorders. As the interface between the fruit and its environment, studies into morphological and developmental aspects of the fruit need to include surface characteristics.

The objectives of this study were to investigate the mango surface with particular reference to the epicuticular wax, to study the nature of lenticel discolouration of mango fruit and to examine the effect of preharvest uncalcined kaolin treatments on both the epicuticular wax and applied commercial wax. The impact of postharvest handling on the packline on the fruit epicuticular wax was also investigated.

To achieve these goals the following focal points were identified:

- Ontogeny and morphology of mango fruit wax
- Morphology of mango lenticels
- Chemical characterisation of mango fruit wax
- Chemical profiles of discoloured lenticels compared to non-discoloured lenticels
- Impact factors on mango fruit wax:
    - Preharvest treatment of mangoes with uncalcined kaolin
    - Effect of mechanical handling on the packline and commercial wax coating.

Each of these focal points is discussed in a separate chapter, which is presented in article format. The conclusions from each chapter will be consolidated in a final discussion, together with an outline of anticipated future research ensuing from this investigation.

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TABLES

Table 1 Statistical overview of tree crop production in South Africa, indicating the number of operational units involved in different commodities, the harvest quantities and the income generated from each (Shabalala *et al*., 2004)

Commodity	Farming operations * (X1000)	Quantity harvested (MT)	Income generated (Former homelands) (R million)	Income generated (Total) (R million)
Mangoes	106	42	119	147
Peaches	94	209	1	364
Pawpaws	81	2.5	2	3
Bananas	80	273	75	741
Avocadoes	66	175	31	197
Guavas	47	61	16	158
Oranges & other citrus	44	500	5	2116
Watermelons & other melons	26	141	3	3810
Grapes	24	1880	-	4586
Strawberries & other berries	17	4.1	-	3
Apples	14	1206	2	649
Litchis	7	4	31	31 †
Pears	5	1100	-	859
Plums	5	113	1	143
Other fruit	4	6.8	-	379
Pineapples	3	79	2	159
Tree nuts	2	1.4	-	3
Total	625	5 790	288	14348

* : The number of operational units is not correlated to the total land area dedicated to each crop; units cultivating mixed crops are calculated per crop, probably including subsistence farmers - thus the number of operational units

†: The figure represented above is derived from information provided by the South African Litchi Grower's Association (SALGA)

FIGURE CAPTIONS

Figure 1 Graph of mango production as a percentage per individual country correlated against the totalled global production (100 % = 25 754 509 MT) (FAO, 2002).

Figure 2 Graph of mango yield (kg/Ha) recorded in some cultivating countries in comparison to the world average figure of 100 kg/Ha (FAO, 2002).

FIGURES

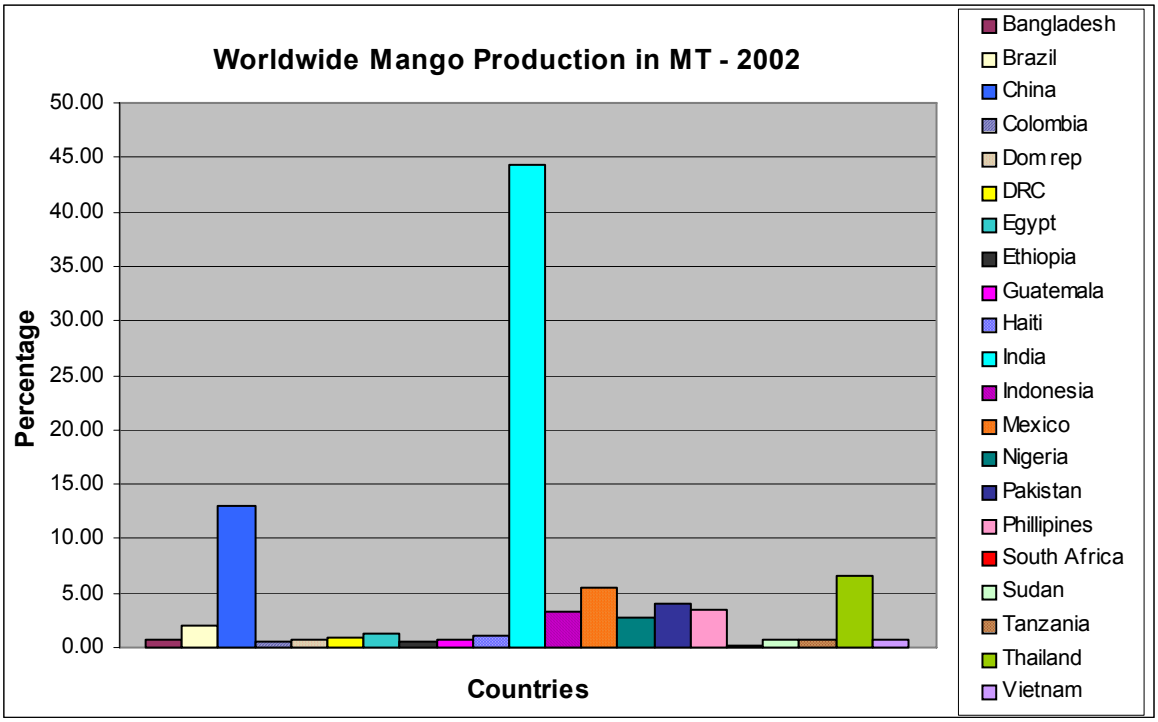

Figure 1

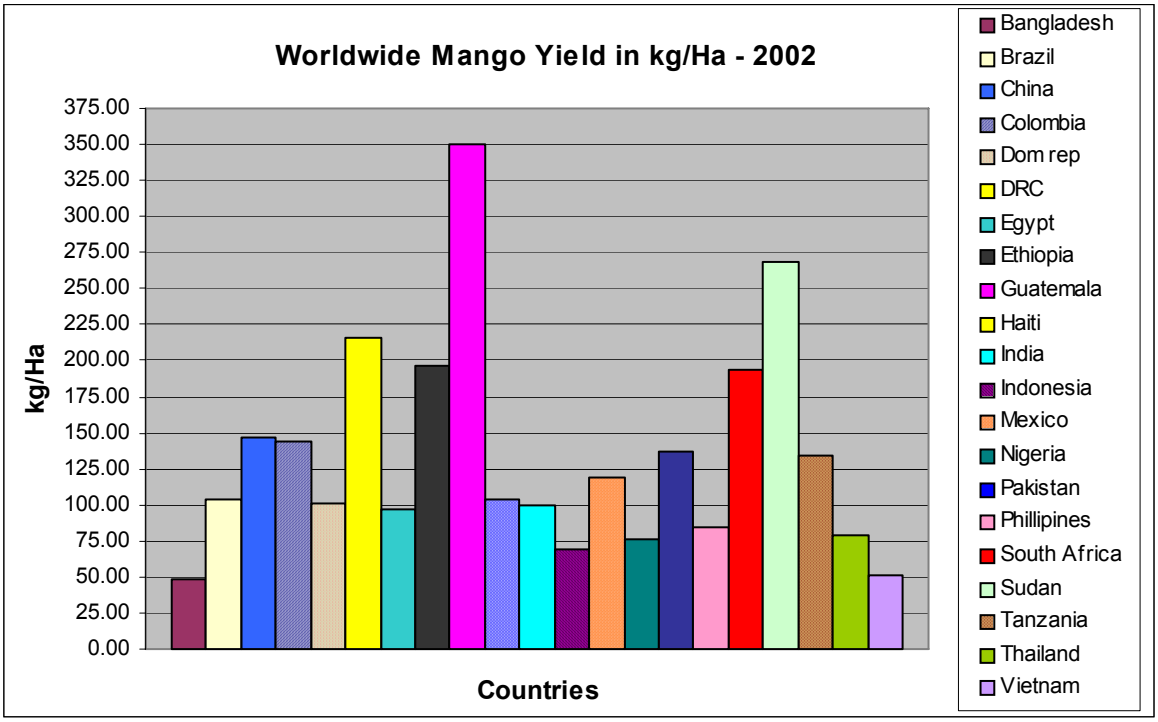

Figure 2

Science is wonderfully equipped to answer the question "How?" but it gets terribly confused when you ask the question "Why?"

Erwin Chargaff (1905-2002)

Austrian biochemist

Chapter 2

THE ONTOGENY AND MORPHOLOGY OF MANGO (*Mangifera indica* L.) FRUIT WAX

2.1 ABSTRACT

The epicuticular surfaces of mango fruit (cultivars 'Keitt', 'Kent' and 'Tommy Atkins') were studied. A complex layer of wax functioning in reflectance and maintenance of physiological processes, bring about the dull bloom observed on developing fruit. The development of the wax is marked by sequential formation of distinct morphologies of crystalloid structures. Initially, there is a non-homogenous layer over the embryonic cuticle and epidermis, while more intricate structures develop soon after initialisation of fruit-set. The growth rate and increase in volume leads to the destruction of the second morphological type, which is pushed to the uppermost boundary of the final wax layer. The final morphology is characterised by a dense cover of delicate sepaline crystalloids, which fuse and eventually collapse as the fruit passes physiological ripeness. Cutin plates formed during lateral expansion of the epidermal layer contribute to the pyramidal, block-like morphology of the wax layer on mango fruit. This morphology is pronounced during fruit set, but less so on mature fruit. Originating from integrated wax and cutin on the ovary, the cuticular layer rapidly increases in quantity and eventually forms an intercellular filling that is saturated with cuticular wax. No apparent relationship between plate boundaries and the subcellular arrangement of epidermal cells could be established. No cracks were observed between the cuticular plates of any of the cultivars used in this study. As far as could be ascertained, this is the first comparative study of the ontogeny and morphology of the fruit wax and cuticle of different mango cultivars from the point of anthesis to physiological ripeness. Inter boundary distances during development of plates during fruit set present a possible answer for periodic poor adherence of preharvest pesticides.

2.2 INTRODUCTION

Fruit are transient aerial plant structures, with their singular importance being agents for propagation, and species survival giving their evolutionary impetus. The fruit surface is a specialised and intrinsic part of this evolution and its characteristics are regarded as species specific and persistent (Hietala *et al*., 1997; Griffiths *et al*., 2000). This implies that the architecture, composition and abundance of surface characteristics are genetically programmed (Lemieux, 1996; Jetter *et al*, 2000; Long *et al*., 2003). Various elements contribute to the complex entity of the fruit surface, but it consists mostly of cutin, cuticular wax and epicuticular wax crystalloids (Holloway, 1982; Jeffree, 1996; Jetter *et al*, 2000). Structural surface features may also include oil glands, a variety of trichomes and stomata (Esau, 1977; Stern, 1994).

As the interface between the atmosphere and the internal components, the importance of the fruit surface cannot be overestimated. The epicuticular wax is the first barrier presented by this interface, protecting the fruit and its physiological processes against injury by high levels of ultraviolet (UV) radiation, microbial attack, predation and mechanical injury (Stephanou & Manetas, 1997). Several wax crystalloid types (Barthlott *et al*., 1998) and chemical compositions (Hietala *et al*., 1997; Griffiths *et al*., 1999; Griffiths *et al*., 2000, Goodwin *et al*., 2003) have been described, attesting to its varied and species-specific nature. The importance of epicuticular wax has lead to its use in several studies, such as monitoring environmental pollution (Bakker *et al*., 1998; Evans *et al*., 2001; Pal *et al*., 2002), development of biological control measures (Rutledge *et al*., 2003; Eigenbrode, 2004) and quantification of physical attributes involved in crop improvement (Beattie & Marcell, 2002; Grant *et al*., 2003).

These studies underline the effects of human intervention, whether through physical control or genetic development of monoculture crops (Hietala *et al*., 1997; Beattie & Marcell, 2002). Such interventions are often part of both pre- and postharvest management of fruit crops. The success and financial feasibility of any intervention forming part of crop management depends on effective and successful implementation thereof, whether genetic, chemical or physical. However, successful implementation not only depends on precise management, but also on innate qualities and characteristics of the plant surface (Bally *et al*., 1996; Barthlott & Neinhuis, 1997; Mouloungui & Gauvrit, 1998; Bauer *et al*., 2004). Due to its hydrophobic properties, the presence of superficial wax layers affects all pre- and postharvest programs that rely on application of chemical or biocontrol measures. In this respect, epicuticular and cuticular wax has a particular role

to play in the regulation of substances moving in or out through superficial plant structures (Chamel & Gambonne, 1997). Various features of the cuticular membrane are all involved in determining the extent to which the fluidity of the carrier matrix and plant wax achieves successful interaction, absorbance, penetration and translocation (Mouloungui & Gauvrit, 1998; Liu, 2004). The physical characteristics of surface wax are as important as the chemical nature thereof, as illustrated by Liu (2004), who found that improvement of the amount of droplet contact area and herbicide absorbance requires matching of surfactant and plant wax properties.

Various interactions with epicuticular and cuticular waxes not only affect disease and pest control, but also plant physiology through its determination of moisture loss (Oliveira *et al*., 2003) and reflectance of UV-B radiation (Rozema *et al*., 1997; Frohnmeyer & Staiger, 2003). Long-term effects of elevated UV-B levels are not yet clear, although Evans *et al*. (2001) proved that UV-B radiation penetrates the mesophyll, damaging cuticular and subcuticular tissue. Rozema *et al*. (1997) reported that UV-B damaged genetic material were excised as part of cellular maintenance, and consider the possibility that physiological damage due to UV-B may surpass negative effects related to predation or competition. Holmes and Keiller (2002) compared the reflectance of a range of plants that were hairless, hairy, woolly or waxy, and showed that epicuticular waxes effectively reflect UV radiation. They concluded that the substantial differences in the ability of waxes to reflect various wavelengths are based on chemical and morphological characteristics of the waxes. This corresponds with findings by Grant *et al*. (2003) that leaf reflectance is directly related to the leaf wax cover, and particularly important in studying the effects of increasing UV-B levels on the biochemistry and physiological processes of plants.

Fruit, as the fertilised ovary, is regarded as a modified leaf (Cutter, 1980), and is biochemically and physiologically complex through its dedicated functionality. To protect these biochemical and physiological processes, allowing embryo maturation and eventual dissemination thereof (Esau, 1977; Stern, 1994), control of external effects such as light and heat is required (Núñez-Elisea & Davenport, 1995). As a subtropical crop, mangoes are subjected to intense solarisation and heat levels (Barkstrom, 2004). A dense waxy bloom covers mango fruit as it develops, giving some protection against these environmental threats. In a study of the surface and lenticels of cultivars ‘Keitt’, ‘Kent’ and ‘Tommy Atkins’, particularly dense and complex epicuticular wax structures were observed. Normal scanning electron microscope (SEM) observations of mature fruit concurred with those made by Bally (1999). He gave an overview of wax ontogeny of the cultivar ‘Kensington’, mentioning cracks in the cuticle proper (*sensu* Holloway, 1982) as

the source of rind discoloration. It is felt that the chronological morphology of various wax fractions needs further investigation, but no other work on wax ontogeny was encountered. This study aims to contribute to better understanding of the ontogeny and morphology of fruit wax from major South African commercial mango cultivars.

2.3 MATERIALS AND METHODS

2.3.1 Plant material

Mango fruit samples of different developmental stages of three polyembryonic cultivars ('Tommy Atkins', 'Kent' and 'Keitt') were collected fortnightly from the orchards of Bavaria Fruit Estates (Hoedspruit, Limpopo Province, South Africa) throughout the 2002/2003 and 2003/2004 seasons. Samples were collected by macroscopic verification alone, necessitating several collections of matching fruit sizes to prevent misinterpretation of wax development due to mismatched data. Each fortnight, five to seven fruit sizes, with five fruit each, were collected per cultivar. Three sections per fruit was sampled, prepared and viewed for each round of collection. Dissections were done immediately upon and at the point of collection. Fruit sizes sampled at each collection were noted (straight line from stem end to stamen end), ensuring an overlap of sizes with the previous round. This assisted in creating a continuous timeline for the developmental events observed.

2.3.2 Methods

Samples for both normal scanning electron microscopy (SEM) (JSM 840, JEOL, Tokyo, Japan) and high-resolution field emission scanning electron microscopy (FE-SEM) (JSM 6000FE, JEOL, Tokyo, Japan) were dissected from fruit at anthesis to mature mango fruit. For normal SEM, sections were viewed at 5 kV and a working distance of 12 mm, while for FE-SEM 5 -10 kV was used.

Samples were prepared using two parallel methods in order to exclude the interpretation of artefacts from preparation. According to the first method, sections (5 x 5 mm) were cut and fixed in a 1:1 mixture of 2.5 % glutaraldehyde and 2.5 % formaldehyde in 0.1 M $NaPO_4$ buffer (pH = 7.3 ± 0.05), postfixed with 1 % aqueous OsO4, and dehydrated in an ethanol dilution series (30, 50, 70, 90 and 3 x 100 %). The material was critically point dried (Biorad E3000, Polaron, West Sussex, UK), mounted on stubs and made conductive in the vapour of a 0.5 % RuO_4 solution (Van der Merwe & Peacock, 1999).

For the second method, small sections (5 x 5 mm) of fresh material were plunge frozen in liquid propane at -180 °C, vacuum dried (Custom built, Tshwane University of Technology, Pretoria, South Africa) at -80 °C and 10-7 mBar for 72 hours, mounted on stubs and exposed to 0.5 % Ruthenium vapour to render material conductive.

To expose the architecture of the cuticle beneath the epicuticular wax, sections of mature 'Keitt' rind (5 x 5 mm) were soaked in 78 % H_2SO_4 for 48 hours, followed by three rinses in distilled water. Thereafter the sections were soaked in chloroform ($CHCl_3$) for 72 hours, air-dried, mounted on stubs and made conductive in the vapour of a 0.5 % RuO_4 solution.

2.4 RESULTS AND DISCUSSION

The description of chronological sequence of events during the formation of fruit epicuticular wax is based on micrographic evidence. Each micrograph, however, represents the observation of tissue that was metabolically active until the moment of fixation. The fixed material therefore gives a visual representation of the momentary structural contribution of the biochemical and metabolic processes.

Determining chronological fruit and, consequently, epicuticular wax development was particularly problematic from post-anthesis to fruit size 50 mm. Structural differences in the epicuticular wax, ontogenetically and morphologically, between the different cultivars, were indiscernible (Fig. 1A - C). All cultivars, regardless of external appearance, had a similar bi-layered epicuticular wax appearance at physiological ripeness (Fig.1D). This confirmed that some intrinsic characteristics of the species are persistent across its cultivars (Knight, 1997), and is not altered by sensorially relevant features such as colour, shape, aroma and sugar content. For this reason, it was decided to report all the results as that of mango fruit as such, and not to relate it to a specific cultivar.

The two methods run in parallel gave complementary results of similar material. This was useful in several respects: analogies pointed out the consistencies and provided necessary repetition of the sampled material, whereas differences in results contributed to understanding and explaining the observations. These differences in the two methods were most pronounced for young material and were not observed in more mature fruit. Plunge frozen sections of very young material showed some shrinkage - up to 15 %, and aldehyde fixed material up to 40 % (Boyde & Maconnachie, 1979). Specific anomalies

encountered in the two methods will be indicated in the discussion of the applicable results.

All the micrographs in figure 2 were of post-anthesis fruitlets, at similar developmental stages, which were harvested and fixed at the same time. Figure 2A and B illustrates the amount of shrinking due to plunge freezing and aldehyde fixation followed by critical point drying (CPD) respectively. Because of the shrinkage, artefacts were created, making the interpretation of results more difficult. Artefact formation is less severe in plunge frozen material. Fractioning of fragile surface structures (Fig. 2C & D) was more pronounced after CPD. The composition of the plant material stayed intact with plunge freezing (Fig. 2E). Soluble components in the material, however, were lost during fixation in preparation for CPD, causing further disturbance of the integrity of the sample material (Fig. 2F). The integrity of plunge frozen material was correlated to the light microscopical findings of Bezuidenhout *et al*. (2005), but in the young fruitlets aldehyde fixation created false structural entities due to uneven shrinkage of fragile or unsupported cellular structures (Fig. 2G & H).

Nevertheless, as illustrated in Figure 3 (A - D), CPD rendered information that was valuable in understanding the chronology of the wax ontogeny. Characteristic development of the epicuticular wax was found to be dependant on the direction of deposition of wax fractions. The sequence and direction of wax deposition illustrated was only visible through the removal of soluble fractions by the aqueous buffer and ethanol dehydration during preparation. Other such instances were the fruit surfaces in Figure 4 (A - D), depicting sections of cutin from 4 - 7 mm fruit. The embryonic wax was removed during sample preparation, making the initials of the more permanent wax visible (A). Globular crystalloids (Barthlott, *et al*., 1998) extruded into nematoid wax crystalloids (B), which seemed to clump together from localised regions (C & D). These nematoid crystalloids coalesce into narrow ribbons (E); in turn, these coalesce to form sepaline lamellae (F & G). Cross-sectional fractures of plunge frozen material often revealed lamellar wax crystalloids that formed after the physical impact (H). This latter type of wax crystalloid structure was only found on fruit exceeding 10 - 15 mm and is structurally fit for light manipulation by the fruit.

An amorphous (Jeffree, 1986) or smooth (Barthlott, *et al*., 1998) wax layer covered fruit from post-anthesis (2 - 3 mm) up to a size of about 10 mm. Initially, it is undulating, adhering to the apical ends of the columnar epidermal cells. This embryonic layer seemed to be a mixture of two immiscible components (Fig. 5A), one of which was indicated as

soluble during aqueous aldehyde fixation. Plunge frozen fruitlets of the same sizes show no such loss of compositional fractions. At post-anthesis, the cuticle thickness averaged < 0.5 µm, in comparison to the primary cell wall thickness of < 1 µm. The embryonic wax was observed as a liquefied moiety that oozed from the cutin (Fig. 5B) and formed a thin film on the surface. Determining a time line of events was impossible, but within a day after fertilisation the fruitlet began to expand, while the wax film seemed to become segmented (Fig. 5C) and the cutin thickened (Fig. 5D). The segment artefact arose from the shrinking of the material, the borders of each segment representing a tangential wall of an epidermal cell. Segmentation became more prominent in fruit > 4 - 5 mm (Fig. 5E). In a cross-sectional fracture, the thickness of the wax layer exceeded that of the cutin and had a longitudinal striated appearance (Fig. 5F).

The stomata of fruit > 3 mm begin to open (Fig. 6A), surrounded by a pliable, and segmented wax film (Fig. 6B). Within a further 2 mm diameter increase, the formation of a new wax fraction was exposed in aldehyde fixed material (Fig. 6C). Due to the removal of the soluble fraction, the embryonic wax layer tends to lift from the surface as a crust. Once segmented, it easily became detached from the cutin during processing. In (D) of Figure 6, granular to globular crystalloids, were observed on raised prominences. Shrinkage of the epidermal cells caused the tangential cell walls to appear in this fashion, giving evidence to the apoplastic transport of the wax precursors to the surface (Kunst & Samuels, 2003). These crystalloids rapidly aggregated, forming an apparently second layer of wax underneath the embryonic wax (Fig. 6E). The presence of amorphous, inter cell wall and cuticular wax (Fig. 6F) was not reflected in the supra-cuticular wax, which appeared as irregular threads after removal of the soluble fraction (Fig. 6F). Once again, this was an artefact, demonstrated by the flaking effect of the wax detaching from the surface in (E), but more so in embryonic wax film segments (Fig. 6G). These became separated due to surface expansion of the growing fruit, forming radial dendrites originating from each segment, similar to cogs of a gear. The resultant space was filled by (at this stage) disorientated platelets that also encroached on the dendritic structures. The dendritic appearance of the laterally separated segments resulted from longitudinal depositions in the now proper wax layer (Fig. 6H).

Aldehyde fixed material of > 10 mm became very prone to disruption of surface structures (Fig. 7A). The irregular margins of the detached segments became the template for the formation of the dendritic structures (Fig. 7B). The disrupted wax film was useful in the determination that the small film segments each became the focus for the development of the radial dendrites, resembling overturned cookie cups. The central area where the

embryonic wax segment was attached seemed free of platelets, with the zones where dendrites have developed containing scattered deposits. The perimeter of each segment enlarged significantly through growth of the dendrites (Fig. 7C), and the total structure increased in height. (Fig. 7D). The wax layer and cutin layer thicknesses were now equivalent, exceeding the cell wall thickness. These structures were also described by Bally (1999). During expansion of the fruit surface, most 'cookie cups' became fractured, indicating the inelasticity of the embryonic film remnants (Fig. 7E). Longitudinal wax deposition still contributed to the increase in thickness, and continued to be structurally simple (Fig. 7F). In contrast, platelets became organised in parallel rows. These were organised into stacked concentric rings around each 'cookie cup', and furrows demarcating the opposing directions of expansion. The result resembled fissured layers (Fig. 7G). The development of a bi-layered epicuticular wax is not true syntopism (Barthlott *et al*., 1998), but rather the sequential appearance of structures from which the multilayered epicuticular wax of mango fruit is finally composed. Similar multilayered epicuticular wax structures were described by Jetter *et al*. (2000) in *Prunus laurocerasus* L. At fruit size > 12 mm the cutin layer (3 - 5 µm), exceeds both the cell wall and wax layer thickness. Up to this point, the epidermal cells were arranged in a very regular layer of columnar cells, (4 - 5 µm periclinal width and 10 - 12 µm anticlinal height) (Fig. 7H).

Between 10 - 25 mm the fruit surface seemed to be covered in 'fissured' layers, but this appearance represented another transitional stage in the development of the epicuticular wax (Fig. 8A). The fissured appearance was only superficial; not affecting the cutin underneath (Fig. 8B); it is better described as furrows. The appearance of this transitional layer was caused by opposing bidirectional wax growth (deposition of amorphous longitudinal wax, lateral deposition of platelets) with sequential rows of platelets produced from, but never filling the furrowed zone. No cracks in the cutin could be observed in any of the cross-sections studied for the three cultivars concerned, although Bally (1999) did describe such for 'Kensington'. It is felt that these were probably similar to the 'fissures' described above. This has implications for the ideas put forward regarding mango skin browning, and may require further investigation.

Although the apparent fissures did not affect the cutin, rapid surface expansion due to volumetric increase of the pulp began to push the epidermal cells out of its previously organised arrangement on top of the parenchyma/mesophyll. An uneven developing cutin layer was the first indication of the gross changes and displacement that the epicuticular layers would undergo (Fig. 8B, D & F). Externally, pyramidal wax plates began to manifest, with pronounced parallel alignment of platelets emphasising the angularity of

each pyramidal structure (Fig. 8C & E). In all probability, the suture-like links between these plates as observed by O'Hare *et al*. (1999) were a pattern due to depositions of wax crystalloids that formed a barrier to protect the rapidly expanding cutin (Fig. 8A, C, & E).

Remnants of the embryonic wax film persisted on the layers developing underneath, and continued to break apart during surface expansion (Fig. 9A). The rate of growth and expansion was not 100 % equally in all directions at all times, giving the effect of lopsidedness in such cases. In (A) the newly exposed facets of the pyramidal structures were still incompletely covered in platelets, again creating an impression of surface cracking. The cross-section in (B) was cut instead of fractured, which resulted in smearing of the abundant cuticular wax. Nevertheless, penetration of the cutin to fill the space created by the displacement of the epidermal cells, were evident. During lateral expansion, the pyramidal sections split open at almost right angles to the previous division, with newly opened sections consistently filled by deposition of platelets (Fig. 9C) that will fuse into sepaline crystalloids. The epidermal cells were continuously deformed and pushed out of alignment (Fig. 9D & F), with the liberated intercellular regions simultaneously blocked by cutin deposits. From fruit size > 60 - 70 mm no further developmental changes were observed other than continued expansion and filling of existing split sites (Fig. 9E). In an abaxial view of an isolated cutin membrane, the cell boundaries were demarcated by convoluted cutin (Fig. 9G), corresponding to the protrusions of cutin observed in Figure 9 H.

The compelling relationship between cutin and wax were demonstrated by fruit with superficial damage caused by thrips (Grové & Pringle, 2000). These microscopically small insects rasp away at the surface of young fruitlets, damaging or destroying the cutin and epidermal cells, penetrating as deep as the mesophyll. The plant counters this attack by signalling living cells to deposit cutin. Thrip damage often extended several cell layers into the exodermis (*sensu* Esau, 1977), as was demonstrated in material depicted in A & B (Fig. 10). The dynamic nature of epicuticular wax became clear in (C), where even remnants of cell walls (underneath which cutin has been deposited) were covered in epicuticular wax. Several of the abnormal cutin surfaces enclosing single mesophyll cells several layers below the defunct epidermal layer were also covered in epicuticular wax (Fig. 10D). The interface of two opposing layers of cutin formed in this way was observed filled with wax crystalloids (Fig. 10E). These have most probably originated from within the enclosed mesophyll cells, since the apoplastic transport route was severely damaged. The signal and mechanism for transport of precursor wax is still unclear (Kunst &

Samuels, 2003), but these findings give support to the idea that it is a genetically programmed event actively regulated by plant cells.

These dynamics were further illustrated by the spontaneous self-assembly of lamellar crystalloids on the exposed surfaces of material cross-sectioned by snapping it while immersed in liquid nitrogen (Fig. 11A). Although structurally different from the amorphous bottom layer and the sepaline crystalloids in the top layer, these lamellae originated in a similar fashion as the other two (ref. Fig. 4). Infrequently, small (10 µm) 'cookie cup'-shaped crusts were found on top of the sepaline wax crystalloids of mature fruit (Fig. 11B). These were probably created when accumulated air bubbles formed during respiration escaped from intercellular air passages filled with liquefied cuticular wax fractions, pushing the wax out. A cross-section of a 'fissure' in the wax layer (Fig. 11C) showed it not extending into the cutin. The cutin was heavily impregnated with cuticular wax, with the globular nature of the cutin demonstrated in figure 11D.

Newly deposited cutin that had not been impregnated with cuticular wax, were found in several samples (Fig. 12). These depositions indicated where active intercellular filling during growth took place (A). The localities indicated by number 1 - 7 correspond to Figures B - H. The first locality was at the transitional zone between the old and newly deposited cutin (B, 1). To the left of the micrograph the old wax is almost completely covered by a dense, viscous layer. A single cutin globule on the right is partially covered, with small subunits of cutin beneath it. A five times higher magnification showed the wax flow in the process of covering the cutin, leaving open areas with no observed wax (C, 2). This was in contrast to the section 3, illustrated by (D, 3), where the new cutin globules and interstitial space was covered more completely. Despite even better coverage in figure 12 E (4), the presence of some holes and pits make the cutin appear more porous. The variably sized pores observed (F, 5) were most likely due to inconsistent and uneven flow when the cutin globules were enfolded with cuticular wax. Scrutiny of a section of older cutin (G, 6) revealed similar pores and openings. At very high magnification and available resolution of the new cutin, no other structural inclusions could be illustrated (H, 7).

Cutin was not only important as the reservoir for cuticular wax, but also determined the external development of morphological epicuticular detail. Bally *et al*. (1996) and Bally (1999) indicated the existence of plate-like structures from which the cutin of mango is compiled. With the information gathered by means of both the normal SEM and FE-SEM we were able to enhance this observation. What appeared as definite sets of

architecturally defined ridged plates forming the base for the pyramidal wax blocks, were the footprints of laterally expanded cutin (Fig. 13A). The geometric wax arrangement therefore corresponds with the geometric cuticular development underneath. Epicuticular wax architecture is therefore dependant on the directional growth patterns of the cutin layer (Fig. 13B & C). The abaxial view of an isolated cuticular membrane (Fig. 13D) revealed that mitotic division is responsible for the formation of the adaxial architecture. The cutin layer itself thus forms a die onto which the wax plate shape is cast. No definite association between the organisation of epidermal and mesophyll cells, and the maxima and minima of the cutin plates could be established (Fig. 13E). Although some wax plates are clearly demarcated by a narrow furrow (H), no break in the cutin between the ridges and valleys could be indicated (Fig. 13G).

2.5 CONCLUSION

Fruit, a rich source of carbohydrates and phytochemicals (Plaami *et al*., 2002), are particularly vulnerable to predation and microbial attack. Protecting the developing embryo until it is ready to be disseminated is therefore important. Mango fruit is a good example of how survival measures against meteorological factors, decay and predators are combined in the epicuticular layers of the fructosphere. These survival measures are paramount to the physiological development of the fruit. Development of pesticide formulations and crop management strategies should therefore not only consider aspects such as meteorology, safety and cost (De Bie, 2004). The uniqueness of the fruit and its requirements for self-protection also needs to be taken into account, accommodating new morphogenetic adaptations in monoculture crops planted outside their natural habitat of evolutionary success.

Understanding the ontogeny and morphology of mango fruit wax more accurately will contribute to the improved management of mango production in South Africa. Epicuticular wax has been established as a self-protection mechanism in plants (Eigenbrode, 2004). For wax ontogeny, events described in this study are not successive in real time, but rather representative of a continuous process. However, collection and sample preparation terminates a very specific moment in the ontogenetic sequence, creating a visual representation of that moment. Interpreting the development or disappearance of the various structures on the fruit surface depends on this reality. Mango fruit wax development is complex from a very early stage, and static images obtained with microscopy must be interpreted to explain a continuous metabolic process. Accurate

judging of the chronological developmental ages of fruit increasing in size is difficult. This is due to the fact that time intervals of individual fruit seldomly match perfectly in terms of biochemistry and development. Fruit that appear to follow sequential increases in size may differ in developmental stage, and therefore the information gathered has inconsistencies that make interpretation of observed features and dynamic changes difficult. This problem was overcome by the large number of samples collected and viewed during the very early stages of fruit development.

A common complaint during preharvest pesticide spraying is the seemingly periodic low wettability of the fruit surfaces. The pronounced angularity of epicuticular wax plates during certain stages of fruit development could present the droplets with too little surface for successful adhesion.

Using different methods in parallel was invaluable in understanding and compiling the chronology of ontogenetic events. The soluble fraction that is damaged during aldehyde fixation is most likely a sugar, or a glycosidically bound terpene or phenolic compound with possible toxic properties. Support for this idea is the observation that thrips only utilise fruit smaller than 8 - 10mm as a food source (Grové *et al*., 1999). The contribution of increasing cuticular wax fractions to thrip deterrence has to be investigated by non-destructive chemical analysis to prevent leaching of cellular content and resin. Cutin can not form part of the nutritional base during feeding, since it is known as an indigestible, inert biopolymer (Jeffree, 1986) and rapidly increases in paradermal dimensions during fruit development.

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2.7 FIGURE CAPTIONS

Figure 1 Wax morphology and ontogeny of the three cultivars studied, with similar typical surface morphology for 'Tommy Atkins' (A), 'Kent' (B) and 'Keitt' (C). Cross-section of frozen material showing bi-layered epicuticular wax (D).

Figure 2 Very young material sections prepared by plunge freezing (A) and aldehyde fixation/CPD (B), showing the degree of shrinkage. Slightly disrupted embryonic epicuticular wax layer present on plunge frozen post-anthesis fruitlets (C). More extensive disruption of the aldehyde fixed material (D). Soluble material present on the surface were retained by plunge freezing (E), with flaking wax due to the compromised composition following aldehyde fixing (F). Cell shape and size of plunge frozen material reflected that of the fresh condition (G). Artefacts created by aldehyde fixation, followed by critical point drying, in young plant material with fragile or unsupported contents (H).

Figure 3 Dendritic wax projections are overlaid with lamellar platelet moieties (A), forming concentric patterns (B). Concentric rings demarcate the horizontal growth of the epicuticular wax (C), at the same time enlarging the initial focus in a 'cookie cup' shape (D).

Figure 4 Wax structures from anthesis (ovaries 3 - 5 mm) to physiologically mature fruit. Globular to granular crystalloids (A). Extruded wax initials forming nematoid crystalloids (B). Clumping of nematoid crystalloids in localities of origin (C). Nematoid and thread-like crystalloids aggregate (D). Fusion of nematoid crystalloids creates ribbon-like structures with entire margins (E). Ribbon-like structures fuse into sepaline crystalloids present on physiologically mature fruit, playing a role in light reflection (F & G). Lamellar wax crystalloids formed on exposed cutin after fracturing of plunge frozen material (H).

Figure 5 Micrographs of the epicuticular surface structures of post-anthesis fruitlets. Thin, smooth, amorphous wax film on periclinal epidermal cell walls (A). The primary cell wall (large arrow) appeared 3 times thicker than the layer that consisted of cutin integrated with embryonic wax (small arrow) (B). Fruitlets, only hours older, had a more substantial wax layer that became

more visible due to shrinkage (C). Older fruitlets also had a better developed cutin layer (D). Segmentation of the embryonic wax film took place on the cell wall boundaries (E). Rapid increase in wax thickness began by longitudinal deposition of an amorphous wax layer (F).

Figure 6 Chronological epicuticular development of the surfaces of post-anthesis mango fruitlets (4 - 10 µm diameter). Stomata, surrounded by an amorphous wax film, beginning to open (A). Pliable appearance of wax film dividing into segments (B). Compromised embryonic wax due to removal of soluble fraction after fixation in aldehyde (C). Wax initials on the raised cell wall sections underneath were made visible by disruption of the wax film (D). Secondary wax deposition assembled into complex structures (E). Impregnation of both the cell wall and cutin by cuticular wax revealed by removal of soluble fraction (F). Plunge frozen, freeze dried material showed the secondary wax deposition integrating with the embryonic wax film (G). Enlarging fruit (6- 8 mm), with cutin growing at a higher rate than that of the wax layer, overtaking it in physical dimensions (H).

Figure 7 Surface structures of fruit exceeding 15 mm in size. Disrupted segments of embryonic wax film revealed platelets forming from beneath (A). The irregular margins of wax segments are the template for development of dendritic structures (B). Overturned 'cookie cup' shapes due to the dendritic extensions radiating from each segment (C). Structural height increase due to longitudinal wax deposition (D). Cracking of the 'cookie cups' signifying the inelasticity of the embryonic film remnants (E). Despite increase in thickness, longitudinal wax structures remained simple (F). Fruit surface with furrows in the wax, giving the impression of fissured layers (G). Cutin thickness of fruit > 12 - 15 mm was up to five times thicker than the wax layer (H).

Figure 8 Surface structures of fruit sizes between 10 - 25 mm. A 'fissure' representing the boundary between two opposing directions of deposition in platelet formation (A). Increasingly irregular cutin layer (8 - 10 µm thick) in cross-sectional views, with distorted epidermal cells (B). Manifestation of pyramidal wax plates (C). Development of complex cutin architecture followed the distortion of the epidermal cells (D). Deposition of wax crystalloids that formed a barrier to protect the rapidly expanding cutin (E).

Increased thickness of epicuticular wax during displacement of epidermal cells (F).

Figure 9 Micrographs of continued surface expansion. Newly opened facets of the pyramidal structures were not as abundantly filled with platelets as those from earlier divisions (A). Cutin deposition was not limited to the apical sides of cells (B). Sequential tearing and splitting of embryonic wax remnants happened at almost right angles (C). Deformed and misaligned epidermal cells with a haphazard appearance (D). Filling of laterally expanded zones and epicuticular changes were visible in fruit sizes > 60 - 70 mm (E). Cross-section profile of fruit > 70 mm with displaced, deformed epidermal cells, a cutin thickness exceeding 10 µm, and a 1 - 2 µm thick wax layer (F). Abaxial view of isolated cuticular membrane section, illustrating the convoluted cutin layer, with no apparent cracks developing (G). Cutin protrusion corresponding to the cell wall convolutions (H).

Figure 10 Thrip damage to the superficial layers of mango fruit was often observed as extending into several layers of exodermal cells, as illustrated by (A) and (B). Cell wall remnants covered by self assembling wax fractions (C). Individual mesophyll cells were often enclosed by cutin containing wax crystalloids (D). Interstitial space between two adjacent cutin layers, filled with lamellate wax crystalloids (E).

Figure 11 Surface structures of fruit close to physiological ripeness. Spontaneously self-assembled lamellar crystalloids on the exposed surfaces of cross-sectioned sample material (A). A ‘cookie cup’ structure developing on top of the parallel platelets due to internal gas build-up pushing liquid wax fractions outward (B). Cross-section of a deep fissure, with no indication of cutin cracks (C). Incomplete coalescence of cuticular wax left sections of cutin globules exposed (D).

Figure 12 (A) Micrograph of a newly formed cutin deposit not yet impregnated with cuticular wax. The localities indicated by numbers 1 - 7 correspond to Figures B - H. Transitional zone between cutin deposits of different ages (B). Several sections of cutin were observed to be apparently without wax impregnation (C). A section with more abundant wax still exhibited pores and holes in the cuticular wax layer (D). The proximity of older, established

cutin contributed to the abundance of wax impregnating marginal sections of the new deposit (E). Observed pores represented incomplete flow of constituent wax fractions (F). An older section of cutin had similar pores and openings (G). Despite varying pore sizes, the presence of tubules could not be illustrated (H).

Figure 13 Adaxial view of a cutin membrane from which all epicuticular wax had been removed (A). Ridged plates formed during lateral expansion of the cutin. The geometric cuticular development corresponded to the geometric wax arrangement (B) & (C). Isolate cutin membrane sections (D) verified the role of mitotic cell division in the establishment of the cutin architecture. The ridges and valleys of the cuticular plates could not be matched indisputably to the placement of subcutaneous cells (E). Narrow furrows demarcated some wax plates (F), but no cuticular breaks or cracks could be illustrated (G).

2.8 FIGURES

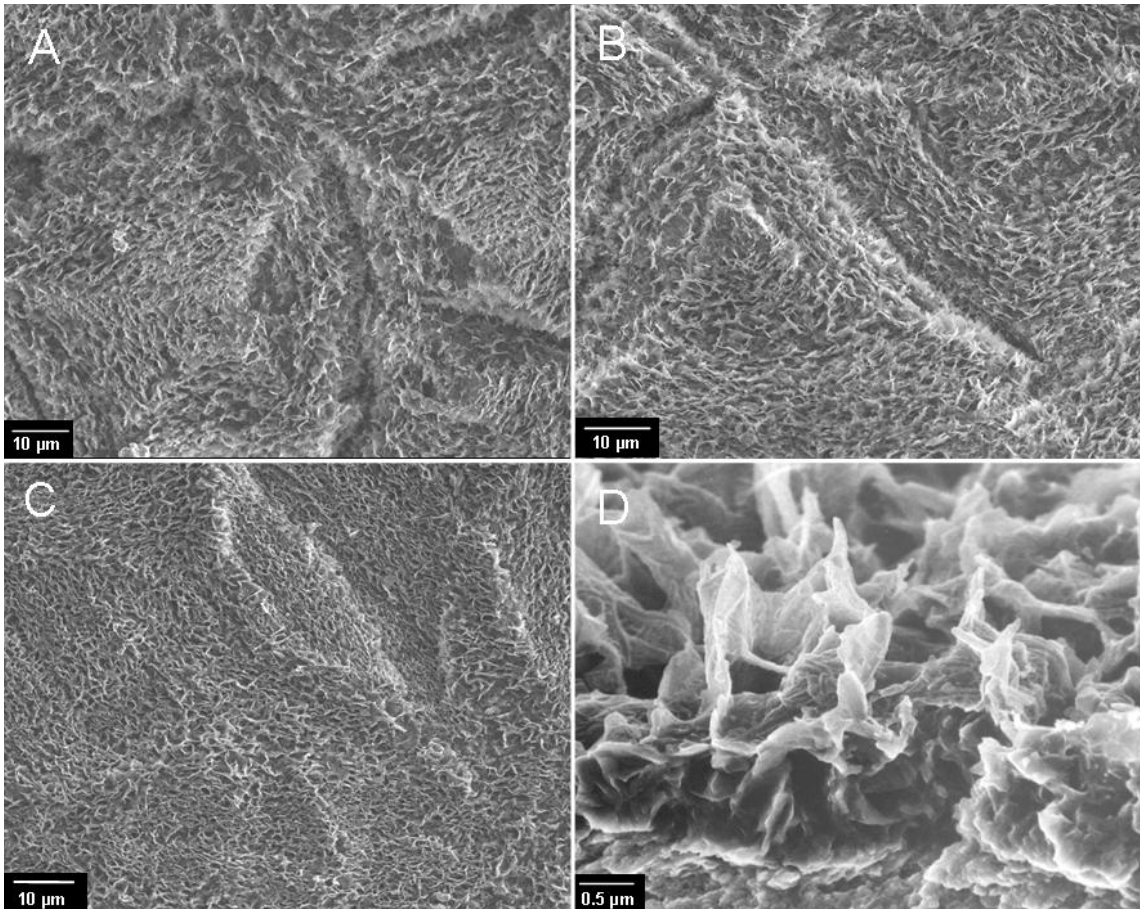


Figure 1

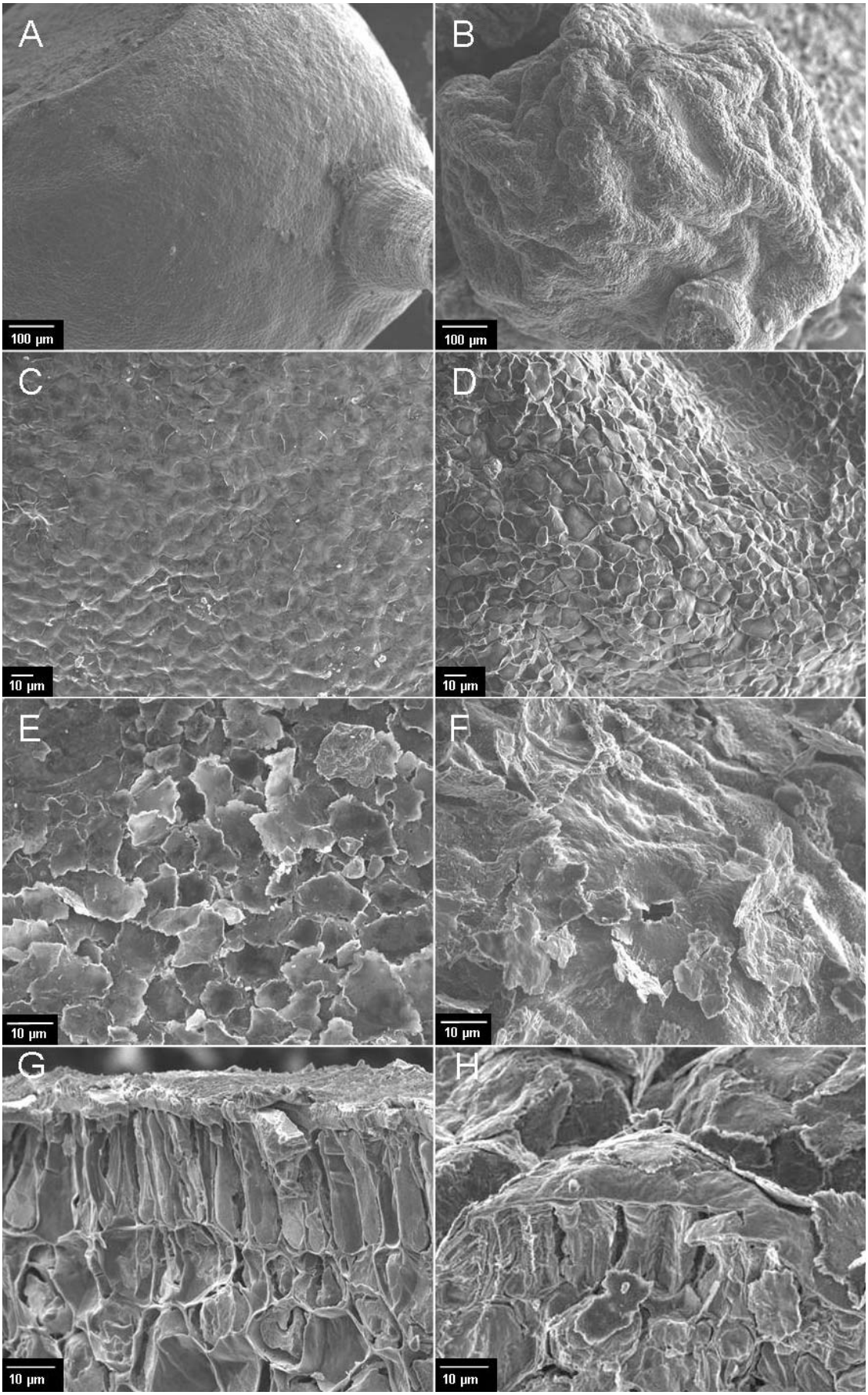
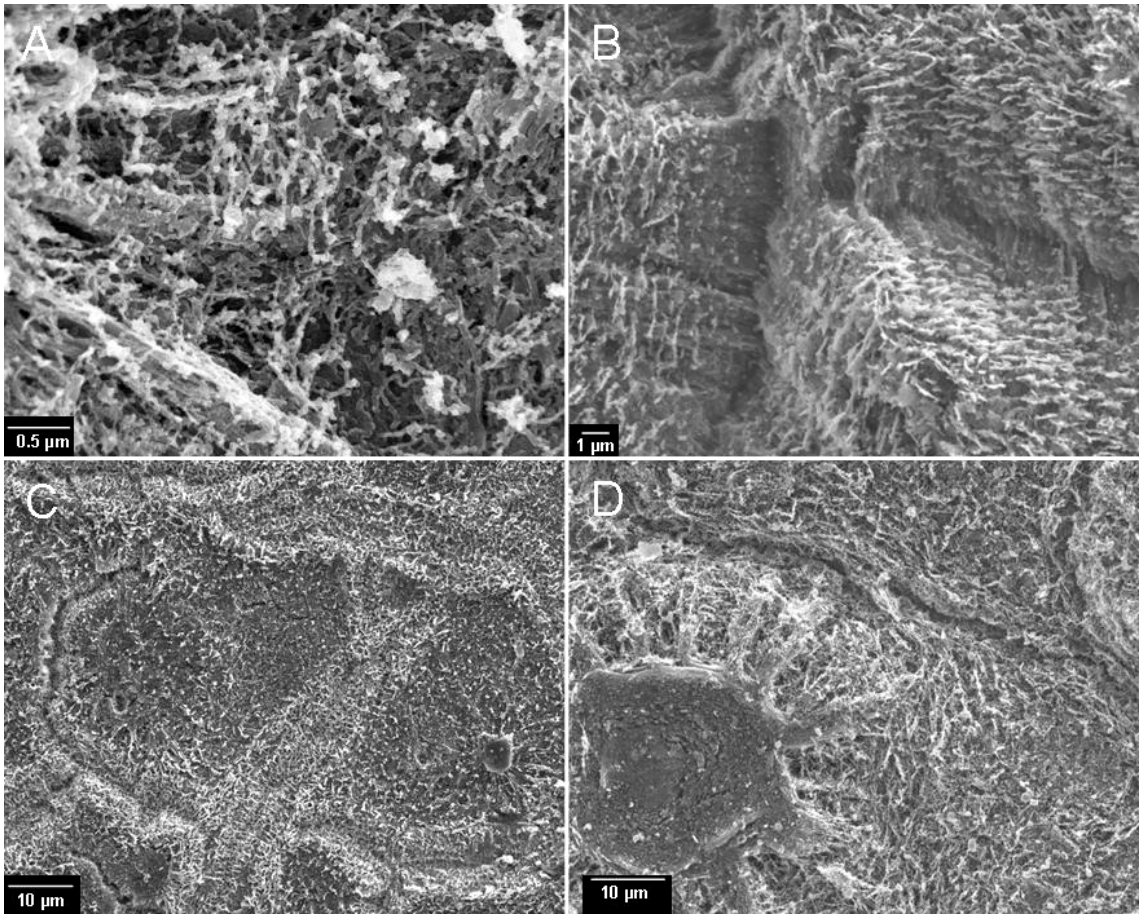


Figure 2



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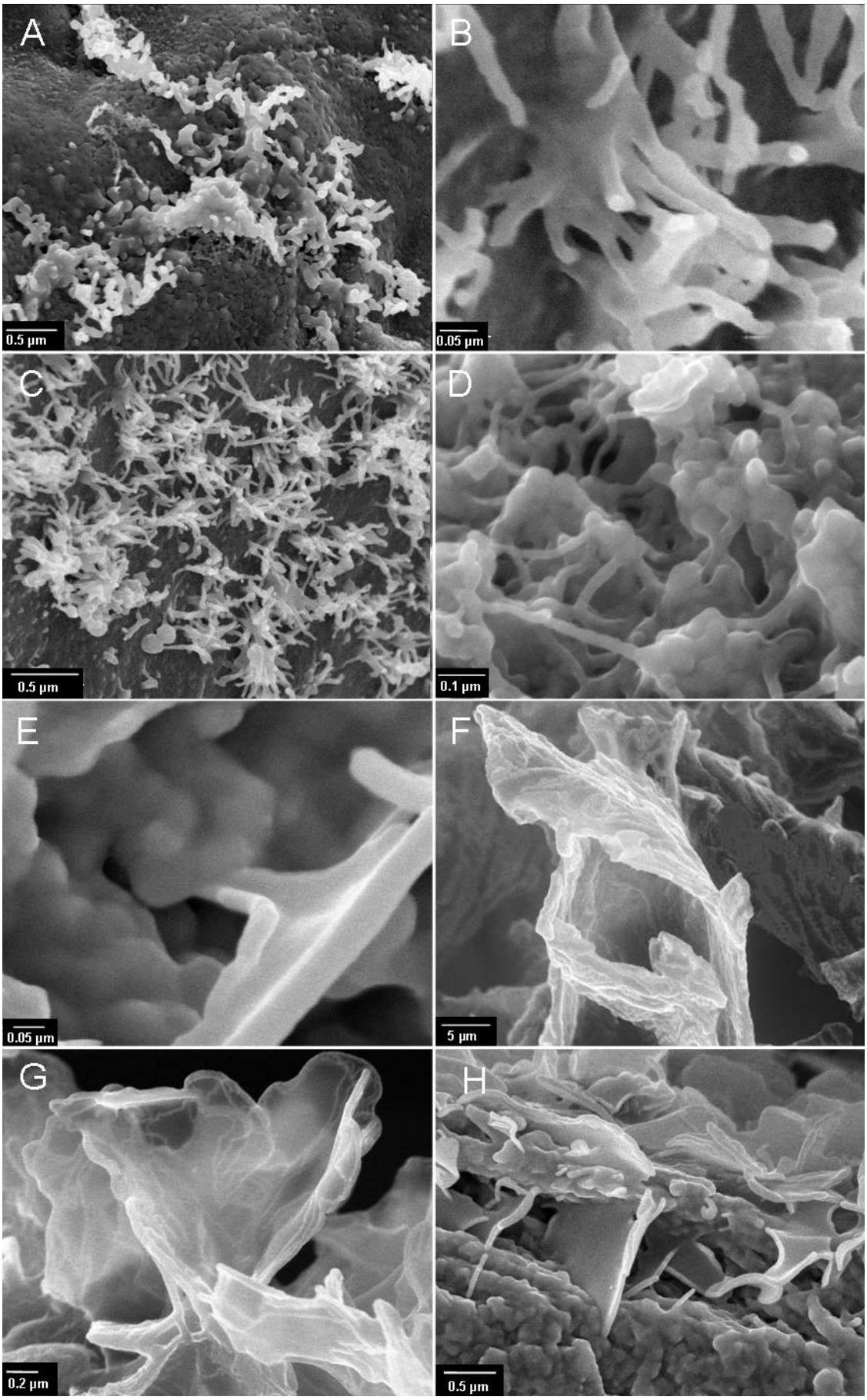

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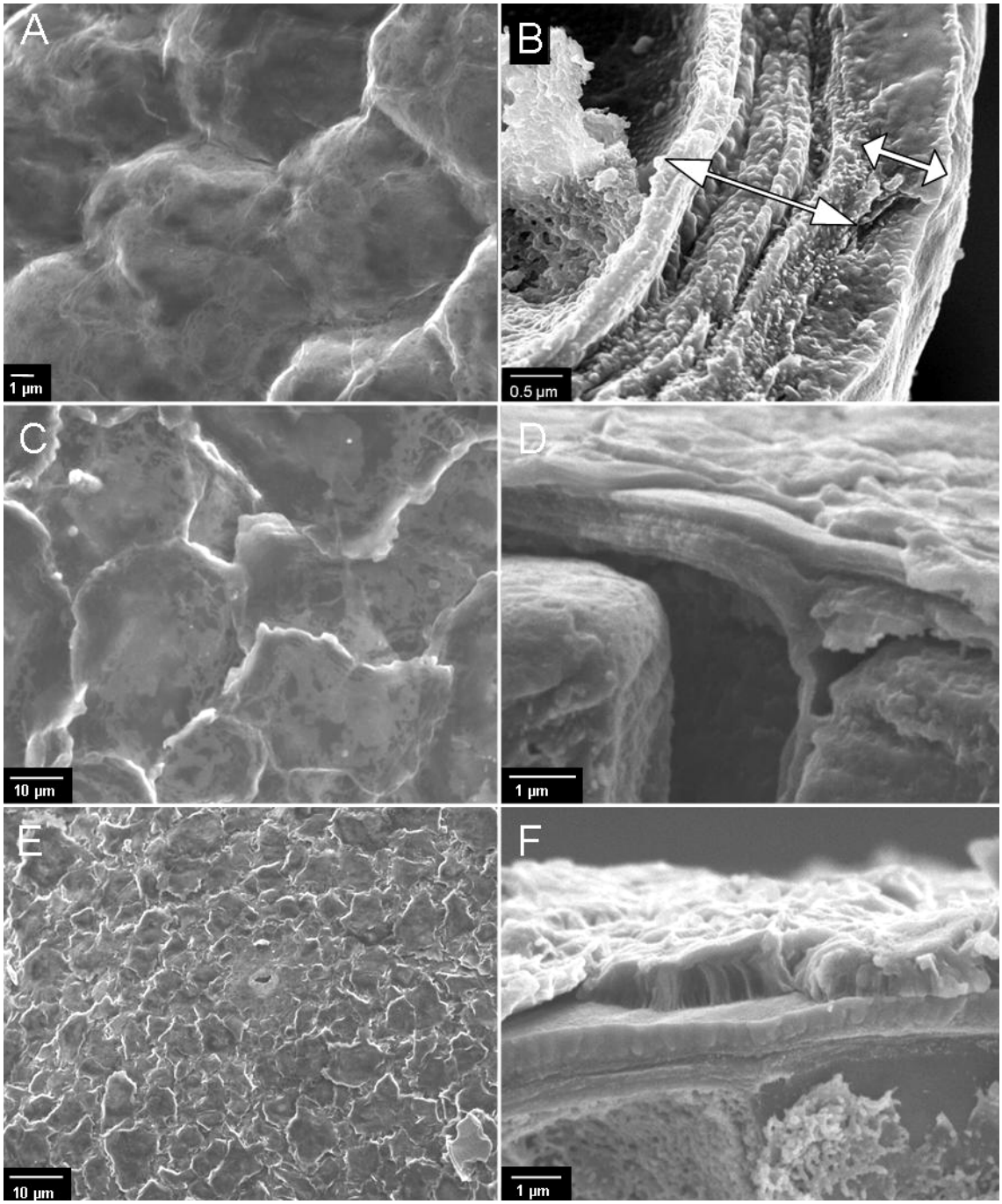

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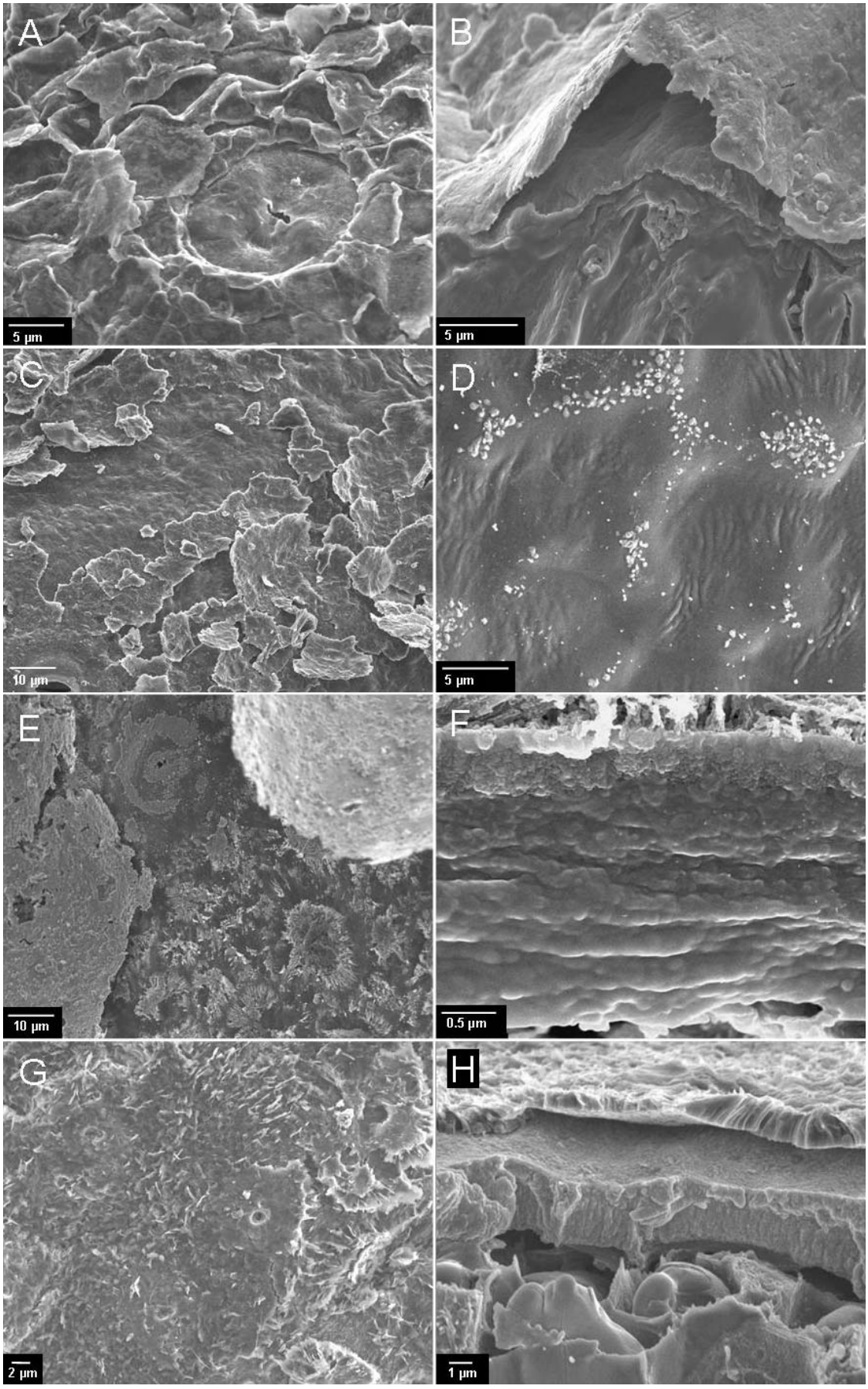

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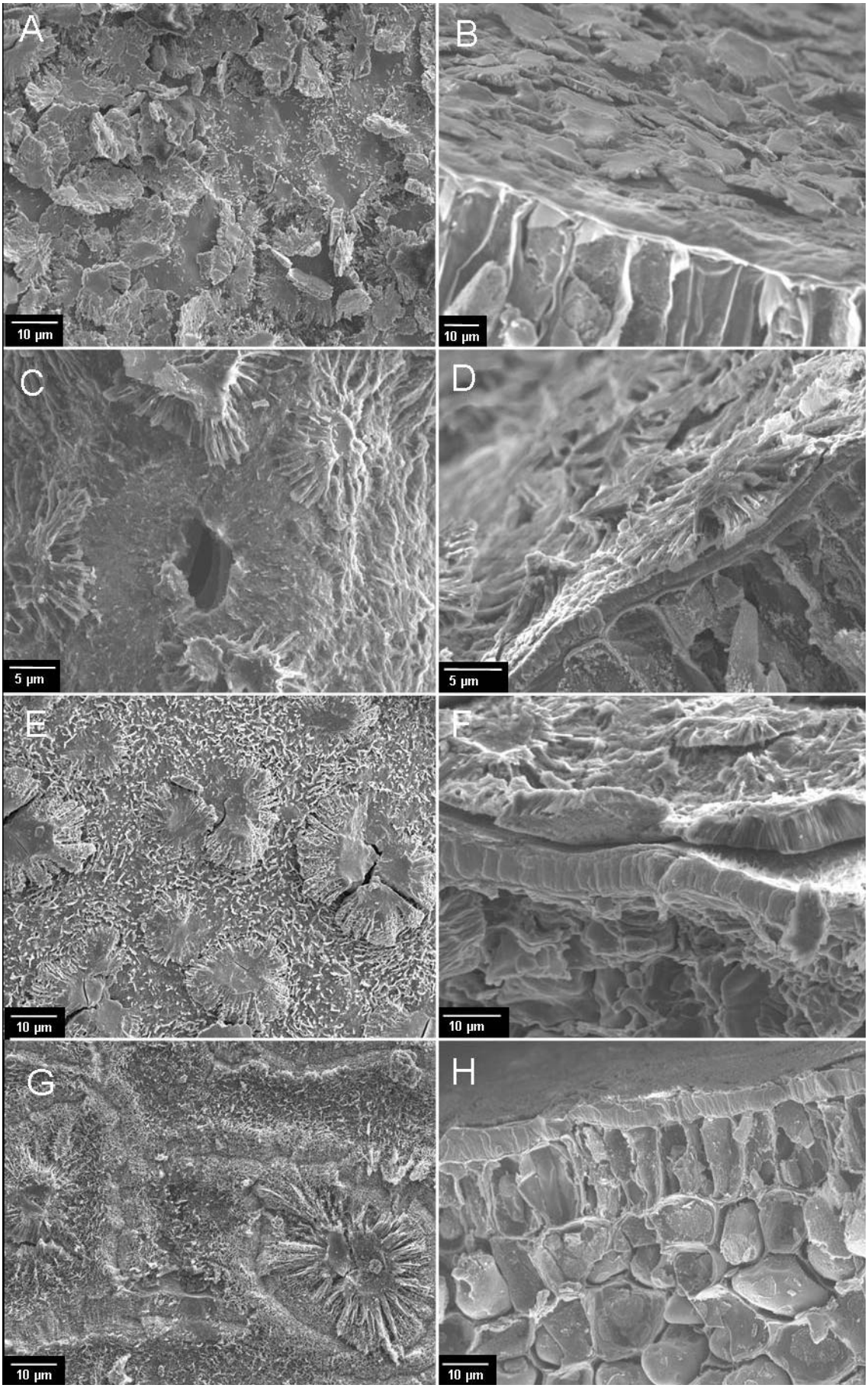

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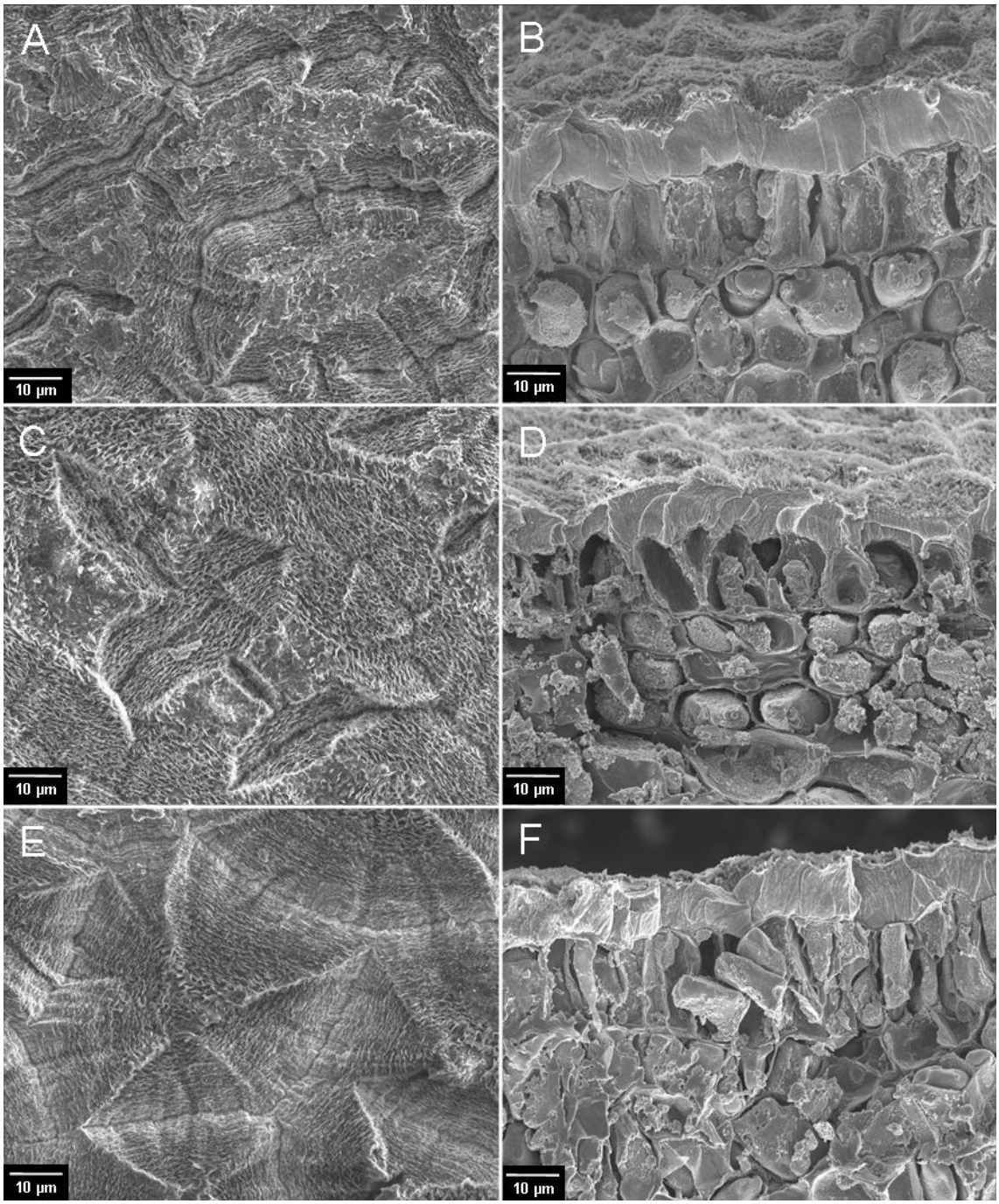

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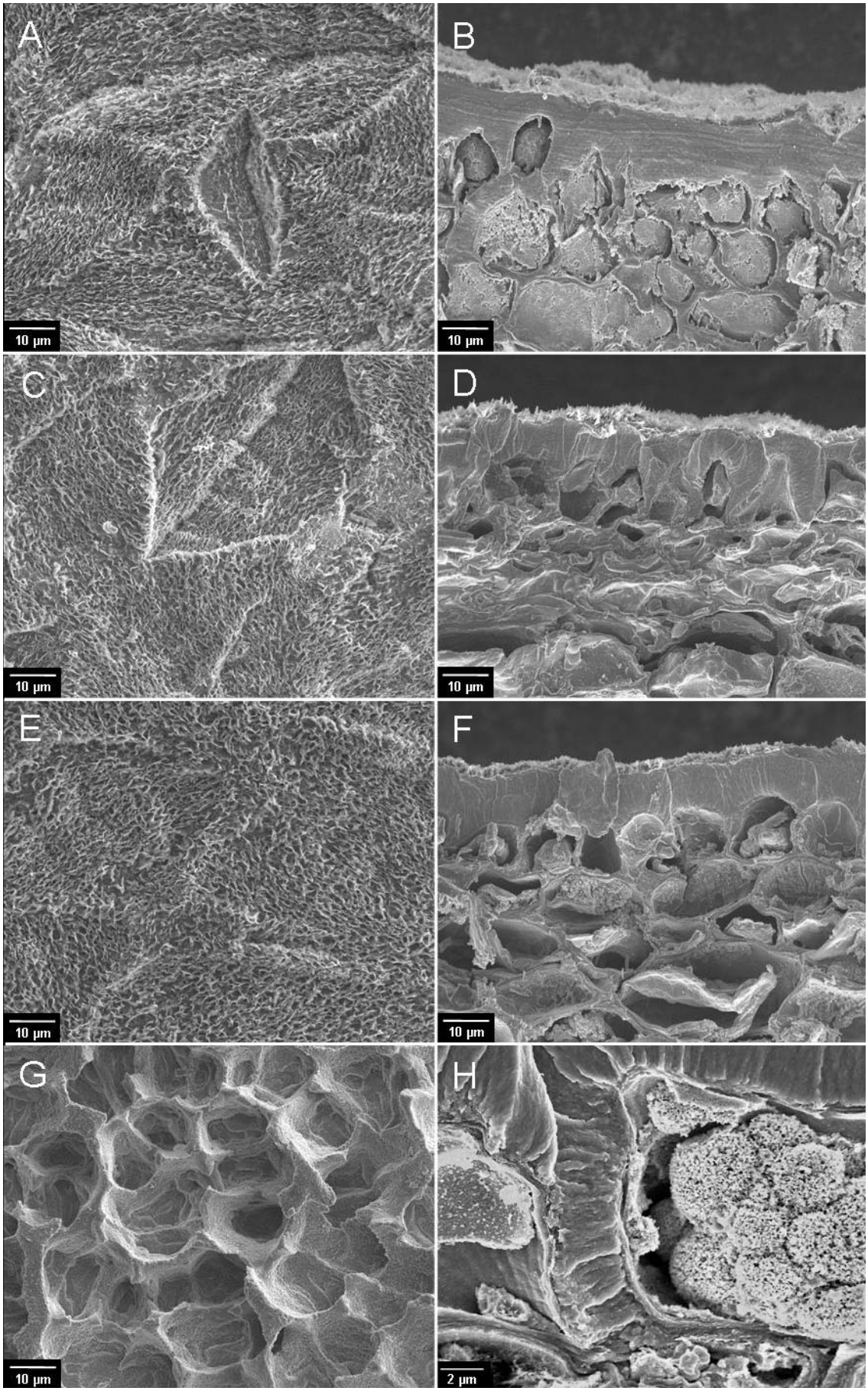

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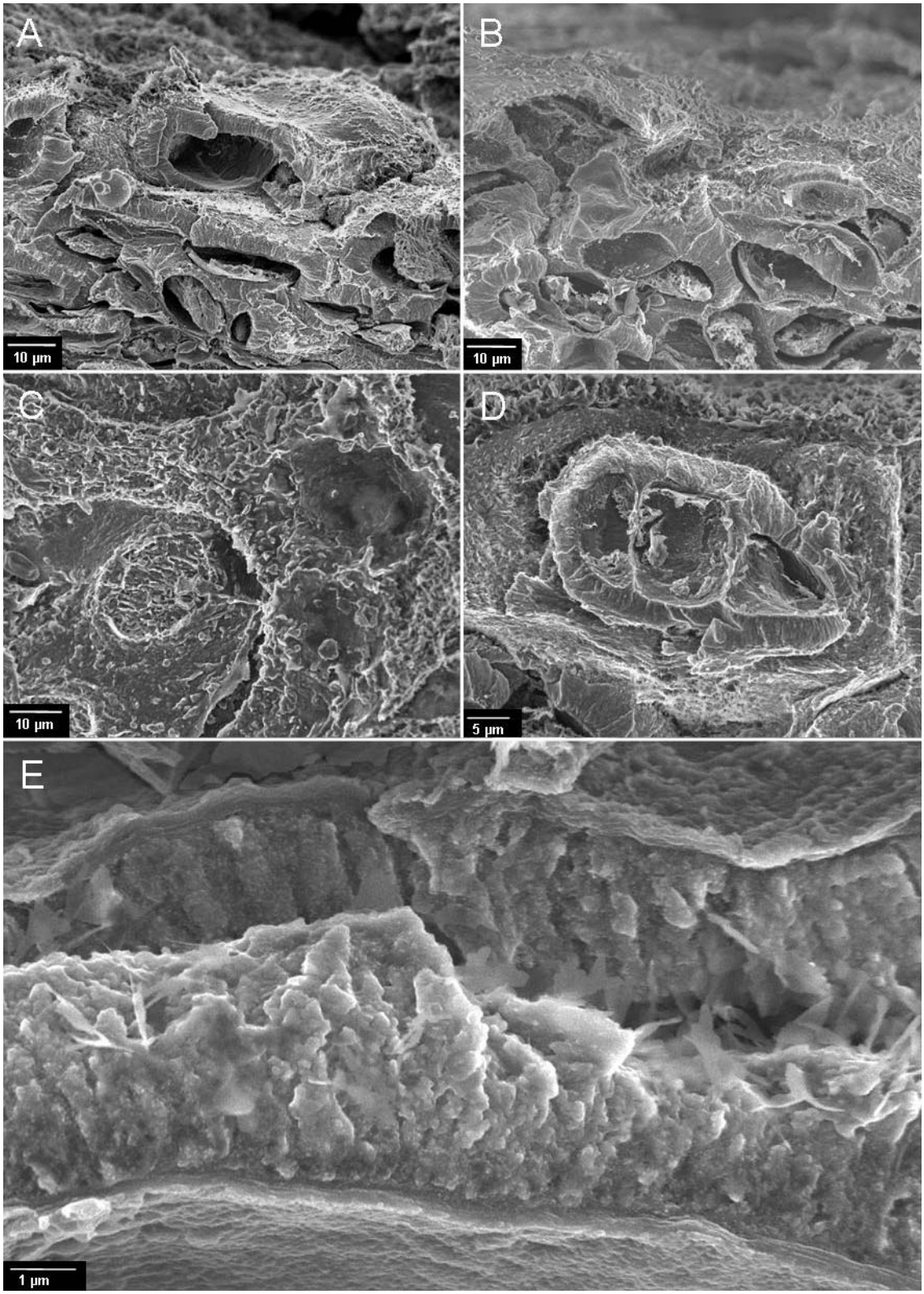
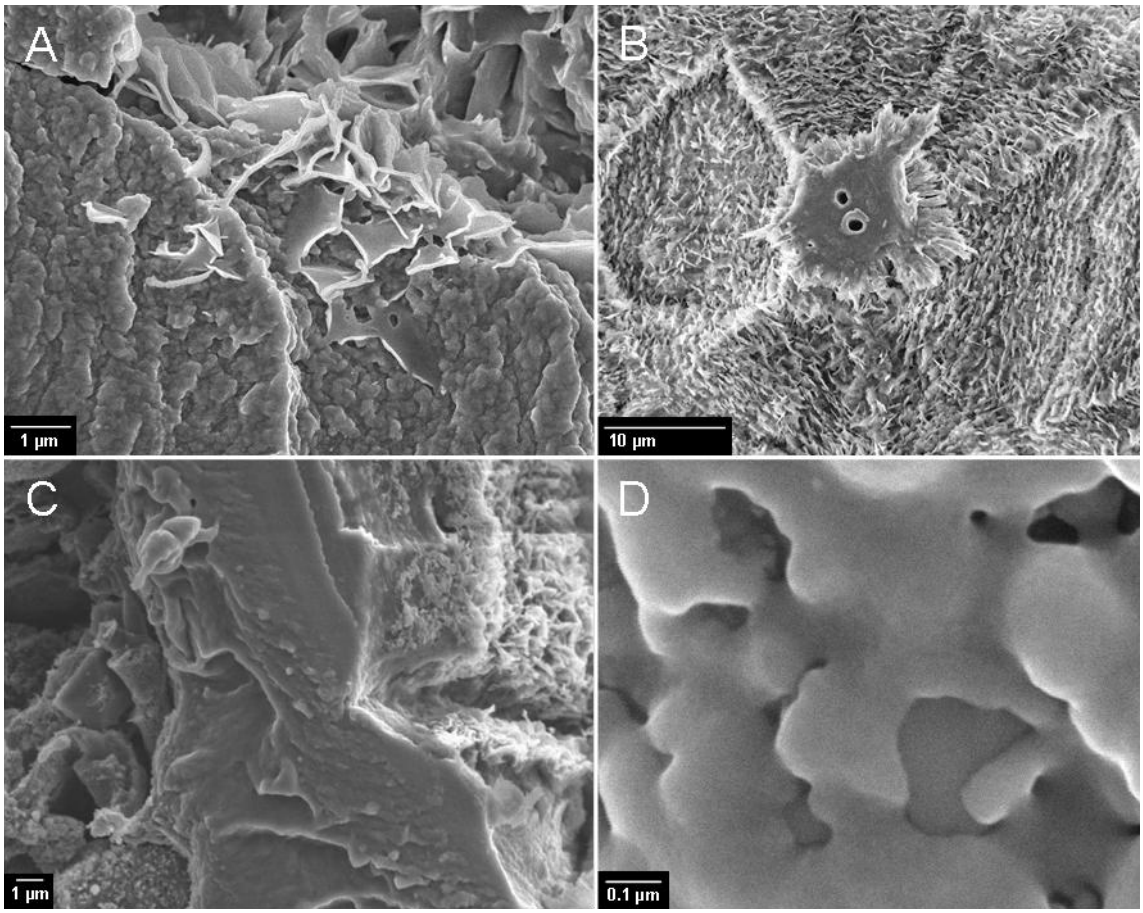


Figure 10



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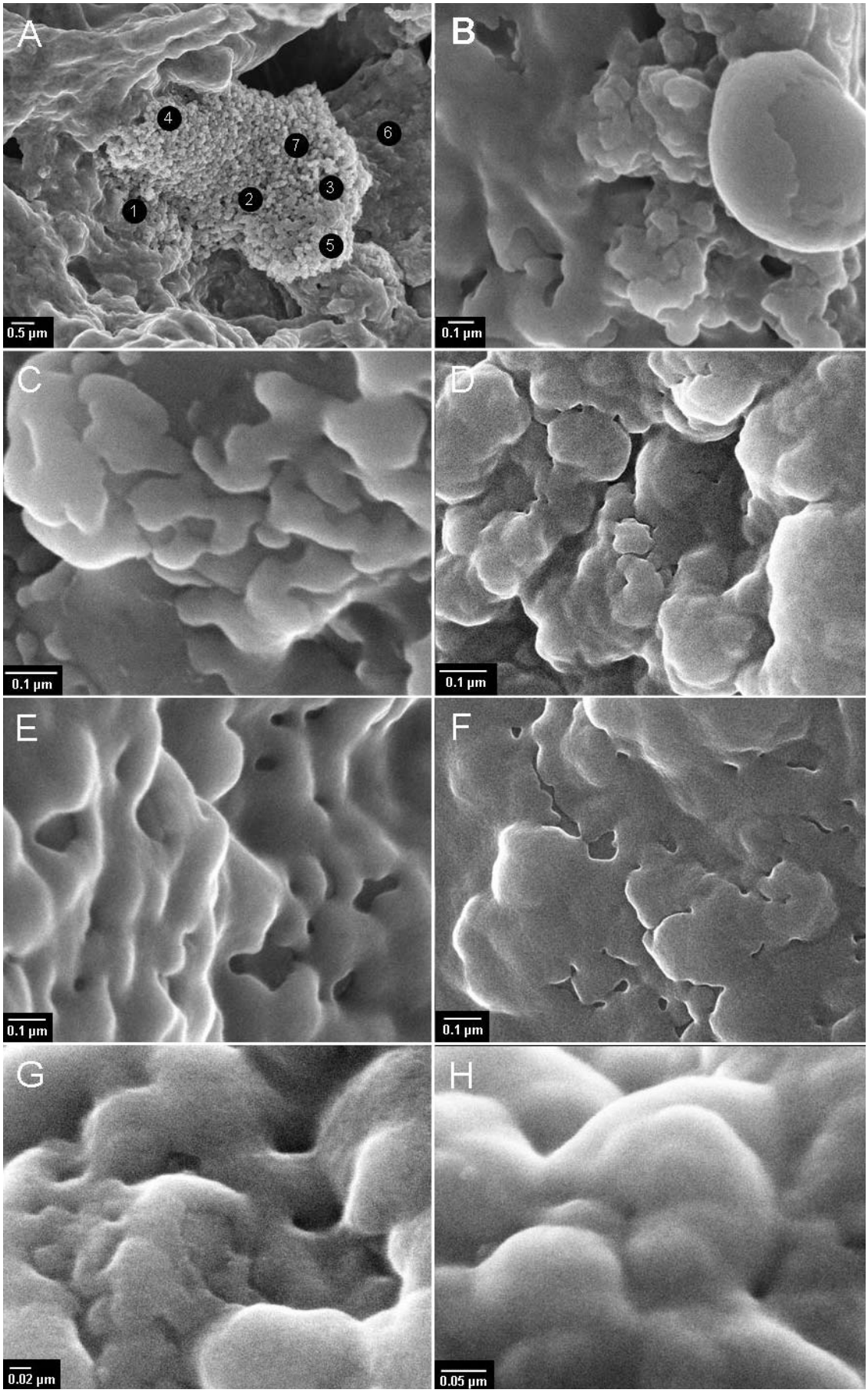

Figure 12

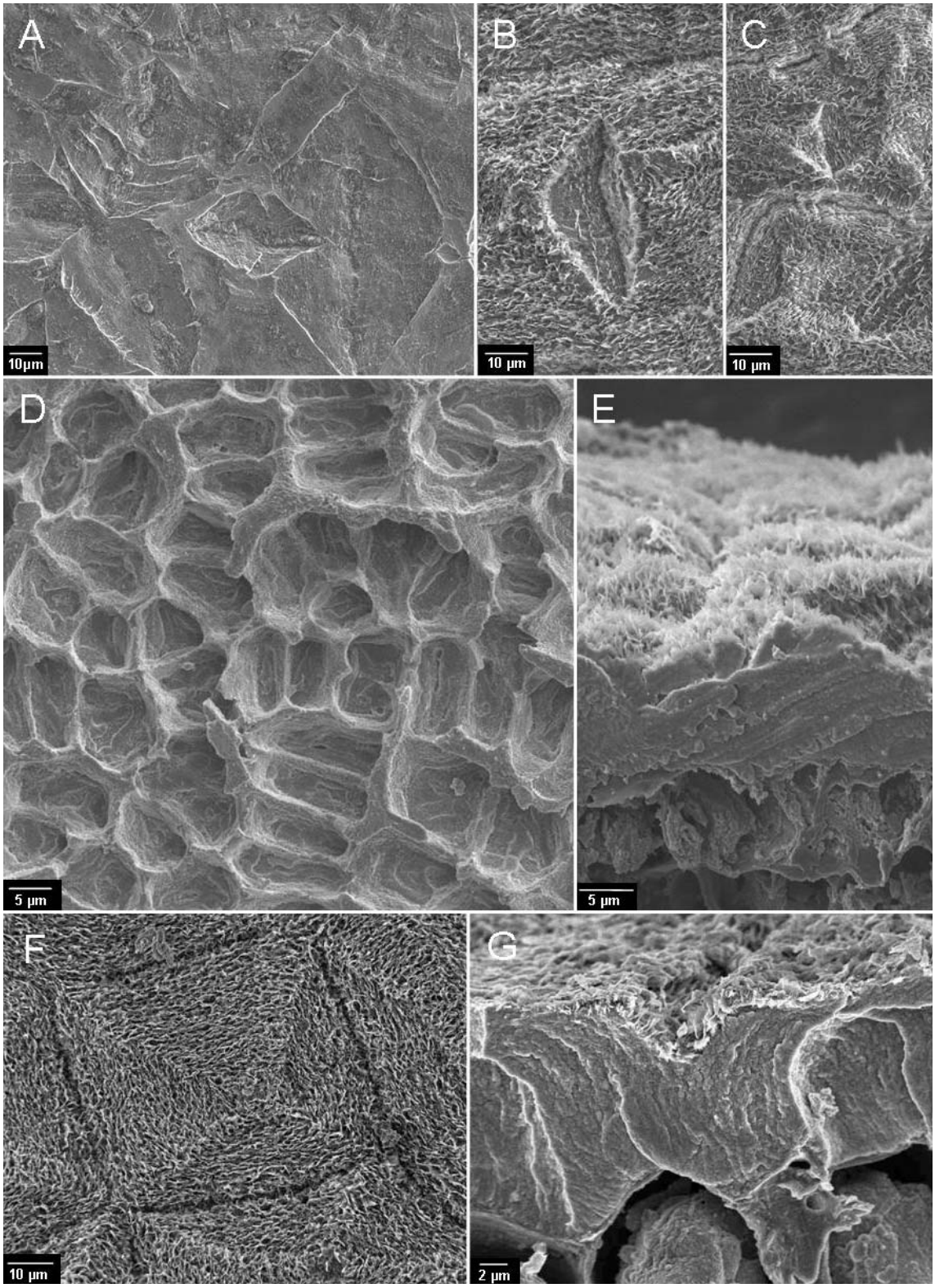

Figure 13

Some circumstantial evidence is very strong, as when you find a trout in the milk.

Henry David Thoreau (1817-1862)

American author, poet and philosopher

Chapter 3

MORPHOLOGY AND CULTIVAR SPECIFICITY OF MANGO (*Mangifera indica* L.) LENTICELS

3.1 ABSTRACT

Lenticel discolouration of mango (*Mangifera indica* L.) fruit annually causes financial loss to growers. Cultivars 'Tommy Atkins', 'Kent' and 'Keitt' were investigated as part of a study into the inducement of this condition. Lenticels occur abundantly on the surface of mango fruit, and are important in regulating temperature and transpiration. They originate from stomata and differentiate as the fruit develop and mature. It was found that the morphology of the lenticels from different cultivars differs in stomate width, lumen depth and abundance of epicuticular wax. Mango lenticels are lined with cutin, and do not have any underlying meristematic tissue. An intra-lenticular layer of wax accompanies the cuticular membrane, with its abundance and complexity distinctive for each cultivar investigated. Sufficiently different morphologies were found between the studied cultivars for lenticels to be considered as a variable in cultivar susceptibility to the development of lenticel discolouration. Although discolouration of lenticels are quite visible with the naked eye and light microscopy, no discernable differences between affected and non-discoloured lenticels could be identified by scanning electron microscopy.

3.2 INTRODUCTION

Lenticels are superficial structures facilitating gaseous exchange between internal plant tissue and the environment, and are found on aerial parts of members from many plant families (Stern, 1994). They usually appear as raised or blistered, corky pores due to suberised tissue and are associated with the woody bark of perennial plants, located in the periderm of trunks, twigs and stems. Descriptions of lenticel morphology are limited to a classic model (Fig. 1) on which textbooks base their discussion of this structure. According to this classic model, a typical lenticel is stratified and consists of epidermal

cells, phellogen (cork cambium) that lines the substomatal chamber, and specialised complementary tissue (phellem and intercellular spaces) (Esau, 1977; Stern, 1994). The growth and development of stem lenticels are marked by meristem activity that initially increases the layers of non-suberised tissue closest to the phellogen. These new layers pushes outward, breaking the barrier tissue from previous growth seasons apart, and becoming suberised later during the same season. During this secondary process of suberisation, the tissue that was pushed into the newly exposed fissure, seals off the nutrient rich, living tissue inside from the atmosphere outside, while the size of the lenticel opening to the atmosphere is expanded (Guirguis *et al*., 1995; Batzli & Dawson, 1999; Rosner & Kartusch, 2003). Lenticels are therefore regarded as cork cambium derivatives. Phellem cells in the cortex are normally tightly packed, tangentially elongated and radially flattened. However, the eruptuous lenticels are differentiated from the rest of the cortex by rounded phellem cells and the resulting intercellular spaces.

Although the occurrence of lenticels on fruit such as pears, avocadoes, apples and mangoes are well documented, it is mostly in conjunction with physiological and pathogen related problems (O'Hare *et al*., 1999; Pesis *et al*., 2000; Amarante *et al*., 2001; Everett *et al*., 2001; Veraverbeke *et al*., 2003a & 2003b; Anonymous, 2004). The ontogeny and anatomy of the mango (*Mangifera indica* L.) fruit lenticels (cultivars 'Keitt' and 'Tommy Atkins') was described in a study by Bezuidenhout *et al*. (2005), confirming their stomatal origin. Other discussions on lenticels from various species are set around its value in identifying horticultural relationships (Guirguis *et al*., 1995; Rosner & Kartusch, 2003), as an indicator of flood stress (Larson, 1991; Kozlowski, 1997; Batzli & Dawson, 1999), and as a functional parameter in reforestation models (Vanclay *et al*., 1997). In each of these instances, the investigation focused on the hypertrophied growth and expression of physiological reactions, and anatomical appearance and frequency of stem and bark lenticels. As a structure functional in gaseous exchange, growth and expansion are important mechanisms in transpiration and temperature control (Kozlowski, 1997; Rosner & Kartusch, 2003). Temperature control is of utmost importance to mango fruit, of which surface temperatures can exceed 56 °C during fruit set and growth (Grové, 2004, pers. com.).

Although fruit up to 25 mm in length (point of peduncle attachment to style end) are dotted with stomata, lenticels of varying shapes and sizes rapidly develop from them (Bezuidenhout *et al*., 2005). Lenticels are a distinctive feature of mango fruit, but are also the cause of economic problems when a condition known as lenticel damage (Bezuidenhout *et al*., 2003; Du Plooy *et al*., 2003) or lenticel spotting (Bally *et al*., 1996)

develops. The condition manifests as red or blackened halos surrounding the lenticels. Discolouration is chronological in terms of colour development, with normal (non-discoloured) lenticels developing a red halo that will eventually turn black. Several investigations into the signalling for the development of the discolouration have been done, but no satisfactory explanation can be offered (Bally *et al*., 1996; O'Hare *et al*., 1999; Pesis *et al*., 2000). Prediction and management of the problem is therefore difficult and uncertain (Table 1). To complicate matters, cultivar susceptibility is highly significant in the development of the condition (Donkin & Oosthuyse, 1996). Based on differences in expression and severity of lenticel discolouration between cultivars, the purpose of this study was therefore to investigate the lenticel morphology of physiologically mature fruit from different cultivars, and to determine the possible connection between discolouration and fruit lenticel morphology.

3.3 MATERIALS AND METHODS

3.3.1 Plant material

Samples of physiologically mature mango fruit exhibiting lenticel discolouration (cultivars 'Tommy Atkins', 'Kent' and 'Keitt') was collected fortnightly from the packhouse of Bavaria Fruit Estates (Hoedspruit, Limpopo Province, South Africa) throughout the 2002/2003 and 2003/2004 seasons. Five fruit per cultivar and three sections per fruit was sampled, prepared and viewed during each collection. Dissections were done immediately upon and at the point of collection.

3.3.2 Methods

3.3.2.1 Microscopy

3.3.2.1.1 Electron Microscopy

Samples for both normal scanning electron microscopy (SEM) (JSM 840, JEOL, Tokyo, Japan) and field emission scanning electron microscopy (FE-SEM) (JSM 6000FE, JEOL, Tokyo, Japan) were dissected from mature mango fruit. For normal SEM, sections were viewed at 5 kV and a working distance of 12 mm, while for FE-SEM 5 -10 kV was used.

Samples were prepared using two parallel methods in order to exclude the interpretation of artefacts from preparation. According to the first method, sections were cut and fixed in a 1:1 mixture of 2.5 % glutaraldehyde and 2.5 % formaldehyde in 0.1 M $NaPO_4$ buffer (pH = 7.3 ± 0.05), postfixed with 1 % aqueous OsO_4, and dehydrated in an ethanol dilution series (30, 50, 70, 90 and 3 x 100 %). Sections were subjected to critical point drying

(Biorad E3000, Polaron, West Sussex, UK), before mounting on double-sided carbon tape on stubs, after which it was rendered conductive in the vapour of a 0.5 % RuO_4 solution (Van der Merwe & Peacock, 1999).

For the second method, small sections of fresh material were plunge-frozen in liquid propane at -180 °C, vacuum dried (Custom built, Tshwane University of Technology, Pretoria, South Africa) at -80 °C and 10^{-7} mBar for 72 hours. Finally, sample material was mounted on double-sided carbon tape on stubs and rendered conductive in the vapour of a 0.5 % RuO_4 solution.

3.3.2.1.2 Light Microscopy

Samples for light microscopy were dissected from physiologically mature fruit from all three cultivars and fixed in a mixture of 2.5 % glutaraldehyde and 2.5 % formaldehyde in a 0.1 M $NaPO_4$ buffer (pH = 7.3 ± 0.05). After standard rinsing and dehydration, the samples were embedded in L.R. White resin.

Thin sections (0.5 - 1.0 µm) of the embedded material were cut on a Reichert Ultracut E ultra microtome (Reichert AG. Vienna, Austria) and heat fixed (60 °C) to glass microscope slides. The material was stained with a 0.5 % aqueous solution of Toluidine Blue (O'Brien & McCully, 1981) while the microscope slides were still warm. Sections were viewed with a Zeiss Axiovert 200 microscope (Zeiss, Göttingen, Germany) fitted with a Nikon digital camera DXM1200 (Nikon Instech Co., Kanagawa, Japan) and the digital images captured with Nikon ACT-1 version 2.

3.3.2.2 Chemical retrieval of cuticular membranes

Cuticular membranes were obtained by enzymatically pretreating small sections of physiologically mature 'Tommy Atkins' and 'Keitt' fruit rind. To remove the subcuticular tissue, sections were submerged for 48 hours, using a 1:1 pectinase:cellulase solution (1mg/ml pectinase, 10mg/ml cellulase, 0.075 M $NaPO_4$ buffer at pH = 7.5 ± 0.05) (Peacock, 2000). After three rinses in distilled water, the sections were soaked in 78 % H_2SO_4 for a further 48 hours, followed by three rinses in distilled water. It was then air dried, mounted on double-sided carbon tape on stubs, made conductive with RuO_4 and viewed with SEM and FE-SEM.

Epicuticular wax was removed from air-dried cuticular membranes retrieved from 'Keitt' material by soaking small sections of previously prepared membranes in chloroform

($CHCl_3$) for 72 hours. It was then air dried, mounted on double-sided carbon tape on stubs, made conductive with RuO_4 and viewed with SEM.

3.4 RESULTS AND DISCUSSION

Although two different sample preparation protocols were followed, data comparison indicated negligible differences in results obtained with material from mature fruit. All micrographs used to discuss the results were obtained by plunge freezing.

General morphology of mango fruit lenticels does not follow the classic building plan of those found on stems and twigs (Fig. 1). Firstly, there is a total absence of the phellogen layer that ensures continuous growth and replacement of cells (Fig. 2A). The stomate chamber or lumen is cavernous and lined living cells that are exposed directly to the outer atmosphere. This is countered by a unique feature in the surface of the exposed cells covered with cutin and epicuticular wax (Fig. 2B). This cutin lining is extensive and completely envelops the central air space (Fig. 3). It also becomes part of the sub-epidermal tissue as a result of the enlargement of the fruit during which epidermal cells are pushed out of the embryonic layer order, and becomes deformed. In all cultivars, expansion during growth leads to tearing and cracking of the cuticular layers surrounding the fruit, because cell division in these layers has ceased long before the volumetric increase of the fruit has reached a maximum. Ridges and convolutions result as the cutin layer fills out the enlarging perimeter of epidermal tissue. Deposition of cutin also extends into the air spaces branching off from the lumen; evidence of hollow tubular structures forming a network beneath the cuticle proper was found (Fig. 4). This proves that the lenticels are linked to one another and share the fate of both environmentally and metabolically produced volatiles.

The wax fractions present in the lenticel lumen is another anomaly, since such an intimate relationship between cutin, cuticular wax and epicuticular wax crystalloids is not normally found in lenticels. The cuticular association and continuation of the epicuticular wax in the lumen is evident from Figure 5. Physical removal of the intra-lenticular wax is not a feasible option at this time, but the observable characteristics of morphology and quantity gave important indications of cultivar specific differences. In the early maturing cultivar ‘Tommy Atkins’, a rapidly diminishing layer of intra-lenticular wax crystalloids are seen on the inside of a chemically retrieved lenticel structure. This layer is also present in the other two cultivars. In ‘Keitt’ (a late maturing cultivar) it was found to have greater abundance

and density. It was the mid-season cultivar 'Kent', however, that had the highest abundance and greatest complexity of the intracuticular wax (Fig. 6). Although carried over to the inside surface from the epicuticular wax on the outside surface, the intra-lenticular wax is not necessarily chemically identical to the outer epicuticular wax, as indicated by Prinsloo *et al*. (2004).

Internally, the size and shape of the lumen varies greatly, while externally the size and shape of the lenticel stoma is also variable. However, these morphological characters have a distinctive depiction for each the three cultivars studied (Fig. 6 & 7). 'Kent' has a large, disorganised stomate with a shallow to intermediate lumen which is often congested by epicuticular wax and cutinised cells. These lenticels have pronounced air passage ways linking the lumens. In all cultivars, expansion during growth leads to some tearing or cracking of the surrounding cuticular layers, because cell division has ceased long before the volumetric increase of the fruit has reached a maximum.

In surface view, the lenticel area is flush with, or slightly depressed into the surface of fresh fruit. The absence of raised structures corresponds to the fact that there is no phellogen, and therefore no continuous growth from beneath. In some material prepared by plunge-freezing a small measure (< 15 %) of shrinkage occurred (Boyde & Maconnachie, 1979), creating a 'hillock'-effect around discoloured lenticels (Fig. 8). This concurs with the hardening effect of the phenolics present in the vacuoles and cell walls (Dai *et al*., 1996) of these discoloured lenticels and is not restricted to a specific cultivar. In the material studied using SEM, no other indications of phenolics and the accumulation thereof could be found. Within all three cultivars, non-discoloured, reddened and blackened lenticels appear structurally similar, both internally and externally.

Comparison of external structures revealed that 'Tommy Atkins' has predominantly small stomata, with limited suberisation taking place. The lenticel stoma has a rounded to irregular perimeter. Internally, the lenticel lumen is lined with intra-lenticular wax fractions that rapidly diminish vertically down the lenticel (Fig. 6 & 9), leaving bare cutin lining the lumen wall of the lenticel. Superficial subsidiary tissue, such as mesophyll cells filling the lenticel lumen, is mostly absent. The combined effect of the observations is that little light is reflected from the stoma, which will inhibit cutin and cuticular wax deposition (Jeffree, 1996), and that the lenticel lumen maintains a larger air volume than those with disorganised lumen contents. It can be deduced from the small stomate, interconnecting air passages and large air volume in the lumen, that the lumen will contain an accumulation of volatile metabolic by-products.

In a study by Bezuidenhout *et al*. (2005), a transverse section through “Tommy Atkins” material stained with Sudan Black B, showed little cutin in the stomate chamber. Their observations are consistent with the finding of this study that the wax layer diminishes. Although Sudan Black B is used to identify cutin, it is a lysochrome, specifically targeting neutral to slightly acidic triglycerides in lipids of the cutin layer (BioGenex, 2004). Embedding in paraffin wax dissolved and dispersed the small fraction of plant waxes present. Consequently, the small wax quantities in deeper lumen areas were no longer discernable.

‘Keitt’ is a cultivar with a medium to high incidence of lenticel discolouration. Lenticel appearance on this cultivar is variable, but present with a large, irregularly torn stomate. Due to the prolonged fruit development of ‘Keitt’ (Table 1), suberisation of its intra-lenticular cells is more prevalent than in any of the other two cultivars. Together with the wax layer partially covering the intra-lenticular, this would contribute to protection of the exposed mesophyll cells beyond the lenticel lumen (Fig. 10). Tracing the differential between the wax fractions in the various parts of the lenticel, regression from dense and structurally intricate epicuticular wax to sparse, simple wax crystalloids, and eventually, nude cutin can be seen. The change is not linear, with a sudden change in the amount of coverage and complexity at about halfway down the lumen. Mesophyll cells at the lower end of the lumen and in air passages leading away from the lumen are covered with a layer of cutin that is almost without crystalline wax. In a cross-section of a lenticel from an immature fruit, the wax fraction extended further into the lenticel cavity, yet still diminished in quantity as the depth increased (Fig. 2B). The lenticel cavity thus expands with the increasing volume and fruit size during growth, but it is uncertain what governs the gradient of wax deposition for cultivars with sparse intra-lenticular wax cover.

Transversely fractured sections of ‘Kent’ material exposed a lumen wall covered with large amounts of wax (Fig. 11). Although the wax layer consists of crystalloids with a more complex architecture than that of the other two cultivars in this study, it still becomes structurally simpler in deeper areas of the central cavity and the air passages leading away from it. Randomly organised, suberised mesophyll cells obstruct the open, more exposed lumen, thereby providing a barrier against harm inflicted from outside. It has a higher abundance of lenticels on the fruit surface than either ‘Tommy Atkins’ or ‘Keitt’ fruit. ‘Kent’ lenticels are also the largest of the three cultivars investigated. Despite these facts and the overall impression of structural disorganisation, this cultivar exhibits the lowest incidence of lenticel discolouration (Donkin & Oosthuyse, 1996).

3.5 CONCLUSION

During fruit set and growth, the surface area of mangoes exceeds a 2400-fold increase in 3 - 4 four months, depending on the cultivar. All structures associated with the fruit and rind must be able to deal with this massive growth rate, with meristematic tissue best adapted to meet the demand. However, the absence of meristematic tissue is one of the distinctive features of mango fruit lenticels. To compensate for this, the dynamic maintenance of cutin and its accompanying waxes ensure that the expansion of the epidermal and mesophyll tissue will not leave the developing pulp exposed to degradation by the environment and pathogens. This intra-lenticular layer of cutin and wax is distinctively different between the three cultivars studied, with 'Tommy Atkins' lenticels least developed, 'Keitt' lenticels variable and intermediary, and 'Kent' lenticels most developed. This finding correlates with cultivar susceptibility to lenticel discolouration. The mechanism for the development of the condition is still poorly understood (O'Hare *et al*., 1999), and other aspects of lenticel morphology may also prove to be influential.

The network of air space and tubular structures between the lenticels correlates well with the lenticel function of gas exchange. Current knowledge indicates that cutin is a product of the epidermal cells formed by an external, environmental signal or combination of signals (Martin & Juniper, 1970; Jeffree, 1996). However, the results of this study indicates a non-epidermal cell type bordering the lumen and air passages. According to Jeffree (1996) though, the presence of air, moisture and light in the lumen are signalling factors for the formation of cutin by the epidermal cells. The description of cutin and epicuticular wax lining the air channels of *Gloriosa rothschildiana* (Ponsamuel *et al*., 1998) support this statement. These signals are present in the cavernous lenticels of mango fruit, and could trigger a cascade of reactions for the formation of cutin by affected mesophyll cells. The amount of light and air that enter the lenticel will be limited by the size of the stomate. External dimensions of lenticel stomates vary considerably between the three cultivars, but can be generalised. Such a generalisation assigns the smallest stomate size to 'Tommy Atkins' fruit, while both 'Keitt' and 'Kent' lenticels are structured with large stomate sizes. The distinguishing features in the latter case are the lumen sizes and the significant differences in wax richness and deposition inside the lumen.

From the results of the microscopy, the amount of cutin present in the lenticel lumen is very similar for all three cultivars. With the epicuticular wax being the prevalent distinguishing factor, the amount of wax crystalloids present in the lenticel may be of far greater consequence than can be determined by visual methods. Barnes & Cardoso-

Vilhena (1996) summarised the importance of epicuticular wax in temperature canopy and surface control of leaves and fruit. A reflective wax coating facilitates lower temperatures, with subsequent lower transpiration rates. This may result in the overall metabolic rate of the leaf being lower, decreasing the production of secondary metabolites such as terpenoids. Such a wax coating also traps some volatile metabolic by-products passing through the cuticular membrane (Schmutz *et al*., 1994). Trapped metabolic derivatives can contribute to the protective nature of the epicuticular wax by enhancing UV protective properties.

A number of terpenoids, representing an array of chemical structures, are emitted as volatile compounds (Lalel *et al*., 2003; Narain & de Sousa Galvaõ, 2004). Most of these, however, contain one or more aromatic functional groups within the molecular structure. Although the presence of some aromatic components in mango wax was indicated by Prinsloo *et al*. (2004), the exact origin of these aromatics is unknown. Furthermore, several aroma volatiles previously described from mango may act as irritants on exposed cellular tissue (John *et al*., 1999). This fact creates another possibility, namely, that the intracuticular wax may trap some potentially harmful terpenoids emitted during normal metabolic action, contributing to lower lenticel discolouration incidence in cultivars with lenticels rich with wax.

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3.7 TABLES

Table 1 Comparison of the susceptibility to lenticel discolouration of mango cultivars 'Tommy Atkins', 'Kent' and 'Keitt' and some of their horticultural characteristics (Knight, 1997; Anonymous, 2000)

Characteristics	Tommy Atkins	Keitt	Kent
Susceptibility to lenticel discolouration	High	Medium to high	Low
External appearance of fruit	Regular ovoid to oblong, orange-yellow covered with red and heavy purple bloom	Ovate with slightly oblique apex and rounded base, pinkish green	Regular ovate, rounded base, greenish yellow with red shoulder
Harvest period (Average time from fruit set)	Early December to February (3 - 4 months)	March to April. (5 - 6 months)	Mid-February to March (4 - 5 months)
Tree characteristics	Medium to large tree; erect; early flush; usually bears well; early maturing fruit	Small to medium tree; erect; open to scraggly; very productive; late maturing fruit	Erect, slender tree; moderate size; early flush; bears well; fruit matures mid-season
Average marketable fruit size	Fruit medium to large 350 - 650 g	Fruit medium to large 350 - 650 g	Fruit large 500 - 750 g
Time of leaf flush	January to February	April to May	March to April
Susceptibility to latex burn	High	High	Low
Susceptibility to gall fly infestation	High	Medium	Low

3.8 FIGURE CAPTIONS

Figure 1 A lenticel depicting the stratified nature of the constituent tissue contributing to the enlargement of the structure (based on Stern (1994)).

Figure 2 Light microscopy of the general morphology of a mango (cultivar Tommy Atkins) lenticel, with no indication of cell differentiation or cambium tissue. The cuticular layers are situated flush over the lumen (1), which is lined with cutin (2) and epicuticular wax (3).

Figure 3 Abaxial view of a enzyme/acid treated cuticle section, with intact lenticel lining, stomate lumen (white arrow) and the network of radially expanding cutin ridges and air passageways traversing between lenticels (A). In (B), where the cutin lining (red arrow) has been torn away, epicuticular wax crystalloids can be seen (white arrow). The red arrow also indicates the convoluted and disorganised development of the cutin between deformed and irregularly spaced epidermal cells.

Figure 4 Close-up view of a tube extending from, and linking some lenticels (A). Damaged areas, as indicated by the arrows in (B), revealed the hollow extensions, giving evidence of tubular passageways.

Figure 5 Wax crystalloids lining the lenticel cavity (white arrow in A) confluent with the adaxial epicuticular wax (white arrows in B), and part of a cutinous membrane (black arrow in A). The cutin in B (indicated by a black arrow) is exposed due to physical handling of the sample material.

Figure 6 The external appearances of the lenticels of the three cultivars compared. ‘Tommy Atkins’ (A) has predominantly small lenticel stomas, with limited suberisation taking place. ‘Keitt’ (B) has stomas of varying sizes, but mostly develops a very large, torn structure with suberisation taking place towards the end of the period of pulp expansion. ‘Kent’ (C) lenticels are the most abundant of the three cultivars investigated, appearing predominantly large, with internal wax visible from outside (arrow).

Figure 7 Diagrammatic representation of the lenticel lumen of each of the three mango cultivars studied. Wax abundance in each type is depicted by the

red areas. ‘Tommy Atkins’ typically has a deep, more organised lumen with a small stomate that is often irregularly fissured. ‘Keitt’ has a deep to intermediately deep, disorganised lumen, and develops a larger stomate. ‘Kent’ has a large, disorganised stomate with a shallow to intermediate lumen.

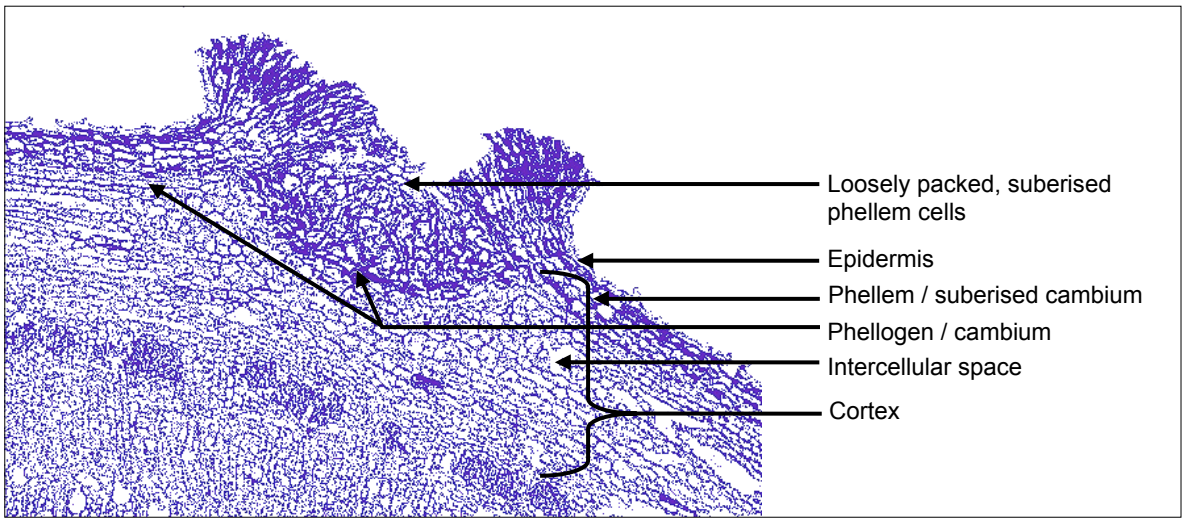
Figure 8 Shrinkage (< 15 %) caused by plunge-freezing of material with discoloured lenticels, created a ‘hillock’-effect due to presence of mesophyll cells hardened by vacuoles and cell walls containing phenolic compounds (A). The material prepared for light microscopy confirms the presence of the phenolics (darkened cells in unstained section) (B).

Figure 9 A transversally fractured lenticel from ‘Tommy Atkins’. White arrows indicate the presence of wax, while the red arrows indicate bare cutin lining the lumen of the lenticel. The extracuticular wax rapidly diminishes and disappears from the cutin layers forming the lumen wall.

Figure 10 A transversely fractured lenticel from ‘Keitt’, with the wax gradient down the lumen wall sampled at points a - d. At point (a), the wax is similar to the epicuticular wax layer, although it appears less densely-packed and architecturally less intricate. Point (b) is on the surface of a mesophyll cell in the lumen, clearly lacking all the complexity of the adaxial wax. At this point, the wax suddenly diminishes in quantity and becomes even sparser. Point (c) is an area deep inside the lenticel, close to an air passageway, with almost no wax present, whilst the cell surface in the air passageway at point (d) clearly still has a cutin layer, but no more wax.
Scale bar (a - d) = 1µm.

Figure 11 A transversally fractured lenticel from ‘Kent’, showing the wax fractions extending deep into the lumen (two upper arrows), as well as air passageways (two lower arrows). The demarcated area indicates the epidermal cells of a resin duct in close proximity to the lenticel.

3.9 FIGURES



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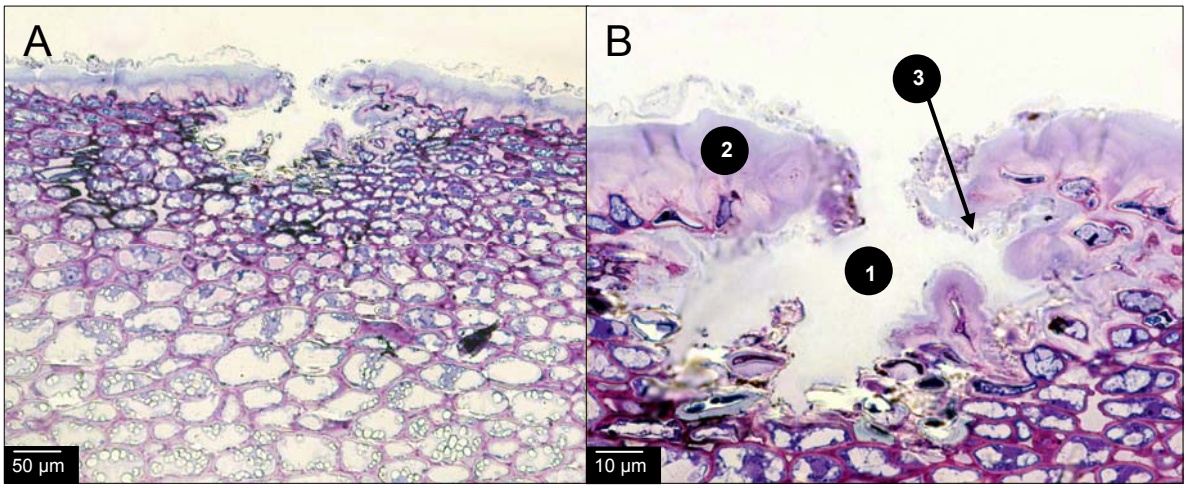

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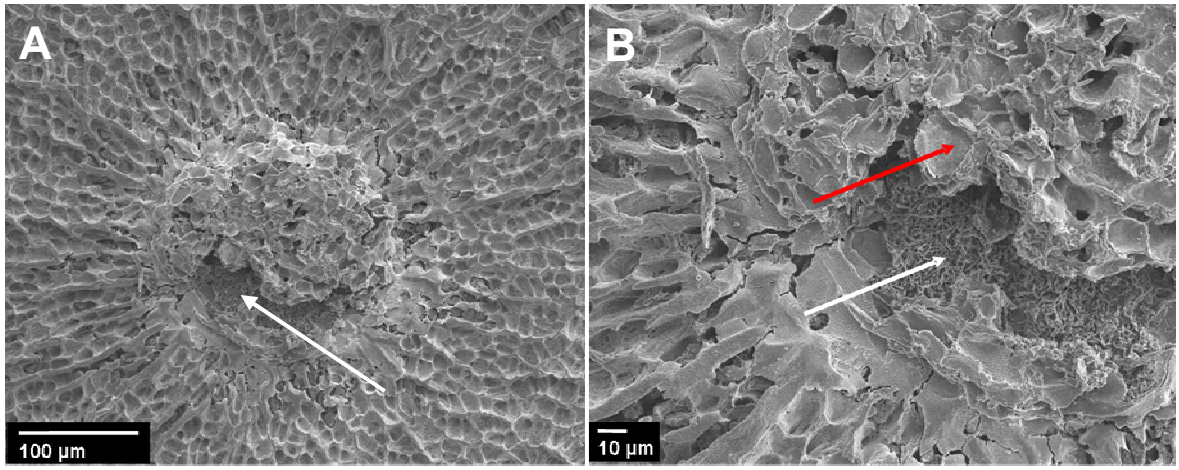

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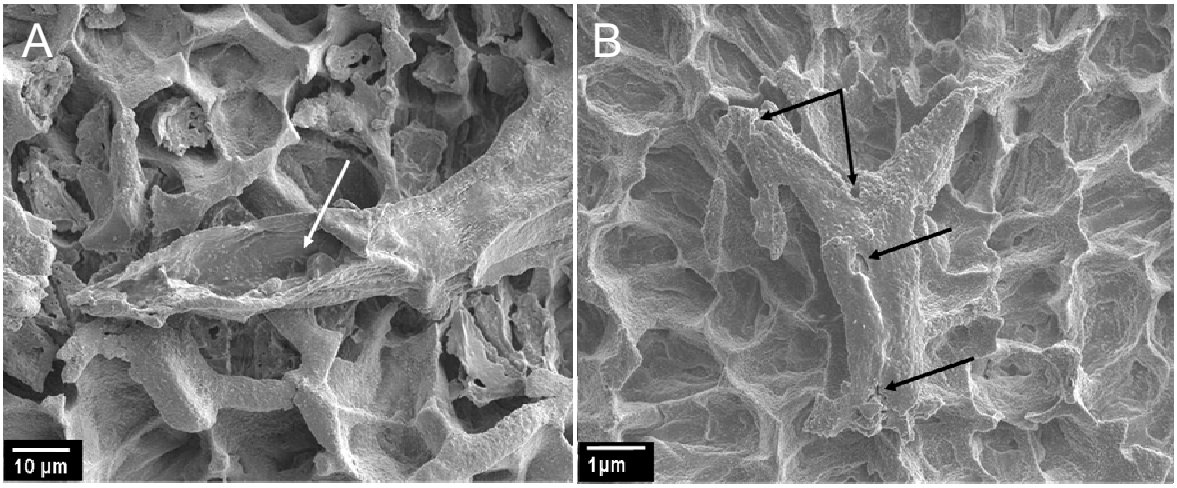

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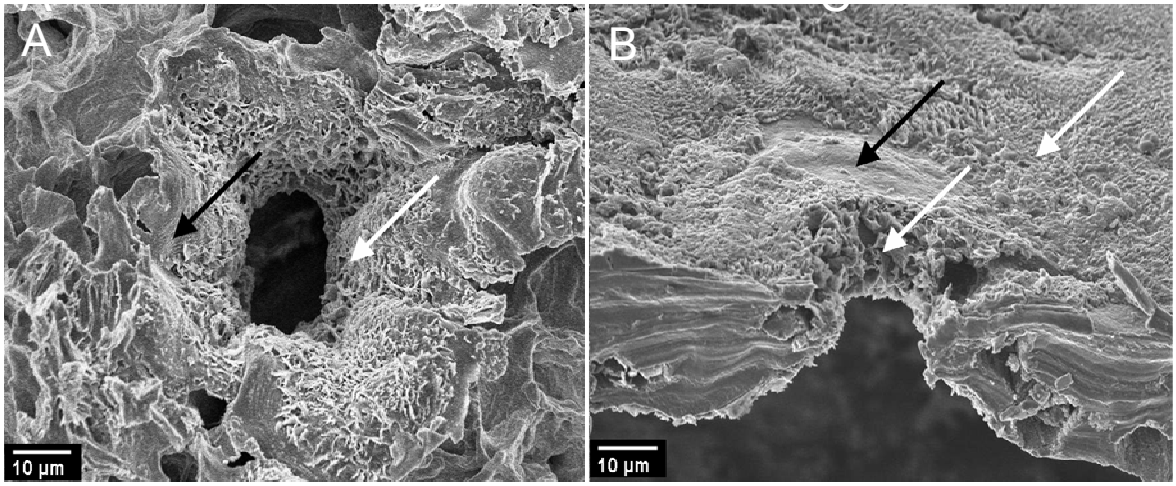

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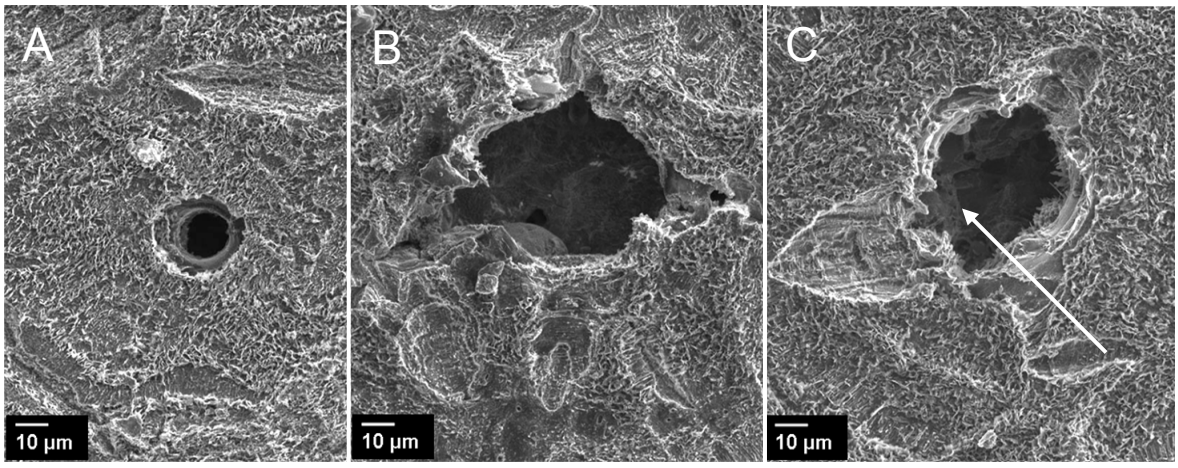

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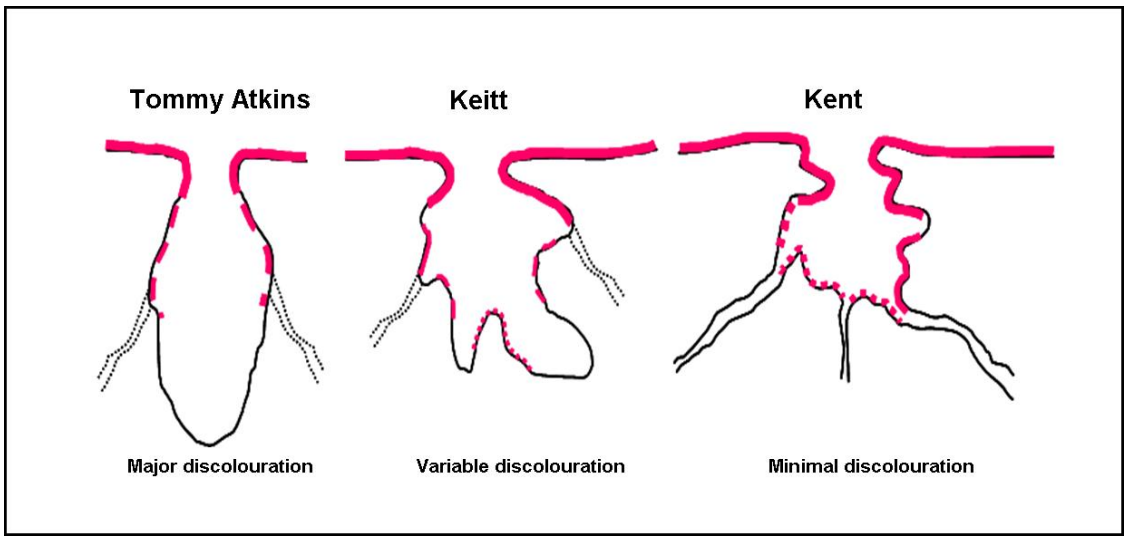

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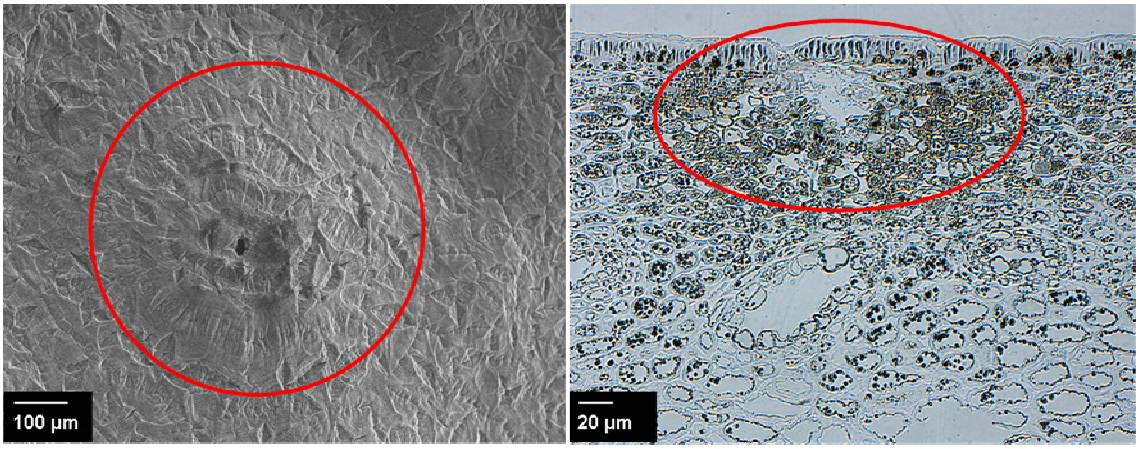

Figure 8

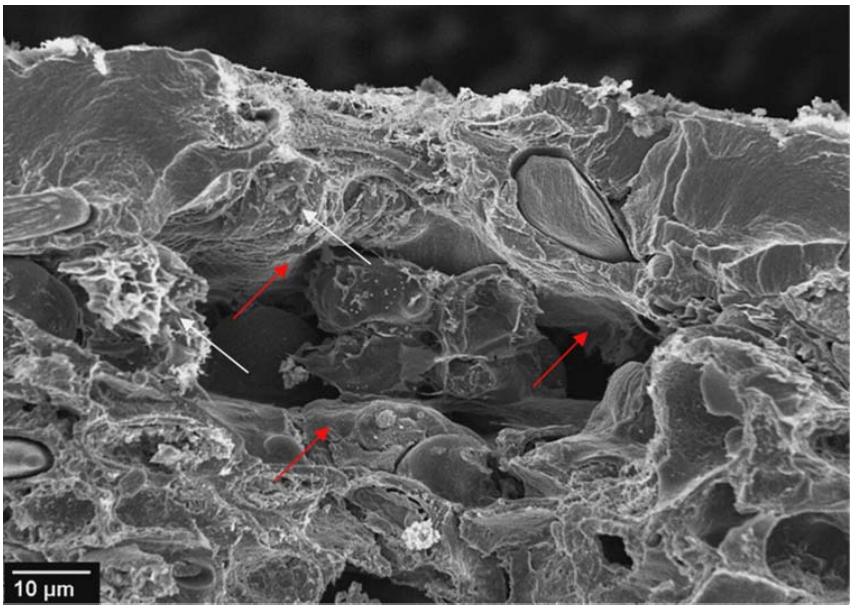

Figure 9

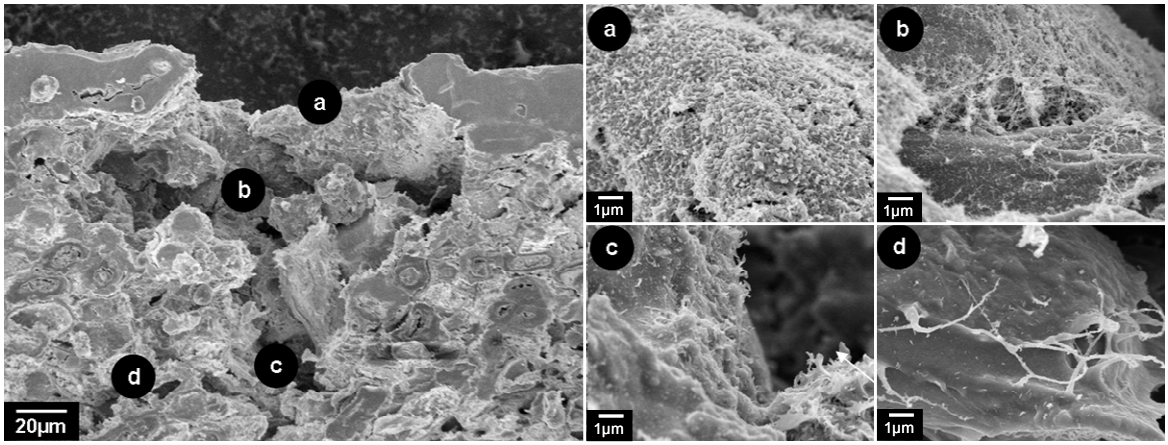

Figure 10

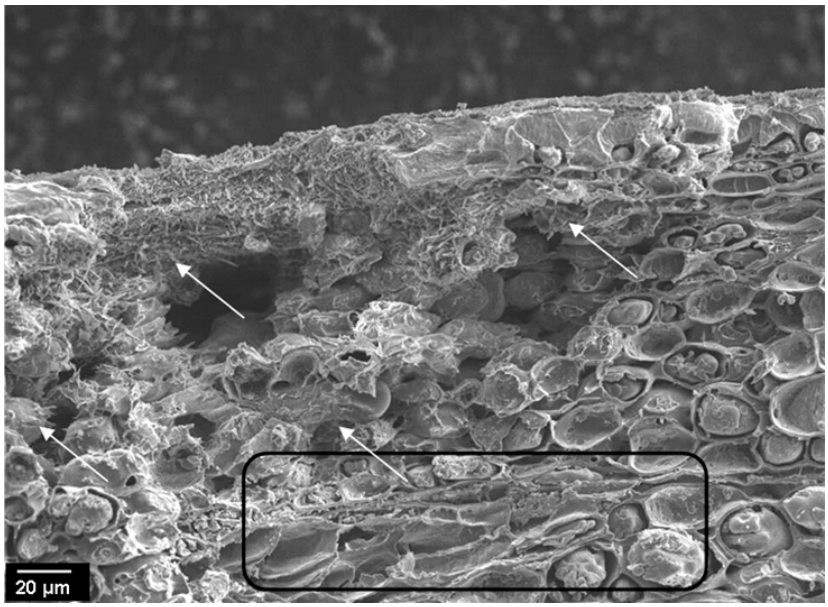

Figure 11

Nothing has such power to broaden the mind as the ability to investigate systematically and truly all that comes under thy observation in life.

Marcus Aurelius Antoninius (121-180 AD)

Roman Emperor

Chapter 4

A RAMAN SPECTROSCOPIC STUDY OF THE EPICUTICULAR WAX LAYER OF MATURE MANGO (*Mangifera indica* L) FRUIT [1]

4.1 ABSTRACT

Raman spectroscopy was used to characterize the epicuticular wax of mature mango fruit. Little is known about the chemical composition of the natural wax layer covering mango (*Mangifera indica*) fruit. Scanning electron microscopy measurements showed two morphologically distinct parts in the wax layer and Raman microscopy determined that they are chemically distinct. Crystalline aliphatic compounds protrude from an amorphous layer in which long-chain aliphatic, aromatic and unsaturated compounds are present. Raman spectra recorded on the perimeter of lenticels indicate an absence of the crystalline aliphatic compounds in this region and this information could assist in the solution of a problem faced by the mango industry due to the discoloration around lenticels, which makes the fruit unacceptable as an export product. The results are supported by Fourier transform infrared spectroscopy. Both techniques are now employed as probes in a long-term project to study dynamic changes of the wax during fruit development and eventual processing. This includes the monitoring of possible changes in carotenoid content and the role of dust particles embedded in the wax.

4.2 INTRODUCTION

Mango (*Mangifera indica* L.) fruit, like most aerial parts of terrestrial plants, is covered by a thin wax layer, which as the outer boundary of the epicuticular layer, forms the interface

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1: This chapter has been published as an article in the *Journal of Raman Spectroscopy* 35: 561 - 567.

between the plant and the atmosphere. It protects the fruit during growth against excessive exposure to UV radiation, plays a role as insect repellent, influences the uptake of foliar applied herbicides, and reduces water loss. It furthermore creates a water resistant surface, allowing dirt and pathogens to be washed away (Balsdon *et al*., 1995; Schwab *et al*., 1995; Barthlott & Neinhuis, 1997; Kirkwood, 1999). The mango skin is also characterised by the presence of lenticels, which are structures of epidermal origin with the function of gas exchange (O'Hare *et al*., 1999). These lenticels are present in large numbers and prone to discoloration that renders the fruit cosmetically unacceptable as an export product (Fig. 1). This leads to financial losses for South African mango industry. Furthermore, world trade in fresh mango fruit is restricted by the highly perishable nature of this fruit, which displays a characteristic peak of respiratory activity during ripening and is therefore considered to have a short storage life.

In order to control financial losses and fruit quality, control measures and improved long-term storage conditions must be employed to expand marketability of the fresh fruit (Jacobi *et al*., 2001). In South Africa, as part of postharvest treatments, the natural plant wax is mechanically altered during packline procedures, such as submersion of the fruit in hot water (± 48°C), which can cause heat injuries to the mango skin and influence fruit ripening (Jacobi *et al*., 2001). The wax is also chemically altered when a commercial wax, which prolongs the shelf life of the fruit by inhibiting water loss, is applied during packing. It is suspected though, that the interaction of the commercial wax with the natural wax can contribute to discoloration around lenticels, which is a manifestation of a physiological response to stress being imposed on the fruit.

Scanning electron microscopy (SEM) has been used extensively in previous studies to study the morphology of the wax layers, while gas chromatography coupled to mass spectroscopy (GC-MS) and high-performance liquid chromatography (HPLC) have been used to determine the chemical composition of plant wax. These studies have shown that the foremost components present in plant waxes are n-alkanes, alkyl esters, n-alkanoic acids, primary and secondary alcohols, aldehydes, mono- and diketones while free and esterified alcohols, and several fatty acids have been identified as some of the lesser components (Jetter, 2000; Kunst & Samuels, 2003).

Fourier transform infrared (FTIR) spectroscopy has been used to identify the presence of carbonyl groups in plant waxes and to study the phase behaviour and crystallinity of cuticular waxes (Merk *et al*., 1998; Dubis *et al*., 1999; Jetter & Riederer, 1999). Fourier transform-Raman spectroscopy was used in a study of the wax on Norwegian spruce

needles and identified carotenoids in micro and macro measurements (Matějka *et al*., 2001). Gas chromatography has also shown that the leaf epicuticular wax of *Prunus laurocerasus* L. occurs in a layered structure with different chemical compositions, which dynamically change during leaf development (Jetter *et al*., 2000; Jetter & Schäffer, 2001).

Raman and infrared microscopy were chosen in this study as possible methods to distinguish chemically between different wax fractions, which have been visually determined with Field Emission-SEM, as sampling size with the Raman microscope can be as small as 1 μm^2, and for the infrared microscope 20 μm^2. This makes it possible to distinguish between small fractions. A further advantage of the two methods is their ability to serve as non-destructive techniques, allowing the material to be isolated and investigated without any chemical change and interference during extraction procedures. We report here on the role of Raman and infrared microscopy in the preliminary part of the study.

In an attempt to understand and improve the postharvest performance of mangoes in general and the problem with lenticel discoloration in particular, this study was undertaken to characterise mango wax, compare it to the commercial wax and determine whether the interaction with commercial wax contributes to the discoloration effect. In so doing, it was necessary to examine the chemical composition and the morphology of the wax layers, since both of these factors contribute to the functional role of the wax.

4.3 MATERIAL AND METHODS

4.3.1 Plant material

Physiologically mature mango fruit were collected from an orchard in the Hoedspruit area, Limpopo Province, South Africa. They were carefully packed for transportation to the laboratory in order to prevent any damage to the fruit surface. Two different cultivars, namely ‘Keitt’ and ‘Tommy Atkins’, were investigated. Since the Raman and FTIR results for the two cultivars hardly differed, only the results for the cultivar ‘Keitt’ are presented.

4.3.2 Instrumentation

Raman spectra were recorded with a XY Raman spectrometer from Dilor, using the λ=514.5 nm laser line of a Coherent Innova 90 Ar^+-laser as exciting line. The spectra were recorded in a backscattering configuration with an Olympus microscope attached to the

instrument. The spectral resolution was at least 2 cm^{-1} for all the measurements. Optimum recording conditions were obtained by varying laser power, microscope objective and size of the confocal hole.

Mid-infrared transmission spectra were recorded of the same samples, using a Bruker infrared microscope (A 590), equipped with a MCT detector, attached to a Bruker 113v Fourier transform infrared (FTIR) spectrometer. The resolution was 4 cm^{-1} and 32 scans were signal-averaged in each interferogram. The 15x objective was used in all instances with a sample aperture size of 80 μm^2.

Powder X-ray diffraction data from soil samples collected in the mango orchard was recorded with a Siemens D501 automated diffractometer equipped with a secondary graphite monochromator. The applied potential was 40 kV and the corresponding current 40 mA. Cu Kα-radiation was used as the primary X-ray beam. A pattern was recorded from 3 to 70° (2θ) in steps of 0.05°. The measuring time was 1 s and the scanning speed 3° (2θ) per minute.

Electron micrographs were obtained using a JSM-6000F scanning electron microscope (JEOL, Tokyo, Japan). The samples were plunge frozen in liquid propane at -180 °C and vacuum dried in a Fisons E6441 High Vacuum Evaporation Unit (Fisons, East Sussex, England) for 72 hours. They were made conductive by exposing it to 0.5 % RuO_4 vapour for 30 minutes (Van der Merwe & Peacock, 1999).

4.4 RESULTS AND DISCUSSION

4.4.1 Microscopic characterisation of mango fruit wax

The cuticle of physiological mature fruit (cv. 'Keitt') is covered with a distinctive layer of wax in which two different regions can be distinguished (Fig. 2). The outermost layer consists of microscopic wax crystaloids (Fig. 2A) that form an irregular surface and protrude from an amorphous wax film underneath (Fig. 2B). Such morphological distinctiveness is due to differences in the chemical composition of the various components contributing to each fraction (Griffiths *et al*., 1999; Griffiths *et al*., 2000; Jetter *et al*., 2000).

4.4.2 *In vivo* measurements

In the first experiments, Raman spectra were recorded *in vivo* on small pieces of mango rind from which the pulp had been removed. It was hoped that the confocal Dilor XY system would make it possible to differentiate between wax layers in the z-direction by changing the depth of focus. However, a strong fluorescence background, which was stronger on the yellow part of the rind than on the green, made it difficult to obtain useable spectra. Decreasing the laser power to 0.5 mW at the sample and using the 50x objective of the microscope reduced the fluorescence sufficiently to obtain the baseline corrected spectrum shown in figure 3. Spectra recorded on the green and yellow parts of the rind respectively were identical and are typical of carotenoid pigments, which occur in many fruit and vegetables. Carotenoid pigments play an important role in photosynthesis as they protect the photosynthetic system from photo-oxidative damage and are accessory light-harvesting pigments, as they absorb light in wavelength regions where the chlorophylls absorb weakly. Raman and resonance Raman spectroscopy have been used extensively to study the molecular structure and configuration of the carotenoid molecules that occur in light-harvesting proteins and reaction centres (Robert, 1999; Ruban *et al*., 2000; Ruban *et al*., 2001).

Carotenoids are very efficient Raman scatterers and most exhibit a resonance or pre-resonance enhancement due to the 514.5 nm laser exciting line. Consequently, any bands that may originate from the wax are dwarfed by the spectrum of the pigment underneath the wax layer. The most intense peaks in the carotenoid spectrum occur at 1524 cm^{-1} (ν_1, -C=C- stretching vibrations), 1156 cm^{-1} (ν_2, -C-C- stretching) and 1005 cm^{-1} (ν_3, $-CH_3$ in-plane rocking vibrations) (Robert, 1999; Ruban *et al*., 2000; Ruban *et al*., 2001; Withnall *et al*., 2003). The ν_4 band at 964 cm^{-1} is attributed to out-of-plane C-H modes, which are formally forbidden for planar molecules, but when carotenoid molecules are twisted around a C-C bond these out-of-plane modes become more strongly coupled to the electronic transitions and their contributions to the resonance Raman spectra become more intense. Bands in this region have proved useful to identify carotenoids in biological samples using different resonance conditions (Ruban *et al*., 2001).

The peak position of the ν_1 band in the Raman spectrum is determined by the number of conjugated double bonds in the molecule, which also determines the colour, ranging from yellow to purple, of carotenoid pigments. The position of the band shifts to higher wavenumbers for *cis*-isomers and the presence of more than one carotenoid, with different resonance effects in biological samples influences the position of the band

(Robert, 1999; Ruban *et al*., 2000; Ruban *et al*., 2001; Withnall *et al*., 2003). It is therefore not always easy to identify carotenoids using the ν_1 value and a spectrum from a carrot slice with known carotenoid content (90 % β-carotene, 10 % α-carotene), was recorded with the same experimental conditions used for the mango measurements, to use as reference. The ν_1 value obtained for the carrot slice at 1519 cm^{-1} is similar to that obtained by Withnall *et al*. (2003) (514.5 nm exciting line) and is 5 cm^{-1} lower than the value of 1524 cm^{-1} of mango fruit rind. Recent studies have identified all-*trans*-β-carotene, all-*trans*-violaxanthin and 9-*cis*-violaxanthin as the major carotenoids present in mango fruit (Rodriguez-Amaya, 2000; Pott *et al*., 2003). It has been determined that the ν_1 value for violaxanthin occurs at 1528 cm^{-1} with 514.5 nm excitation. Thus the upwards shift of the ν_1 band in the carrot spectrum at 1519 cm^{-1}, with only α-carotene and β-carotene present, to 1524 cm^{-1} in the mango spectrum is probably caused by the contribution of both isomers of violaxanthin.

4.4.3 Freeze-dried samples

The rind from the mature fruit, which was plunge-frozen and freeze-dried for SEM studies gave the same fluorescence problem experienced with the fresh fruit and could not be used to obtain spectra from the surface wax.

In order to eliminate the strong resonance enhanced signal from the β-carotene and the fluorescence background, subsequent wax samples were then manually removed from the mango surface with a surgical scalpel and placed on microscope slides. This was done in progressive layers in order to make it possible to differentiate between the top and bottom layers of the wax (Fig. 2 A & B).

4.4.4 Outermost wax layer

The Raman and infrared spectra of waxes may be considered in two wavenumber regions, namely: 600-1800 cm^{-1}, which contains functional groups, as well as information about skeletal backbone vibrations, and 2700-3600 cm^{-1}, which is dominated by C-H, N-H and O-H stretching bands.

Three Raman spectra (Fig. 4), recorded from different parts of the fruit epicuticular wax layer, are shown in the region 1000-1700 cm^{-1}. The simplicity of the spectrum of the uppermost wax layer (Fig. 4 A; Table 1) is typical of long-chain aliphatic compounds, with the very strong band at 1295 cm^{-1}, representing $-(CH_2)_n-$ twisting vibrations, the most prominent band in this region. The main peak of the bending vibrations of the CH_2 moiety

is observed at 1439 cm^{-1} and a weak wagging vibration at 1370 cm^{-1} (Lin-Vien *et al*., 1991; Cao *et al*., 1995; Schrader, 1995; Coates, 2000).

In the region between 1000 and 1100 cm^{-1} various bands representing C-C stretch vibrations occur and the two sharp bands at 1161 and 1130 cm^{-1} have been assigned to all-trans chain segments (Cao *et al*., 1995).

The infrared spectrum of the same region is shown in Figure 5 and the in-phase rocking mode at 719 cm^{-1} confirms that the wax is an alkane with n>4. On closer examination it is seen that both the $-(CH_2)_n-$ scissoring mode (1462-, 1472 cm^{-1}) and in phase rocking mode (719-, 729 cm^{-1}) are split. This is similar to the values for polyethylene and is ascribed to factor-group splitting because of interaction between molecules in orthorhombic crystalline areas (Coates, 2000). The strong band in the infrared spectrum at 1709 cm^{-1}, is assigned to a C=O vibration and confirms the presence of long-chain aliphatic ketone or fatty acid (Dubis *et al*., 1999). Lack of coincidence of Raman and infrared bands in the spectra are typical of polyethylene type of compounds, where a local centre of symmetry exists in the middle of $-CH_2{-}CH_2-$ groups, making the rule of mutual exclusion active (Schrader, 1995).

The stretching vibrational modes of CH_2- and CH_3- groups occur in the region 2700-3600 cm^{-1}. In Figure 6 A the bands in this region of the outermost wax layer is shown and is dominated by the symmetric (in-phase) and anti-symmetric (out-of-phase) stretching vibrations of the methylene chain at 2846 and 2881 cm^{-1} respectively. Smaller peaks at 2904 and 2930 cm^{-1} are attributed to motions of terminal CH_3 groups. The peak-height intensity ratio I_{2846}/I_{2881} is very sensitive to chain packing and has been used to determine inter-chain interactions, whilst I_{2930}/I_{2881} measures effects from intra-chain interactions (Levin & Lewis, 1990). The spectra in this region were deconvoluted with the Labspec software supplied with the Dilor Raman instrument and it was found that I_{2846}/I_{2881} = 0.8 and I_{2930}/I_{2881} = 0.05 for the aliphatic layer. The high value of I_{2846}/I_{2881} is an indication of an ordered system and therefore high crystallinity, with little interaction between the chains, which is supported by the low value of I_{2930}/I_{2881}. The infrared spectrum of this layer (Table 1) exhibited the strong symmetric and asymmetric CH_2 stretch vibrations at 2848 cm^{-1} and 2918 cm^{-1} respectively.

4.4.5 Wax layer adjacent to the cuticle

The Raman and FTIR spectra have identified the wax crystals, seen on the outer boundary (Fig. 2 A), as long-chain alkanes, most probably ketones or fatty acids. The

Raman and FTIR spectra of the layer adjacent to the cuticular layer display the same bands mentioned above (Fig. 2 B), but also exhibit new bands in the 600-1800 cm^{-1} region at 1607 and 1632 cm^{-1}. The band at 1607 cm^{-1} is very typical of the conjugated C-C vibrations of aromatic rings, while the band at 1632 cm^{-1} occurs at a position usually associated with isolated C=C vibrations (Coates, 2000). Evidently the two wax layers are chemically distinct, with crystalline long-chain aliphatic compounds the main constituents of the outermost layer, whilst the layer beneath is a mixture of aliphatic, aromatic and unsaturated compounds. In the infrared spectrum of this layer a new band has appeared in the C=O stretching region at 1734 cm^{-1}, which could be an indication of the presence of esters in this wax fraction (Dubis *et al*., 1999).

In figure 6 B the Raman spectrum of this layer is shown in the region of the C-H stretch vibrations. The two bands at 2846 and 2881 cm^{-1} have slightly shifted towards higher wavenumbers namely 2850 and 2883 cm^{-1} respectively, which suggests that the disorder of the chains has increased (Cao *et al*., 1995). The amount of methyl groups has increased as the small bands observed as shoulders at 2904 and 2930 cm^{-1} due to stretch vibrations of CH_3 -groups are more intense. The ratio I_{2850}/I_{2883} decreased from 0.8 in the top layer to 0.7 and the ratio I_{2935}/I_{2883} increased from 0.1 for the top layer to 0.4 in the layer containing the aromatic compounds. This is an indication of a decrease in crystallinity and an increase in inter-chain interactions, which would imply a more disordered amorphous structure. The infrared bands in this region have previously been used to study the temperature related phase behaviour of plant wax (leaves from *Hedera helix* L. and *Juglans regia* L.) (Merk *et al*., 1998), but we did not observe the same sensitivity to phase as with our Raman measurements in this region and therefore only used the Raman data to determine qualitatively, crystallinity.

4.4.6 Analysis of wax around the lenticels

Raman spectra of the wax covering the area surrounding a lenticel were obtained in vivo on samples where the fruit rind had been carefully removed from the lenticel, but with the basic structure still intact. One of these spectra can be seen in Figure 2 C and it is clear that the peaks ascribed to the aliphatic long-chain compounds have disappeared almost completely as best illustrated by the dramatic decrease in intensity of the $-(CH_2)_n-$ twisting mode at 1295 cm^{-1}. The C=C stretch peaks around 1600 cm^{-1} are now the most dominant peaks in the spectrum. The ratio indicating crystallinity, I_{2850}/I_{2883} =0.8 is similar to that of the amorphous layer, but the ratio I_{2930}/I_{2883} increased from 0.4 in the aromatic/aliphatic layer to 0.7, which indicates more inter-chain interaction as well as the presence of more $-CH_3$ end-groups. The absence of the nematoid crystals, consisting of long-chain aliphatic

compounds, around the lenticels is visually confirmed in Figure 7. The chemical and physical characteristics of the wax covering around lenticels are clearly different from the surroundings and postharvest treatments of the mango might affect this area differently than the rest of the rind.

4.4.7 Analysis of industrial wax

Raman spectra of the industrial wax used to cover mangoes in South Africa could not be obtained due to fluorescence. The FTIR spectrum of unblended Carnauba wax, derived from the leaves of Carnauba palms and commonly used as commercial wax, is shown in Figure 8. It is clear that the spectrum is very similar to that of the mango wax, but the splitting of the bands at 720 and 1470 cm^{-1} (indicative of crystallinity and a high degree of regularity for the backbone structure) observed in both fractions of mango wax, is not present. The FTIR spectra of two other commercial products namely Mangoshine® and Biocote® did show this splitting and therefore consists of more crystalline fractions than unblended Carnauba wax. One of the aims of the long-term project would be to establish if there is a difference in performance of the different commercial waxes and if the presence of a crystalline fraction in the commercial waxes prevents or contributes to lenticel damage.

4.4.8 Dust particles on mango surface

Small crystallites were observed between the wax crystals during the in vivo Raman measurements. The 100x objective of the Raman microscope made it possible to record spectra of these crystals individually and the results are presented in Figure 9. In the uppermost spectrum (Fig. 9 A) the most prominent peak at 462 cm^{-1} clearly indicates the presence of α-quartz in a reddish crystallite and the two smaller peaks at 219 and 290 cm^{-1} belong to Fe_2O_3, which is present in small quantities in most South African soils and responsible for the reddish colour of the α-quartz (Gillet, 2002). The middle spectrum (Fig. 9 B) with peaks at 290, 475 and 506 cm^{-1} resembles the spectrum of feldspar (albite form) and the bottom spectrum (Fig. 3 C) is assigned to TiO_2 with the very intense peak at 143 cm^{-1} and peaks at 396 cm^{-1}, 514 cm^{-1} and 640 cm^{-1} characteristic of the anastase phase. It can be deducted that this outermost wax layer, with its microscopically rough surface, entraps ultra-fine dust particles from the surroundings and in this way protects against penetration to the inner layer and the fruit itself. X-ray powder diffraction (XRD) data confirmed that dust collected in the vicinity of the mango trees used in this study were a mixture of mainly α-quartz and feldspar in the albite form ($NaAlSi_3O_8$), with small percentages of muscovite, orthoclase, sepiolite and kaolinite present. The spectrum of

TiO_2 was not obtained on a crystallite, but on the wax itself as can be seen by the presence of the characteristic peaks of the aliphatic wax layer in the spectrum. However, since this was the only instance in which the particular spectrum was obtained, it too is suspected to be contamination and not an intrinsic part of mango wax. Although TiO_2 was also not detected with XRD, it is present as a trace compound in many South African soils and the very strong Raman bands make small quantities easy to detect.

4.5 CONCLUSION

Epicuticular waxes are complex mixtures that consist of a great number of components, which vary greatly between plant species. Many of these constituents have very similar Raman and infrared spectra and differ structurally only in chain-length and a functional group or two. It is thus impossible to identify all the components of the wax using Raman spectroscopy alone, but we have shown that Raman spectroscopy could chemically distinguish between morphologically different wax fractions as observed with SEM measurements and that FTIR measurements fulfil a complimentary role in the identification of some functional groups.

It has previously been shown that the morphology and chemical composition of the epicuticular wax changes dynamically during the growth of mango fruit (Bally, 1999). In the forthcoming mango season, Raman microscopy will be linked the morphological changes to chemical changes in the wax layer that occur during fruit ripening and so elucidate the biosynthetic pathways of the wax formation.

The enhancement of the Raman signal of most carotenoid pigments with the 514.5 nm line makes Raman microscopy also a useful tool to study pigment distribution and formation during mango fruit development. In the long term project, the use of different laser lines is envisaged to help in the identification of different carotenoid pigments, as well as the possible enhancement of chlorophyll bands to study the modification of both pigments during the growth and ripening processes. The pigmentation around lenticels will be of special interest, and in combination with fluorescence microscopy and HPLC will contribute to a better understanding of the discoloration problem.

The microscopic identification of dust particles on the wax surface makes it possible to study the influence of external factors, which may be harmful. At this stage, it is not clear if the dust particles play any role in the discoloration around lenticels, but the amount and

presence of the particles shall be monitored. The detection of such small quantities of foreign matter holds particularly useful potential in the surveillance of pre-harvest control measures such as the detection of fungicidal copper compounds.

A better understanding of the chemistry and morphology of the wax surface can contribute to the improvement of postharvest treatments, which could lead to financial benefits for the mango industry.

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4.7 TABLES

Table 1 Wavenumbers (in cm^{-1}) and assignment of main Raman and infrared bands of crystalline and amorphous wax fractions

Aliphatic layer[a]	Aromatic/unsaturated[a]	Assignment
719 (s) IR 729 (s) IR	719 (m) IR 729 (m) IR	$-CH_2-$ rocking, $n > 3$
1036 (w) IR		C–C stretch
1107 (w) IR	1105 (w) IR	C–C stretch
1061 (m) R		C–C stretch
1130 (m) R		C–C stretch
1170 (w) R	1170 (m) R 1169 (sm) IR	Ring in-plane CH deformation
1295 (s) R		$-CH_2-$ twisting
1300 (w) IR		
1416 (m) R		CH_2 wagging
1439 (m) R	1462 (br) R	Anti-sym. CH_3 bending
1462 (m) IR 1471 (m) IR	1462 (m) IR	CH_2 scissoring
1586 (m) R		C=C stretch
1607 (s) R		Aromatic C=C stretch
1632 (m) R		Unsaturated C=C stretch
1709 (s) IR	1709 (s) IR	C=O stretch
1734 (s) IR	C=O stretch	
2846 (vs) R, 2848 (vs) IR	2850 (s) R, 2848 (s) IR	CH_2 symmetric stretch
2881 (vs) R, 2917 (vs) IR	2883 (s) R, 2918 (s) IR	CH_2 asymmetric stretch
2902 (w, sh) R	2902 (m) R	CH_3 stretch
2930 (w, sh) R	2930 (m) R	CH_3 stretch

[a] s = Strong; m = medium; w = weak; v = very; br = broad; sh = shoulder; R = Raman; IR = infrared

4.8 FIGURE CAPTIONS

Figure 1 Mango fruit with lenticel damage

Figure 2 Image of mango wax showing two distinct wax layers A) and B) and the cuticle C)

Figure 3 Spectrum of carotenoid pigment, recorded in vivo on mango fruit rind.

Figure 4 Raman spectra of mango wax of different parts of the wax: A) crystalline outermost layer, B) smooth layer next to cutin and C) area around lenticel.

Figure 5 Infrared spectra of mango wax: A) crystalline outermost layer and B) layer adjacent to cutin.

Figure 6 Raman spectra of mango wax in the region of the C-H stretch vibrations: A) outermost wax layer B) layer next to cutin and C) area round lenticel.

Figure 7 Micrograph of epicuticular surface around lenticel.

Figure 8 FT-IR spectrum of Carnauba wax.

Figure 9 Raman spectra of inorganic compounds recorded on mango wax A) Fe_2O_3 and α-quartz B) feldspar and C) TiO_2 (anastase) together with aliphatic wax layer.

4.9 FIGURES

Figure1

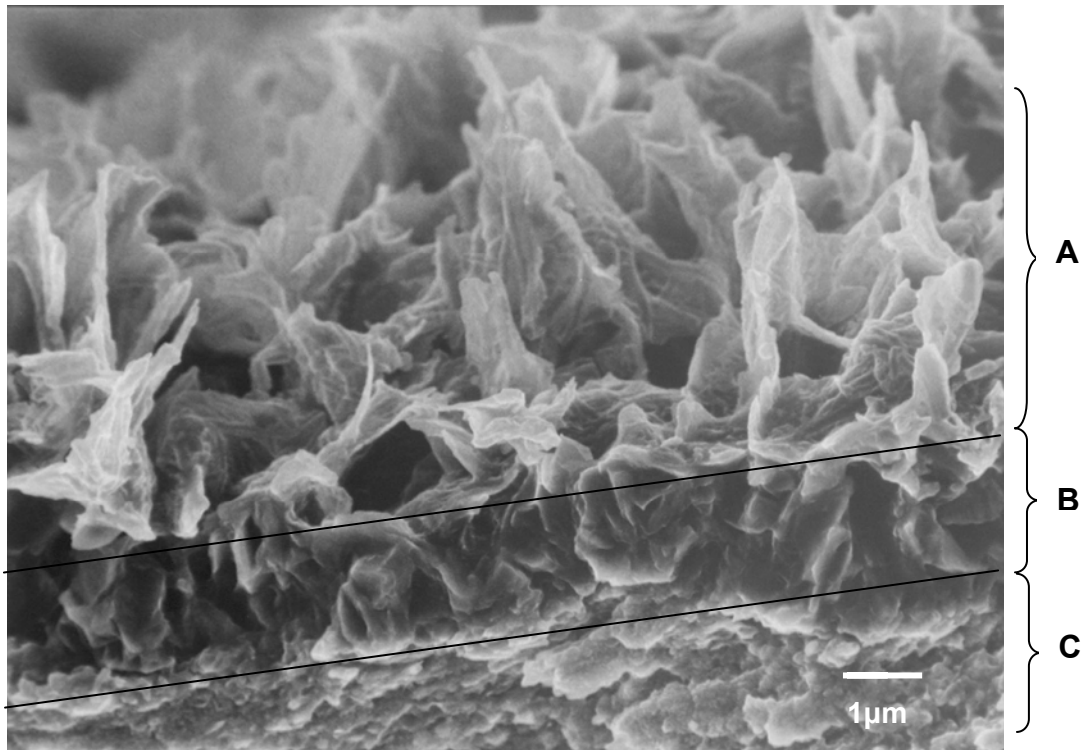

Figure 2

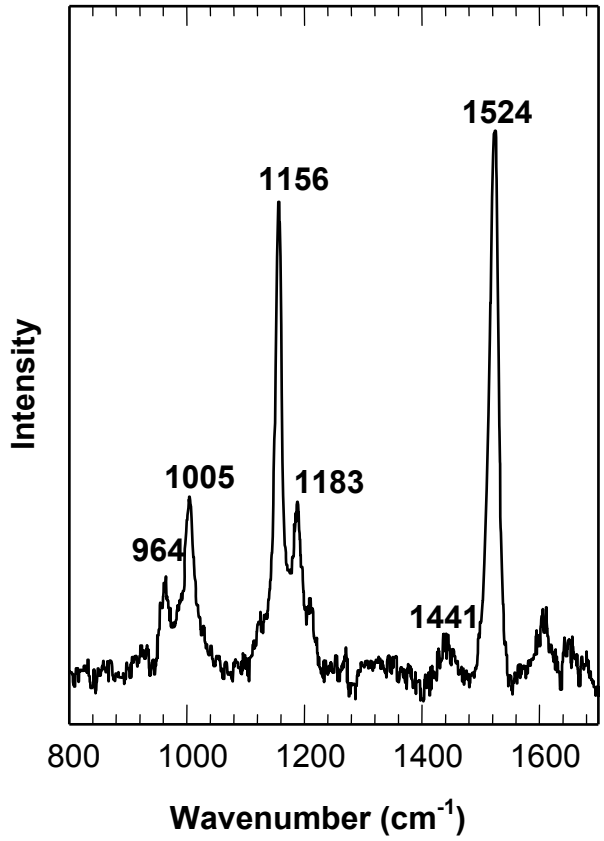

Figure 3

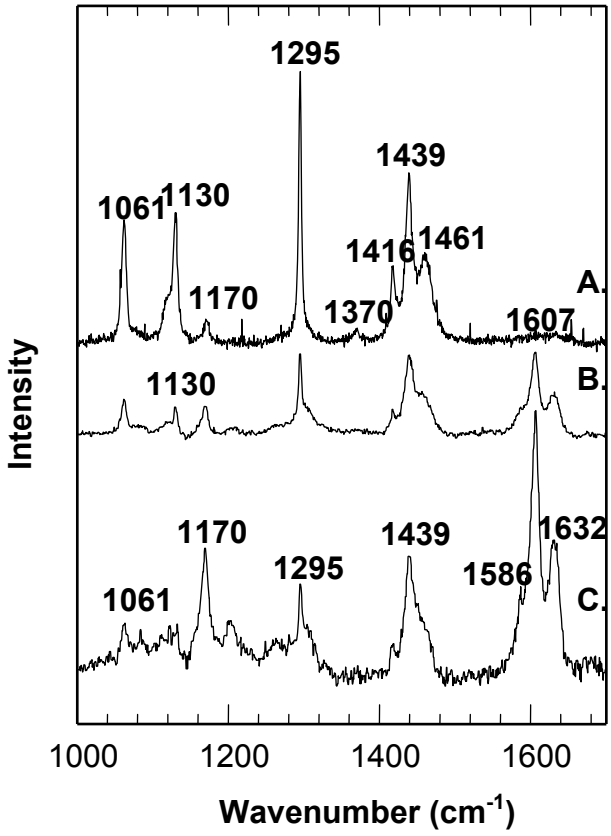

Figure 4

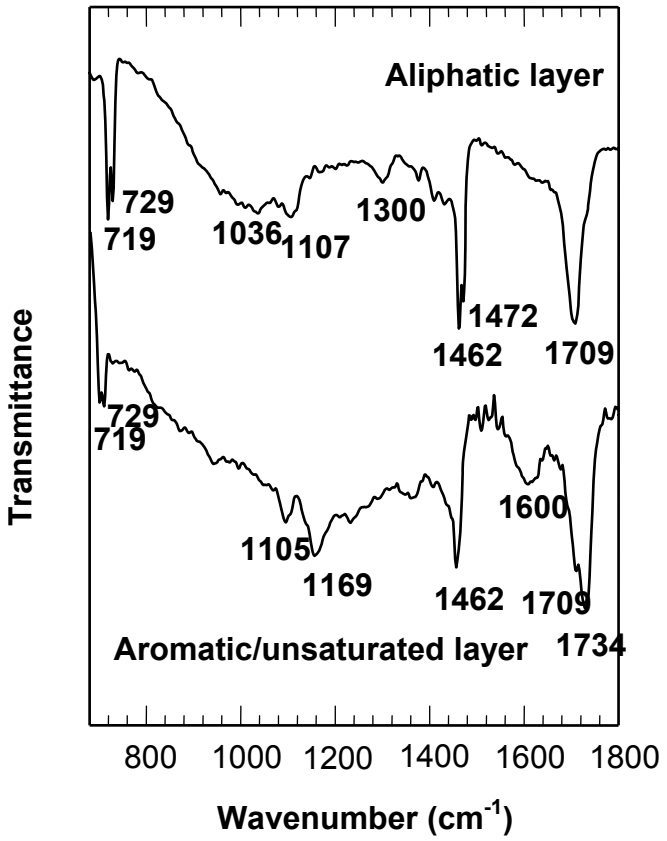

Figure 5

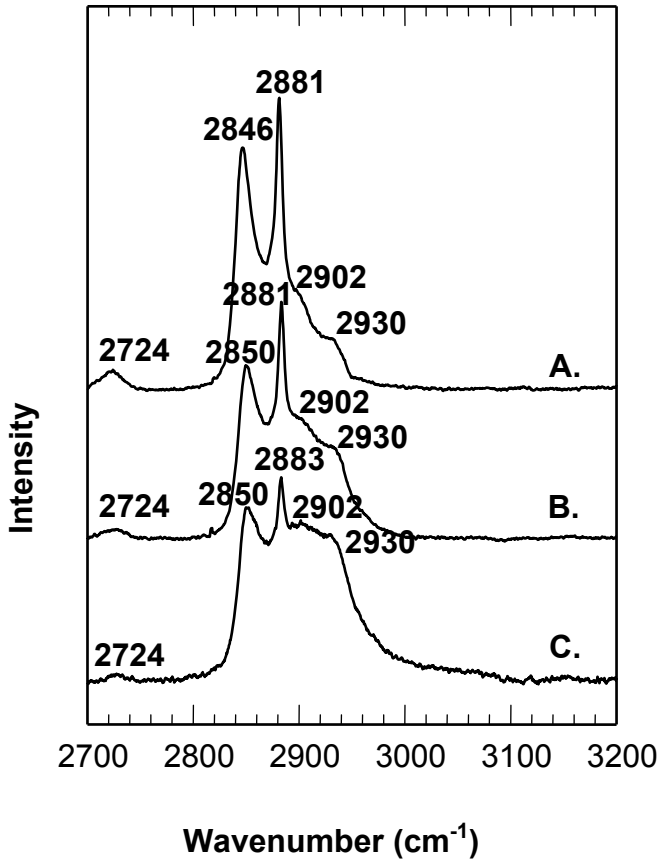

Figure 6

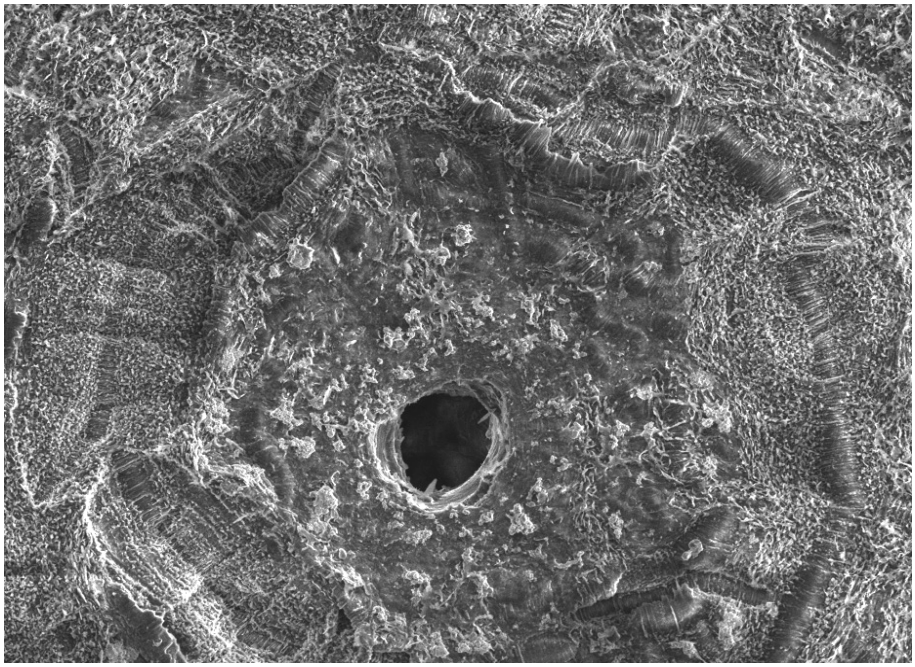

Figure 7

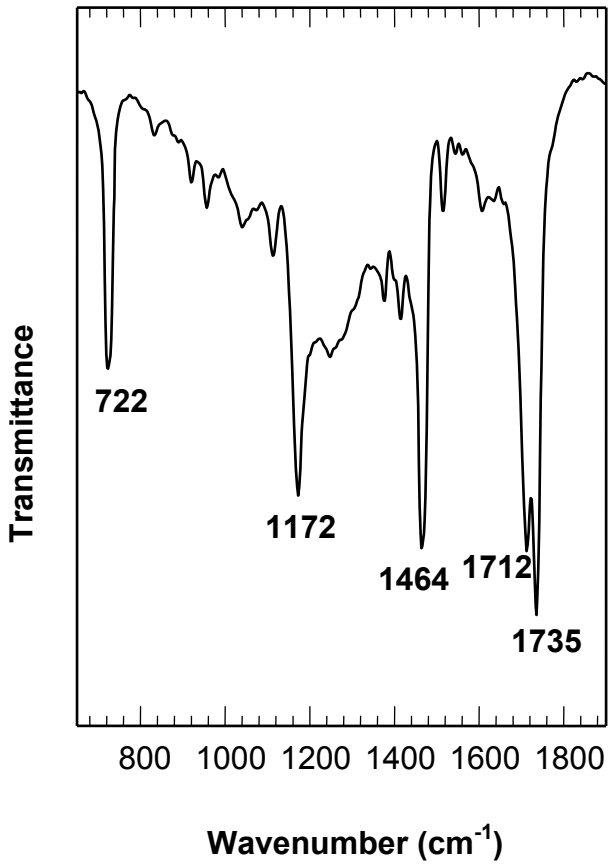

Figure 8

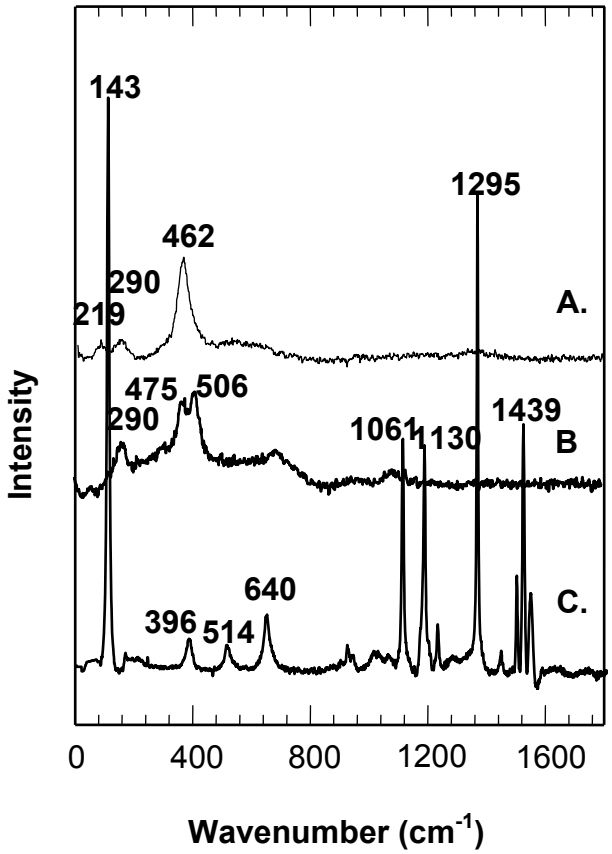
143
1295
462
290
219
A.
475
506
290
1061
1130
1439
B
640
396
514
C.
Intensity
0
400
800
1200
1600
Wavenumber (cm-1)

Figure 9

The most exciting phrase to hear in science, the one that heralds new discoveries, is not 'Eureka!' but 'That's funny...'

Isaac Asimov (1920-1992)

Science fiction writer

Chapter 5

LENTICEL DISCOLOURATION OF MANGO (*Mangifera indica* L.) FRUIT EXPLAINED BY CHANGES IN PHENOLIC PROFILES

5.1 ABSTRACT

The chemical and cytological nature of lenticel discolouration was investigated as part of a physiological study of mango fruit. It is a visible self-defence response by the fruit, manifesting with unacceptable reddened to darkened spots. Differential extracts of phenolics from discoloured areas were analysed by means of reverse phase-high performance liquid chromatography. Discernable shifts in profiles of the phenolics correspond to the progressive colour changes of the lenticels. Fluorescence microscopy of fixed material revealed the localisation and transition of various forms of phenolics, as associated with various degrees of discolouration after staining with vanillin-HCl. The results confirmed that the pigments responsible for the colour of the lenticels are flavonones rather than anthocyanidins. Conservation of cellular structure and metabolic activity were shown using transmission electron microscopy. Reverse phase-high performance liquid chromatography results also correlate with transmission electron microscopy of the lenticels, which indicated that the accumulation of cell wall-bound phenolics do not result from vacuolar collapse or membrane disintegration. Although lenticel discolouration is considered a cosmetic defect, it is an indication of an inherent self-defence mechanism.

5.2 INTRODUCTION

Fresh mango (*Mangifera indica* L.) fruit enjoys popularity worldwide. This has lead to development of cultivars that must satisfy several specifications regarding its horticulture and market access (Bompard, 1993). Meeting these requirements, however, is not enough to assure success. Consumer preference is an important and demanding factor, with appearance, regardless of aroma or taste, the first sensorial criterion of fresh produce

(Pollack, 2001). It is of particular importance for export markets, where competitive agents exploit cosmetic preferences to manipulate market access of suppliers, with resultant financial consequences. Lenticel discolouration, a superficial blemish that affects some cultivars by causing the fruit to exhibit a speckled appearance, illustrates this point. Such fruit is branded as less desirable and downgraded, even rejected, despite the fact that the speckled appearance does not affect the internal quality of the fruit. Blemish development is limited to the lenticel perimeter and immediately adjacent area, not extending deeper than the outermost layers of the fruit rind. The development of a red halo is the first indication of discolouration, with the redness sometimes intensifying and ending in the eventual localised darkened appearance of the lenticel. Lenticels on unaffected fruit display a confluent colouration with the normal rind colour of each cultivar.

Fruit rind, or more correctly, exocarp (*sensu* Esau, 1977) is a structure that is primarily composed of natural wax, cutin, epidermal layer, sub-epidermal tissue and structures such as stomata and trichomes. The stomatal origin of mango fruit lenticels gives the best indication of their morphological characterisation, since they are structurally different from the classic lenticels present on other plant species (Tamjinda *et al*., 1992; Du Plooy *et al*., 2003). Lenticels on mature mango are epidermal structures that form part of the exocarp due to their superficial nature and integration with sub-epidermal layers. By comparing only the cuticle and epicuticular wax layers, mango cultivars cannot be distinguished with certainty at microscopic level. However, combinations of macro morphological lenticel characteristics can be attributed to specific cultivars. Combined with the intricate matrix of resin ducts in the mango peel (Joel, 1980) and the resin chemistry (John *et al*., 1999), it could give some explanation why some cultivars are more prone to lenticel discolouration.

Reasons for the development of the condition have been investigated from various horticultural and post harvest management points of view (O'Hare & Prasad, 1992; Du Plooy *et al*., 2002; Willis & Duvenhage, 2002). Tamjinda *et al*. (1992) described the micro morphology and histochemistry of the discoloured lenticels on the cultivars Namdokmai and Falan. Du Plooy *et al*. (2003) and Bezuidenhout *et al*. (2003) investigated the development of this condition on 'Keitt' and 'Tommy Atkins', two mango cultivars that are commercially important to South African growers, with particular susceptibility to lenticel discolouration. The red colouration is linked to either anthocyanin (Kangatharalingam *et al*., 2002) or flavonoid (Dixon & Paiva, 1995) production and is suspected to be a mechanism of self-protection. Both groups of biochemicals originate from phenylalanine, a product of the Shikimate pathway. Secondary metabolites from the subsequent flavonoid pathway have a role in plant responses associated with biotic and abiotic stress signals

(Dixon & Paiva, 1995; Dixon *et al*., 2002; Robards, 2003) (Fig. 1). Flavonoids, for example, have been implicated in deterrence of herbivory (Berenbaum & Zangerl, 1992; Simmonds, 2003). Flavonoids comprise of a total of 12 subgroups, including the flavanoids. Anthocyanocides can be further subdivided into proanthocyanidins and anthocyanidins (Dixon & Paiva, 1995; Dixon *et al*., 2002; Robards, 2003). Several flavonoids and anthocyanins have been indicated as physiologically functional in screening against UV-A and UV-B by scavenging for reactive oxygen species (Robards *et al*., 1999; Heim *et al*., 2002). This functionality stems from the diversity of molecular structures that originate as hydroxylated derivatives of the benzo-γ-pyrone nucleus. These can undergo further conjugation between the aromatic rings, glycosidic units and methoxy groups. The number of structural elements creating the complexity of a flavonoid group is linked to its physiological functionality. It is proposed that lenticel discolouration is primarily a cosmetic condition (Du Plooy *et al*., 2003), and probably due to a physiological stress response by fruit tissue surrounding the lenticel entrance (Grassmann *et al*., 2002). Insight into the chemical nature of lenticel discolouration will give a better understanding of mango fruit physiology. This study is an investigation into the development and changes of the phenolic profiles of discoloured lenticels (in comparison to unaffected lenticels) and the corresponding cytological changes.

5.3 MATERIALS AND METHODS

5.3.1 Plant material

Physiologically mature mango fruit exhibiting varying intensities of lenticel discolouration (cultivars 'Tommy Atkins' and 'Keitt') was collected fortnightly from the packhouse of Bavaria Fruit Estates (Hoedspruit, Limpopo Province, South Africa) throughout the 2002/2003 and 2003/2004 seasons. For the cytological study and analysis of the phenolics, lenticels were collected in three predetermined colouration groups, namely non-discoloured (no visible discolouration), reddened and darkened lenticels (intense or dark red colouration).

5.3.2 Chemicals

Vanillin and phenolic substances used as authentic standards were purchased from Sigma Aldrich, (Munich, Germany). The electron microscopy reagents were purchased from SPI Supplies (SPI Supplies, West Chester, PA, USA). Solvents used in the extraction and HPLC were of analytical or HPLC grade and obtained from Merck Chemicals (Merck, Halfway House, South Africa).

5.3.3 Experimental

5.3.3.1 Microscopy

Samples for fluorescence microscopy and transmission electron microscopy (TEM) were dissected from mature mango fruit and immediately fixed in a mixture of 2.5 % glutaraldehyde and 2.5 % formaldehyde in a 0.1 M $NaPO_4$ buffer (pH = 7.3 ± 0.05). After standard rinsing and dehydration, the fluorescence microscopy samples were embedded in L.R. White resin, whilst the TEM samples were post fixed in OsO_4 and imbedded in Quetol 651 (Van der Merwe & Coetzee, 1992).

5.3.3.1.1 Fluorescence microscopy

Thin sections (0.5 - 1.0 µm) of the material embedded in L.R. White were cut on a Reichert Ultracut E (Reichert AG. Vienna, Austria) ultra microtome. Sections were heat fixed (60 °C) to glass microscope slides. Fluorescence was achieved by staining the tissue with Vanillin-HCl (1 % in concentrated HCl, w/v) (Guerin *et al*., 1971). Sections were viewed with a Zeiss Axiovert 200 (Zeiss, Göttingen, Germany) microscope fitted with a Nikon digital camera DXM1200 (Nikon Instech Co., Kanagawa, Japan). Digital images of emission at 397 nm, 515 nm and 590 nm were captured with Nikon ACT-1 version 2.

5.3.3.1.2 Transmission electron microscopy

Lenticel material fixed in Quetol 651 was cut on hte Reichert Ultracut E ultra microtome. Sections (ca. 90 nm thickness) were contrasted in 4 % aqueous uranyl acetate and Reynold's lead citrate (Reynolds, 1963) and viewed with a Philips EM301 transmission electron microscope (TEM) (Philips, Eindhoven, Netherlands) set at 20kV.

5.3.3.2 Extraction of phenolics

For the extraction of phenolics, discoloured lenticels were dissected from fresh fruit and immediately plunged into liquid N_2 after dissection, to terminate enzyme activity (polyphenol oxidase) (PPO), and freeze dried under vacuum (Vacutech Dura-Dry II™ MP, FTS Systems, Stone Ridge, NY, USA) for 72 hours. Freeze dried lenticels were ground in a marble mortar and pestle, keeping the three predetermined discolouration groups separate. For each discolouration category 0.05 g of powder was weighed into an Eppendorf tube and 1ml of a cold mixture of methanol: acetone: water (7:7:1, v:v:v) added and maintained at 4 °C to prevent oxidation. No antioxidant (ascorbic acid or $Na_2S_2O_5$) was used, since it would interfere with the Folin-Ciocalteau reagent used for determination of total phenolics (Regnier, 1994). The combined solvent/powder sample was placed in a vortex (Heidolph REAX, Jencons Scientific Ltd, Leighton Buzzard, UK) for 1 min, before

placing in an ice bath on an orbital shaker at 200 oscillations per min. After 30 minutes the supernatant containing the extract was separated by centrifuging at 10 000 rpm for 3 minutes. This extraction procedure was repeated three times and the supernatant fractions pooled. Organic solvents in the solvent mixture were removed by evaporation under N_2 flow and the volume adjusted to 1 ml with cold methanol. Free acid, glycosidically bound phenolics, ester bound phenolics and cell wall bound phenolics were isolated according to De Ascensao and Dubery (2003), and the fractions stored at 4 °C until further use.

5.3.3.3 Quantification of phenolics by the Folin-Ciocalteau method

The reaction mixture used was scaled down to facilitate the use of an ELISA plate technique for the quantification of phenolics (Table 1) (Sivakumar *et al*., 2005). According to this method, gallic acid was used to determine the equivalent phenolic acid content, which is a modification to the Folin-Ciocalteau method as described by Regnier and Macheix (1996). Spectrometric measurements of the phenolic concentrations in the various extracts was calculated from a standard curve ($y=0.0013x + 0.0177$, $r^2 = 0.9982$) determined by ANOVA and expressed as mg gallic acid equivalent per gram of dry weight (Table 2).

5.3.3.4 Reverse phase - high performance liquid chromatography

Thin layer chromatography gave non-distinctive results (data not shown); therefore HPLC was used as the preferred method of determination of phenolic constituents. Extracted phenolic fractions were analysed by means of reverse phase - high performance liquid chromatography (RP-HPLC). Triplicate separations of each extract were run without the addition of an internal standard, because overlapping could not be excluded.

The system for the HPLC analyses consisted of a Varian ProStar 230 solvent delivery system with manual injection and a Varian ProStar 310 UV-Vis detector fitted with a Degassit on-line solvent degasser (Varian, Walnut Creek, California). System control and results analysis was done using the Varian Star version 5.5 software. An excess injection volume of 25 µl of each sample was used in a 20 µl loop. The column was a C-18 reverse phase Inertsil ODS-3 column (250mm length, 4.6mm inner diameter, 5µm particle size) with an integrated guard column (Varian, Walnut Creek, California). The column was kept at 25 °C, with elution performed using a gradient program (Table 3) at a flow rate of 1ml/min.

Samples were run using two solvents and following the gradient program in Table 3. Mobile phase A consisted of distilled water acidified with trifluoroacetic acid (TFA) (pH = 3.58 ± 0.02) and mobile phase B of acetonitrile without any added modifier. After each run, the column was re-equilibrated with the initial conditions for 10 minutes. The detector was programmed for peak detection at 280 nm, which, although not optimum for ferulic acid and its derivatives, allowed simultaneous detection of hydroxybenzoic and hydroxycinnamic acids and their derivatives (Zhou *et al*., 2004). Identities of some peaks were by suggested by comparing their retention times to those of authentic standards run at the same gradient and conditions (Table 4).

5.4 RESULTS AND DISCUSSION

5.4.1 Microscopy

5.4.1.1 Fluorescence microscopy

Higher plants efficiently utilise phenylpropanoids such as hydroxycinnamic acid, cinnamoyl esters, and flavonoids, including flavones and flavonols, and anthocyanins to provide UV-A and UV-B screening (Dixon & Paiva, 1995; Dixon *et al*., 2002). Each species has a specific range of flavonoids responsible for UV screening, with combined capabilities more effective than screening by a single compound. The presence of these compounds in plant tissue was detected using Vanillin-HCl as a fluorescent dye to locate phenolics specifically (Guerin *et al*., 1971; Regnier, 1994).

No pronounced auto fluorescence could be observed in any of the unstained sections, although distinctive blue colouration was visible in cell walls (Fig. 2). The shorter excitation wavelength (365 nm) with emission of 397 nm gave the most useful distinction between different types of phenolics. Phenolics such as ferulic acid, caffeic acid and flavonoids were visible as bright white, blue, and red to brown-red zones (Fig. 3 - 5), with colouration indicative of different phenolic classes encountered (Table 5). No fluorescence in yellow and orange hues to signify anthocyanins, were detected. The limited auto fluorescence observed was attributed to the use of resin-stabilised material. At the time of sampling, it was not possible to provide fresh sections. However, L. R. White resin did not quench the effect of fluorescent stains, and the results were satisfactory.

Tissue surrounding non-discoloured lenticels exhibited minute amounts of red fluorescent phenolics (flavonoids). These were primarily located intracellular in vacuoles, but traces of cell wall bound red fluorescence were also present. Bright white fluorescence of the wax

fraction lining the cavity was indicative of simple phenolics such as gallic acid trapped in lenticular wax fractions (Fig. 3). No other fluorescence was detected in visible light, corresponding to the non-discolouration of the lenticel and the tissue surrounding it.

The visible discolouration of reddened lenticels was due to an accumulation of phenolics in the cell walls and vacuoles of the tissue surrounding the lenticel cavity (Fig. 4). The cell walls were mostly affected in areas of tissue associated with the lenticel and adjacent resin ducts. Cells that constituted the lenticel cavity walls also showed an accumulation of soluble ester bound phenolics. Deep red fluorescence, indicative of the presence of flavonoids, was limited to the cell walls.

In the darkened lenticels, some cells still contained fluorescent cytoplasm, indicating that the vacuoles were still intact. A bright white perimeter of the vacuole in some cells represented the protoplasm. The cell walls still fluoresced red, but less brightly so, with an orange to brown tone, indicating the deposition of polymeric phenolics (Guerin *et al*., 1971; Regnier, 1994), that tended to mask the red of the flavonoids. On the fruit surface, this masking effect was clearly seen as pertinent dark lenticels. In the final stages of discolouration, lenticels appeared darkened, and may become blackened. Growers and traders regard such darkened lenticels as necrotic lesions, giving rise to the use of the misnomer ‘lenticel damage’.

5.4.1.2 Transmission electron microscopy

Transmission electron microscopy gave a visual representation of the chronology of events and chemical changes during discolouration. Cell walls of mesophyll cells in the immediate vicinity of the lenticel lumen were investigated. In non-discoloured lenticels (Fig. 6A & B), cell walls were characteristically striated and granular, corresponding to multidirectional layering of cellulose in the secondary cell wall (Swanson & Webster, 1977). There was no evidence of development of any electron dense material, which would indicate deposition of secondary metabolites in the cell wall. Cellular contents consisted of large vacuoles, plastids, nuclei, endoplasmic reticulum, ribosomes and other micro-organelles, and Golgi apparatus. Vacuolar contents were granular and highly dispersed, often with a somewhat coagulated appearance due to precipitation during fixation (Scalet *et al*., 1989).

Membrane integrity and subsequent continuation of metabolic activity was clear through the presence of intact endoplasmic reticuli and Golgi apparatus visible in both reddened (Fig. 7A & B) and darkened (Fig. 8A & B) lenticels. This indicated that the intensifying

discolouration was not due to a release of PPO when phenolics accumulate (Beckman, 2000), which was contrary to previous conclusions (Bezuidenhout *et al*., 2003). Rather, phenolics were incorporated into the cell wall and storage compartments of the implicated cells. Lenticel discolouration, therefore, was observed as a controlled process whereby the lenticel cavity was encapsulated with cells containing phenolic-laden vacuoles (Fig. 7A & 8A) that were prepared for resistance against pathogen attack (Dixon & Paiva, 1995; Beckman, 2000). A diversity of factors has been observed as the trigger or signal for this self-protection mechanism, which has made lenticel discolouration difficult to manage (Loveys *et al*., 1992; Taminja *et al*., 1992; Willis & Duvenhage, 2002; Bezuidenhout *et al*., 2003). In this study, phenolic deposition was only observed in, and therefore limited to, tissue in the vicinity of the lenticel cavity. Evidence from some sections viewed suggested that resin ducts may act as the transport route for elicitors of the phenolic reactions associated with cell wall deposition (Diaz *et al*., 1997). Furthermore, electron-dense material in the cell wall gradually increased from the outside of the cell wall to the inside (Fig. 7B & 8B), indicating the arrival of cell wall linking reactants through the apoplastic route.

5.4.2 Quantification of phenolics by the Folin-Ciocalteau method

Values obtained through quantification of phenolics by the Folin-Ciocalteau method were indicative of the presence of soluble phenolics. Total phenolics were highest in darkened lenticels, which meant that the soluble phenolics present in vacuoles of cells in dissected lenticel tissue have increased. This concurred with findings reported in the review by Beckman (2000) that specialised phenolic storing cells are located at localities where infection or stress can be contained. As were seen from the TEM micrographs, there was no membrane damage signalling the liberation of PPO. The increasing reddening and eventual darkening of the lenticels is therefore due to the build-up of phenolics in the vacuoles (Regnier & Macheix, 1996; Wink, 1997). A build-up of phenolics could be toxic to adjacent tissue, and cells containing vacuoles filled with phenolics were isolated by cross linking of polymeric ferulates (insoluble) in the cell wall (Beckman, 2000). Leaking phenolics are contained by the denser, strengthened walls (Fig. 8A & B).

5.4.3 Reverse phase - high performance liquid chromatography

Extraction of the phenolics from both non-discoloured and discoloured lenticels yielded highly concentrated samples. The modified Folin-Ciocalteau method (Sivakumar *et al*., 2005) for the determination of phenolic acid content was particularly suited to the small quantities of sample material available for this study. Apart from a crude extract, four targeted extractions were done to obtain free phenolic acids, glycoside bound phenolic

acids, ester bound phenolic acids, and cell wall bound phenolic acids. A gallic acid equivalent calibration curve (Fig. 9) was used determine the abundance of each fraction in the sample material (Fig. 10). These values are representative of the relative abundance of each fraction in the extract. Non-discoloured lenticels had lower concentrations of all the phenolic fractions, followed by red discoloured and lastly, dark discoloured lenticels. This is in agreement with phenolic acid functionality as discussed by several studies (Dixon & Paiva, 1995; Beckman, 2000; Dixon *et al*., 2002; Grassmann *et al*., 2002; Zhou *et al*., 2004). Although high, crude extract values does not reflect the combined values of the four other phenolic acid fractions extracted with more specific hydrolysis reactions.

Fluorescence microscopy gave some indication of phenolic acids that could be expected after extraction. Derivatives of cinnamic and ferulic acid would be polar and hydrophilic, while polymeric phenolics would be less polar and more hydrophobic. Any co-extracted biopolymers such as terpenes would be very hydrophobic (Guerin *et al*., 1971; Regnier & Macheix, 1996). Effective separation by gradient elution was achieved by exploiting the differences in the hydrophilic/hydrophobic nature of the mobile phase constituents, as well as the polarity of eluted molecules. Phenolics were adsorbed onto the stationary phase at low solvent strength through Van der Waals interactions, and were selectively released according to their decreasing ability to participate in hydrogen bonding at distinct solvent strengths (Cunico *et al*., 1998).

Results from the RP-HPLC investigation could not be quantified to satisfaction and were therefore be presented qualitatively only (histograms in Fig. 11 - 15). Representative chromatograms for non-discoloured, reddened and darkened lenticels extracts were included for each group of isolated phenolics (chromatograms in Fig. 11 - 15). Despite the small quantity of tissue that was used for extraction, highly concentrated extracts were obtained. Previous studies into mango phenolics involved the fruit pulp only, and the results from this study could not be related directly to these studies.

Changes in the phenolic composition within each colouration group were referenced against a single major peak present within all three chromatograms of the compared extraction set (histograms in Fig. 11 - 15). Bar heights in the histograms, therefore, did not indicate absolute peak sizes, but rather relative changes in the ratio of compared peaks. That meant that higher bars indicated bigger numerical differences. In this way, trends of changes in concentrations within a set of extractions were indicated by changes in the heights of the histograms for each peak investigated.

From each of the extracts evaluated, the more polar, labile phenolics such as gallic acid, proto catechuic acid and its derivatives, epicatechin and *p*-coumaric acid (Rodríguez-Delgado *et al*., 2001) were eluted from the column within 23 minutes. After this time, the gradient increased from favouring predominantly hydrophilic compounds to favouring more hydrophobic oligo- and polymeric compounds such as quercetin and kaempherol (Regnier & Macheix, 1996; Markham *et al*., 2000; Rodríguez-Delgado *et al*., 2001). All extracts from non-discoloured lenticels had the lowest concentrations of the more hydrophobic oligomeric phenolics, whereas reddened lenticels had the most oligomeric phenolics compounds. This indicated that free acids became increasingly incorporated into complex phenolics through metabolically driven defence strategies (Dixon & Paiva, 1995; Beckman, 2000; Passardi *et al*., 2004). Moreover, increases in polymeric phenolics were correlated with decreases in the concentration of soluble and glycosidically bound phenolics, such as feruoyl glycoside, in the vacuoles. Ferulic acid in particular becomes bound to the cell wall structure (Lozovaya *et al*., 1999), with analogous situations described by Beveridge *et al*. (2000). This shift could be seen in increased numbers and complexity of peaks between 30 - 50 minutes. Increased retention time was indicative of a decrease in analyte polarity and thus solubility. The result correlated with previous findings on the physiological activity of various flavonoids and phenolics (Ketsa & Atantee, 1998; Dixon *et al*., 2002). Some peak broadening that occurred may be alleviated, and the sensitivity increased, by adding TFA to the acetonitrile as well. The TFA acts as an ion pair agent and reduces the pH of the mobile phase, thereby minimising the interaction of the phenolics with the silanols of the column packing (Cunico *et al*., 1998). However, not all peaks were affected and sufficient differences could be identified.

Crude extracts (Fig. 11) of non-discoloured, reddened and darkened lenticels pointed towards the presence of both polar and less polar compounds. Individual separation of the latter peaks was difficult to achieve due to their polymeric nature. Nevertheless, distinct differences between the phenolic profiles of non-discoloured, reddened and darkened lenticels were detected. According to retention times from table 3, peak *d* at t_r = 14.212 min was chlorogenic acid (chromatograms, Fig. 11). The ratio of this peak to the first three polar compounds in all crude extracts was the biggest in that of the *d:a* ratio of reddened lenticels. Due to the increase in concentration of chlorogenic acid for reddened lenticels, and a simultaneous decrease of the compound at t_r= 26.109 min, there was a marked increase of the *d:e* ratio. The *d:h* ratio for both reddened and darkened lenticels, relative to this ratio of non-discoloured lenticels, indicated that there was an increase in the compound at t_r= 32.161 min in both types of discoloured lenticels. Finally, the chlorogenic acid concentration in the crude extract of both reddened and darkened lenticels exceeded

that of non-discoloured lenticels. This result correlates with previous findings in which chlorogenic acid has been associated with induced stress before (Mayr *et al*., 1994; Robards *et al*., 1999).

In the extract of non-conjugated phenolics (free acids, Fig. 12), all the peaks were compared to the chlorogenic acid peak (*c*) at t_r= 14.467 minutes. There were significant changes between the ratios of initial peaks and those associated with oligomeric phenolics. The *c:a* and *c:b* ratios (i.e. ratios of small, polar free acids) for reddened lenticels were markedly different from the corresponding ratios for non-discoloured and darkened lenticels. Furthermore, decreasing ratios between more complex compounds and chlorogenic acid in non-discoloured and reddened lenticels indicated that these compounds were increasing in darkened lenticels (Kahn, 1985; Beveridge *et al*., 2000). Non-discoloured lenticels had very large ratios for *c:h*, *c:i* and *c:j*, indicating a predominance of free acids to other phenolics. These free acids were less prevalent in reddened lenticels (chromatogram, Fig. 12) than in non-discoloured and darkened lenticels.

Hydrolysed extracts of cell wall bound compounds from non-discoloured, reddened and darkened lenticels yielded two distinctive peaks (Fig. 13). Peak *d* (t_r= 24.837 min) and peak *e* (t_r= 26.055 min) were present in a similar ratio for all three groups of extracts. However, when peak *d* was compared to other peaks from each extract, the metabolic translocation of polar components to the cell wall became clear (Passardi *et al*., 2004). Hydrolysis depolymerised the glycans bound to the cell wall matrix, which were present in higher concentrations in discoloured lenticels than in non-discoloured lenticels (histograms in Fig. 13). In discoloured lenticels a small, polar fraction at t_r= 4.602 min was absent from non-discoloured lenticels, indicating the functionality of this molecule in cell wall deposited phenolics (Lozovaya *et al*., 1999). Oligo-and polymeric phenolics were present in stable quantities in all three groups of extracts, which is a further indication that the disassembled polar compounds originate from the primary components that contribute to cell wall reinforcement (Kahn, 1985; Lozovaya *et al*., 1999; Beckman, 2000). Redistribution of the constituent compounds in cell wall bound phenolic extracts was most noticeable in reddened lenticels, as were seen from differences in the *d:a*, *d:b*, *d:c*, and *d:f* ratios.

For the chromatograms of glycoside bound extracts from non-discoloured, reddened and darkened lenticels (Fig. 14), peaks *a-g* within each colouration set were compared to peak *b*, a small, polar phenolic acid at t_r= 6.852 min that corresponded closest to gallic acid.

Removal of the more soluble glycoside moieties by mild hydrolysis liberated the phenolic free acids at these early retention times. Within each of the three lenticel colour groups, the peak sizes showed relatively small changes in the peak ratios (histograms in Fig. 14). This corresponds with enzymatic hydrolysis during cellular utilisation of glycoside bound phenolics (Ferreres *et al*., 1997; Heim *et al*., 2002), with increasingly bound phenolics showing in more dense vacuolar contents (Fig. 6 - 8).

Apart from free acids, ester bound phenolics are the most labile and is reflected as such in the chromatograms in Fig. 15. Peaks *a* - *i* within each set of lenticel extracts were compared to peak *c* (t_r = 14.416 min), with the peak ratios of *c:a*, *c:f* and *c:l* in non-discoloured lenticels markedly higher than the same ratios in the discoloured lenticels (histograms in Fig. 15). This was indicative of the conversion of these compounds into less labile (cell wall bound) compounds (Lozovaya *et al*., 1999; Beckman, 2000; De Ascensao, & Dubery, 2003) that contribute to cell wall reinforcement (Ketsa & Atantee, 1998; Russel *et al*., 1999).

5.5 CONCLUSION

Lenticels are points vulnerable to possible infection in ripening, senescent fruit. Phenolic accumulation presents rapid and efficient blocking and protection against such infection (Matern, 1994; Wink, 1997; Robards, 2003). Discolouration was morphologically characterised by vacuolar phenolics as well as accumulated phenolics in the cell walls of the mesophyll cells surrounding the lenticel cavity and proximal tissue. The resultant externally visible differences between non-discoloured, reddened and darkened lenticels were substantiated by both fluorescence microscopy and TEM. Chronological cell wall discolouration was illustrated by TEM results as increased electron density. The discolouring process happens from the outer boundaries of the cell wall towards the inside of the cell, showing apoplastic transport of the reaction elicitors. There was a progression in bound phenolics in the cell walls and the vacuoles as the intensity of lenticel discolouration increased. The reddening of the encapsulating tissue surrounding the lenticels were related to the build-up of soluble phenolics in the vacuoles of mesophyll cells in the lumen walls (Robards, 2003). Deposition and cross linking of phenolics in cell walls further contributed to darkening of the lenticels. Initial vacuolar inclusions consisted of simple phenolics that appeared granular in TEM micrographs due cross-reaction during fixation. In reddened and later, darkened lenticels, the vacuolar contents become increasingly coagulated, electron-dense and hardened, with corresponding changes in the

fluorescent qualities. Although hardening of phenolics could be described as an artefact of the fixation reactions, their chemical reaction to fixatives is linked to increasing complexity of the phenolics in the vacuoles as they become more conjugated in advanced stages of lenticel discolouration (Scalet *et al*., 1989). The observations were correlated by the results of the total phenolics determination, where an increase in colour is directly related to an increase in total soluble phenolics.

Cell wall bound phenolics form red zones with Vanillin-HCl staining. The red, however, was slightly masked by the brown colouration of quinone-related compounds in discoloured lenticels (Guerin *et al*., 1971; Regnier, 1994). Nevertheless, it is still possible to deduce that the conversion of soluble phenolics into cell wall bound polymeric compounds ultimately produced discoloured lenticels. No evidence could be found that the red discolouration is related to anthocyanins accumulating in the lenticel and surrounding tissue, since the results in this study indicated the presence of hydroxycinnamic acid derivatives and flavonols.

Blackened lenticels that are sometimes observed may be the result of cell death when toxic phenolics are released from ruptured vacuoles. Permeation or infiltration of such phenolics would be restricted by the reinforced cell walls. The lenticel, immediate surrounding tissue and sub-cuticular mesophyll are protected from infection by an efficient barrier of toxic phenolics and a process of structural reinforcement.

Extracts from isolated lenticels rendered fewer flavonoids than anticipated. Comprehensive arrays of peaks representing a wider collection of phenolics were only obtained from the extracts for non-conjugated phenolic acids and ester bound derivatives. RP-HPLC results indicated a high level of reproducibility between lenticel extracts of similar colouration, but extraction validation was inadequate, with consequent unsatisfactory quantification. This was due to the following reasons:

- lenticels were dissected from fruit by hand.
- lenticel tissue was not cut from fruit of similar acidity, ° Brix and firmness.
- slow progress in dissection meant that lenticel tissue was not cut in the same time period. Fruit physiology was therefore not exposed to the same environmental conditions before and during sample collection.
- dissection by hand could lead to poor reproducibility of the quantity of fruit pulp included in each section surrounding the lenticel.

- small quantities of lenticel tissue sampled (0.5 g) will amplify any interference from pulp tissue.
- internal standards were not initially added to extracts, since peak overlapping could not be predicted, and could not be added and accurately quantified after initial removal of any volume of extract for qualitative sampling.

HPLC results confirmed that the discolouration is a self-defence strategy to protect the lenticel and immediate surrounding tissue (Dixon & Paiva, 1995; Robards *et al*., 1999; Beckman, 2000; De Ascensao & Dubery, 2003) and correlated with results from fluorescence microscopy and TEM of the lenticels. No disrupted membranes were observed, and discoloured lenticels were still alive; this supports the idea that lenticel discolouration is the manifestation of an active self-defence mechanism, due to environmental stress being applied to the fruit. The signal for stress related phenolics to develop, and the cultivar sensitivity towards this signal is still unclear. Lenticel discolouration can not be eradicated, but could be controlled through adjusted management of horticultural and packhouse practices.

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5.7 TABLES

Table 1 Table with absorbance values of gallic acid used as a phenolic standard to construct a calibration curve (y=0.0013x + 0.0177, r2 = 0.9982) (De Ascensao & Dubery, 2003)

c (ug/ml)	A
0	0.000
10	0.033
50	0.091
100	0.153
150	0.221
200	0.287
300	0.402
350	0.476
400	0.541

Table 2 Table with gallic acid equivalents of the phenolics acid fractions present in each of the three colour groups for the lenticels. Values for phenolic concentration is expressed as gallic acid equivalent per gram dry weight

Phenolic fraction for which gallic acid equivalent was determined	Colour grouping used for lenticels		
	Non-discoloured lenticels	Red discoloured lenticels	Dark discoloured lenticels
Cell wall bound	0.021	0.167	0.330
Ester bound	0.143	0.554	0.420
Glycocidically bound	0.268	0.830	0.702
Free acids	0.132	0.745	0.485
Crude extract	0.825	2.421	2.116

Table 3 Gradient program used for the HPLC analysis of phenolics extracted from mango lenticels

Time	Flow Rate (ml/min)	%A	%B
Initial	1.0	90	10
20.00	1.0	80	20
33.00	1.0	75	25
38.00	1.0	65	35
43.00	1.0	53	47
55.00	1.0	30	70
57.00	1.0	20	80
59.00	1.0	20	80
60.00	1.0	90	10

Table 4 Table with retention times for selected standards, with suggested occurrence of these phenolics in investigated lenticel extracts. Non-conjugated phenolics are referred to as free acids

Standard	Class of Phenolics (Robards *et al*., 1999)	Retention time (minutes)	Lenticel extract occurrence
Gallic acid	Phenolic acid	7.866	Crude extracts, Ester bound extracts, Glycosidically bound extracts
Chlorogenic acid	Cinnamic acid ester	14.966	Crude extracts, Free acid extracts, Ester bound extracts, Glycosidically bound extracts
Mangiferin	Xanthone	17.690	Glycosidically bound extracts
Caffeic acid	Cinnamic acid	19.283	Crude extracts (red, dark only), Free acid extracts
Quercetin	Flavonol	23.501	Cell wall extracts
Sinapic acid	Cinnamic acid	27.592	Crude extracts (red, dark only), Free acid (non-discoloured only), Ester bound extracts, Glycosidically bound extracts (red, dark only)
Ferulic acid	Cinnamic acid	29.037	Crude extracts, Free acid (non-discoloured, red only), Glycosidically bound extracts (non-discoloured, dark only)
Coumaric acid	Cinnamic acid	40.201	Crude extracts, Free acid (non-discoloured, dark only), Ester bound extracts (non-discoloured, red only), Glycosidically bound extracts (non-discoloured only), Cell wall extracts

Table 4 Table of fluorescence colours and the associated phenolics groups (Guerin *et al*., 1971; Regnier & Macheix, 1996)

Fluorescence colour	**Group of Phenolics**
White	Gallic acid / Caffeic acid
Blue-white	Coumaric acid
Light Blue	Hydroxycinnamic acid & Ferulic acid derivatives
Red	Flavonoids
Brown	Quinone
Deep blue / Purple	Not phenolic - Biopolymers, Terpenoids

5.8 FIGURE CAPTIONS

Figure 1 Overview of the biochemical pathway along which anthocyanocides and flavonoids are formed. Only key intermediates and enzymes, and products of interest are indicated.

Figure 2 Weak auto fluorescence in the unstained sections still exhibited distinctive, albeit faint, blue colouration in cells that were filled with phenolics (a).

Figure 3 Fluorescence of a non-discoloured lenticel sectioned through the lenticel cavity. Insignificant build-up of phenolics in the cell walls of surrounding mesophyll tissue (a), and some vacuolar phenolics were visible inside cells (b). Intra-cavital wax phenolics were visible as bright halos on the outer surfaces of cells lining the lenticel walls (c).

Figure 4 Fluorescence of reddened lenticel sectioned through the area adjacent to the lenticel cavity. Random build-up of phenolics in the cell walls of surrounding mesophyll tissue was visible (a). The contents of cells forming part of the cavity wall was differentiated from that of cells further away by the blue fluorescence (gallic and ferulic acid derivatives) (b), with cytoplasmic cinnamic acids evident as white fluorescence on their inner surfaces (c) (Guerin *et al*., 1971).

Figure 5 Fluorescence of darkened lenticel sectioned through an area adjacent to the lenticel cavity. Build-up of phenolics in the cell walls of surrounding tissue became more confluent (a), with encapsulation of the lenticel evident. Fluorescence of the vacuolar content of cells lining the cavity wall changed from blue to blue-white, indicative of caffeic acid (b). The white fluorescence due to cinnamic acids in the cytoplasm surrounding the vacuoles was visible (c). Deposition of polymeric phenolics in the cell walls caused the red fluorescence to have a brownish tinge (Guerin *et al*., 1971).

Figure 6 Transmission electron micrograph of cells from the wall of the cavity of a non-discoloured lenticel. Organelles and membranes are intact, with the endoplasmic reticulum indicated by the black arrows (A). There is no indication of electron dense areas developing in the cell walls (B). Vacuolar contents appeared granular (white arrows in A & B) due to some precipitation of phenolics during the fixation process.

Figure 7 Transmission electron micrograph of cells from the wall of the cavity of a reddened lenticel. All intracellular structures were intact, with abundant endoplasmic reticulum visible (black arrows, A). Deposition of electron dense cell wall material was visible from the outer boundary of the cell wall (B), indicating transport by the apoplastic route rather than secreted from inside the cell.

Figure 8 Transmission electron micrograph of cells from the wall of the cavity of a darkened lenticel. Dense and phenolic rich vacuolar content is signified by uneven cutting marks typical of the phenolic contents hardening during fixation of the material (1 in A, arrow in B). Metabolic functionality was apparent from the intact cellular structures (2 in A). The cell walls were impregnated with electron dense material (3 in A).

Figure 9 Calibration curve of gallic acid as a phenolic acid standard for determination of equivalent phenolic concentrations in the sample material.

Figure 10 Graph of gallic acid equivalents of each isolated phenolic fraction in the dried sample material.

Figure 11 Chromatograms of crude extracts from non-discoloured, reddened and darkened lenticels, with the combined histogram of the changes in peak ratios between the different lenticels. Peaks a - h within each colouration set were compared to peak d.

Figure 12 Chromatograms of non-conjugated phenolics (free acids) extracted from non-discoloured, reddened and darkened lenticels, with the combined histogram of the changes in peak ratios between the different lenticels. Peaks a - j within each colouration set were compared to peak c.

Figure 13 Chromatograms of extracts of cell wall bound components from non-discoloured, reddened and darkened lenticels, with the combined histogram of the changes in peak ratios between the different lenticels. Peaks a - j within each colouration set were compared to peak d.

Figure 14 Chromatograms of glycosidically bound extracts from non-discoloured, reddened and darkened lenticels, with the combined histogram of the changes in peak ratios between the different lenticels. Peaks a - g within each colouration set were compared to peak b.

Figure 15 Chromatograms of ester bound extracts from non-discoloured, reddened and darkened lenticels, with the combined histogram of the changes in peak ratios between the different lenticels. Peaks a - i within each colouration set were compared to peak c.

5.9 FIGURES

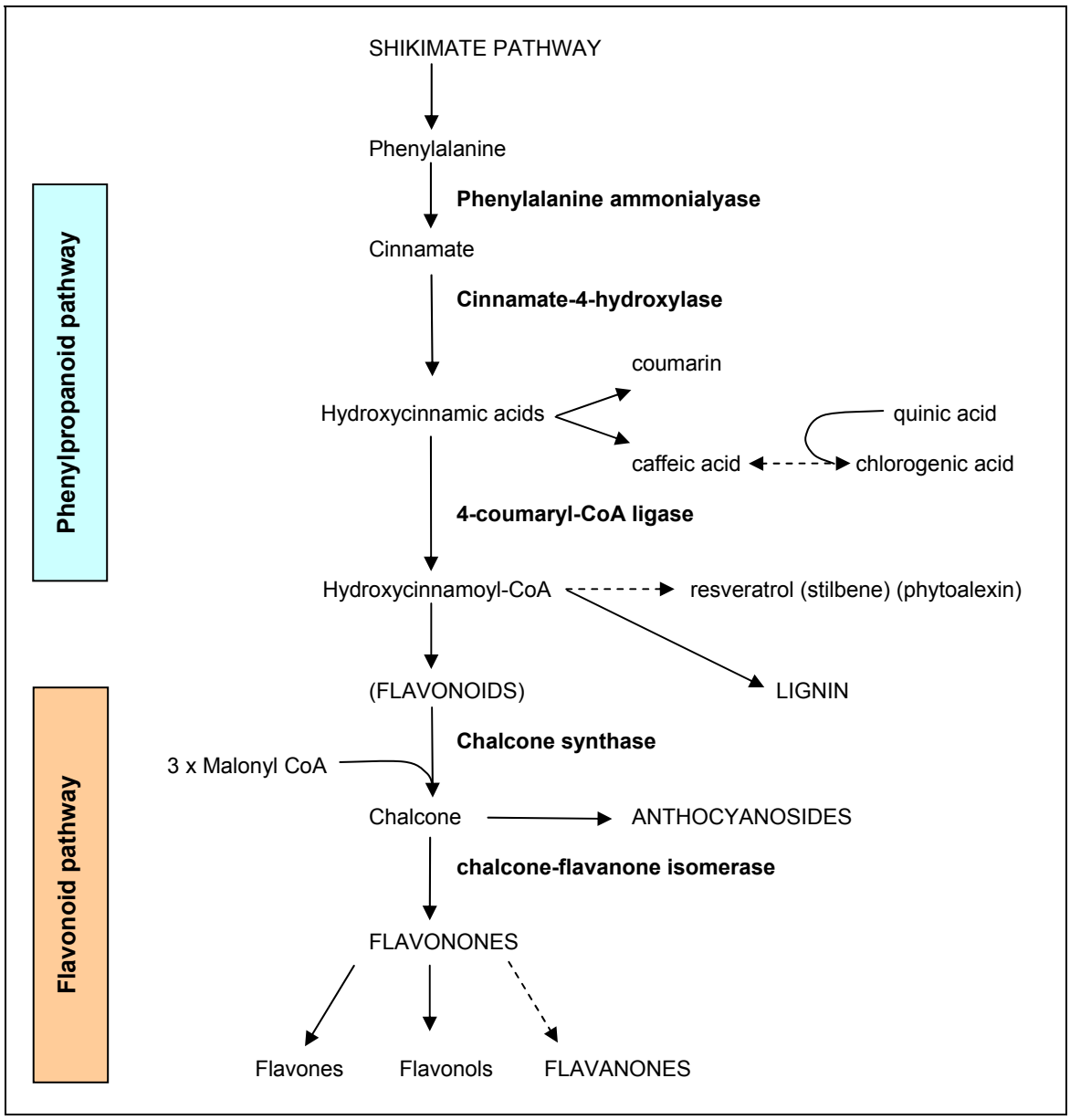


Figure 1

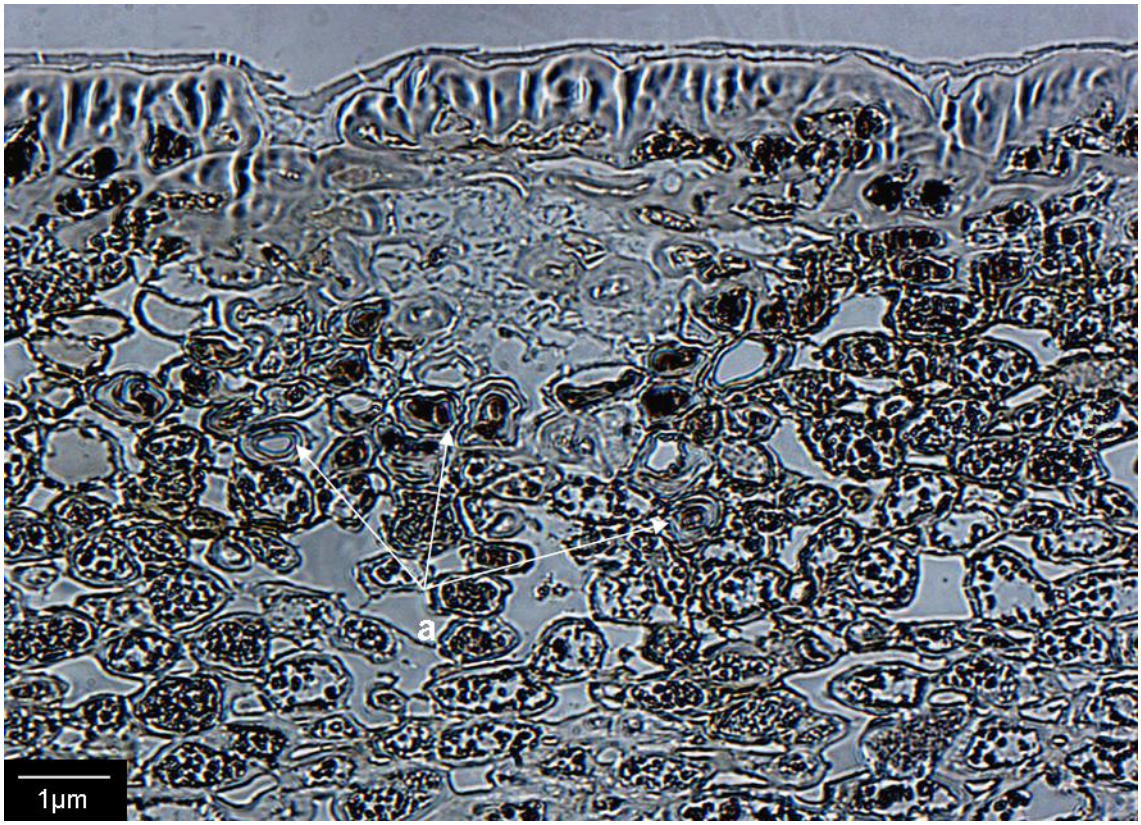
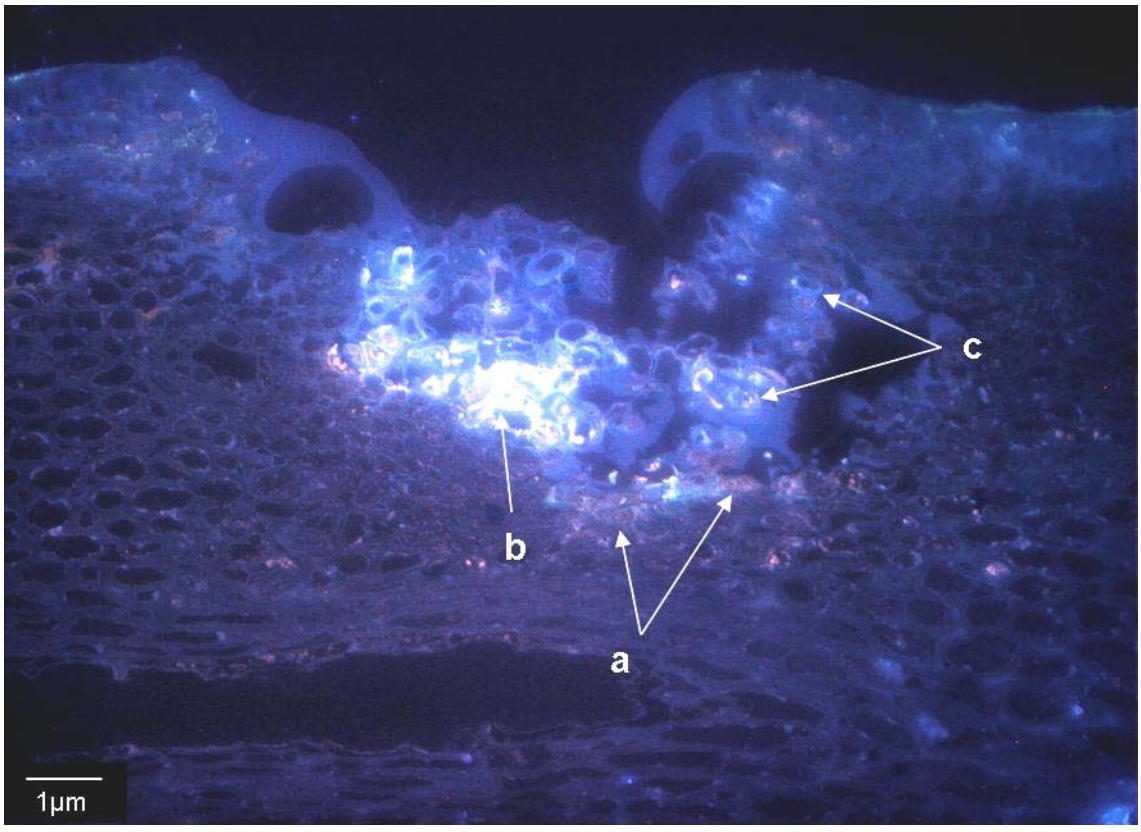


Figure 2



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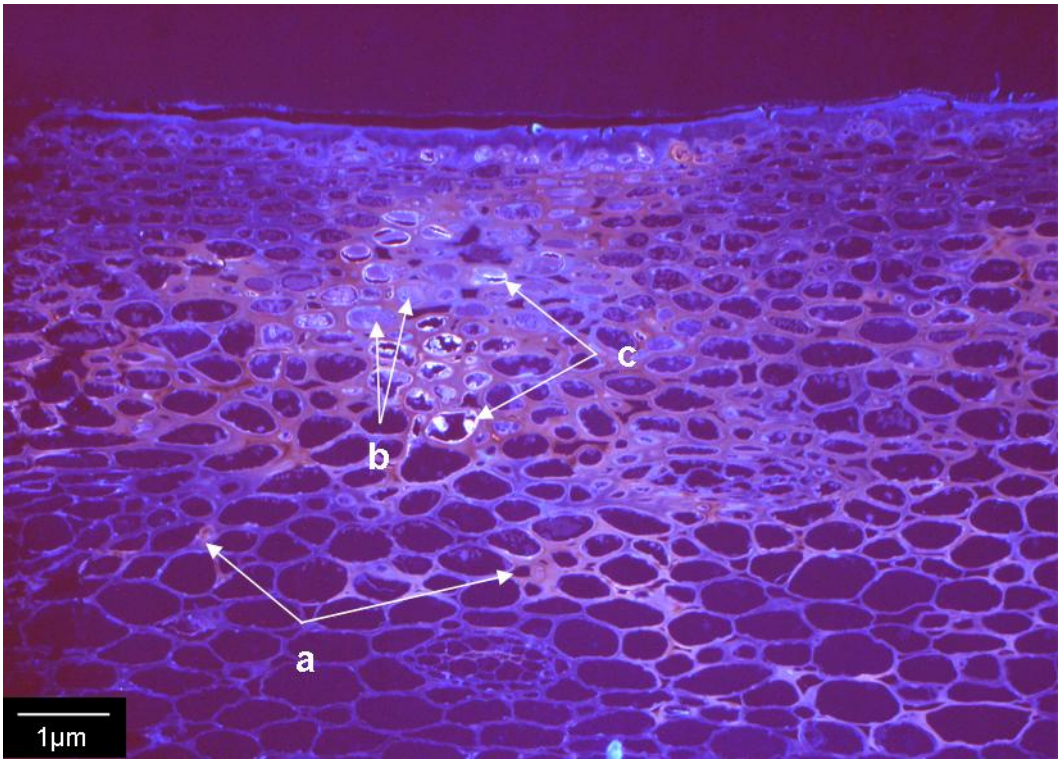

Figure 4

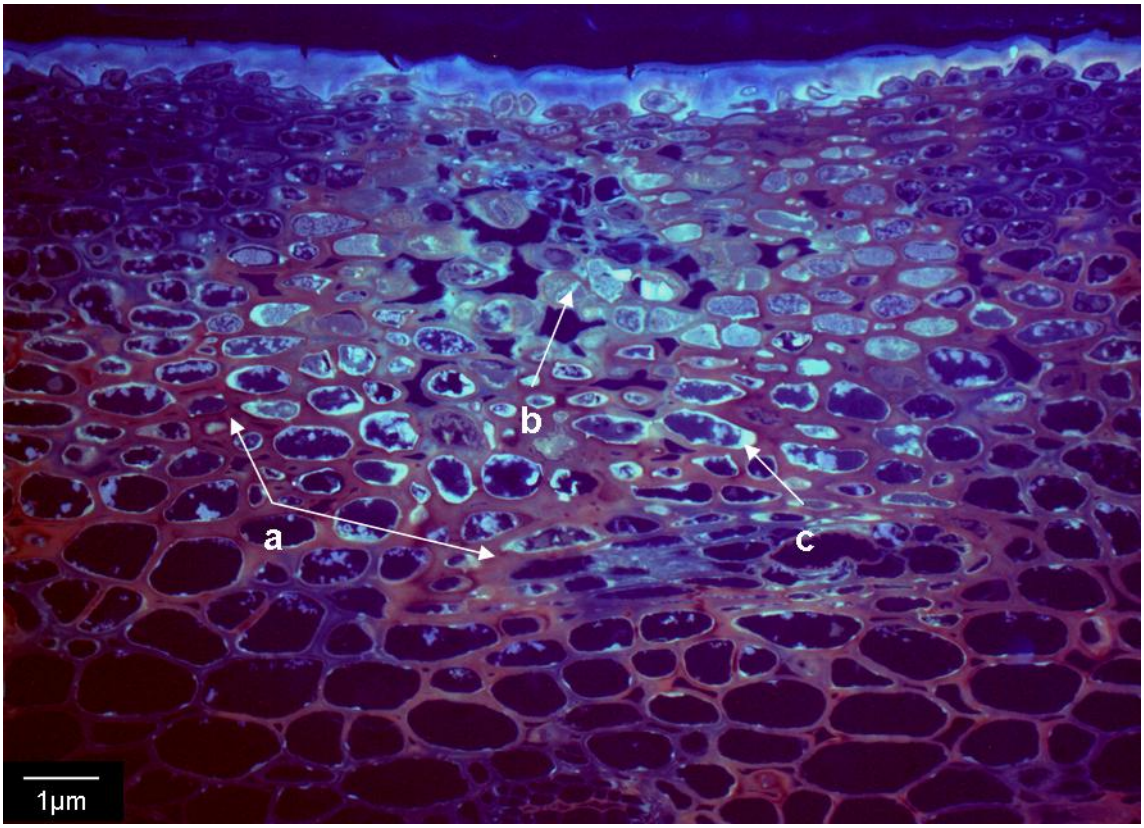

Figure 5

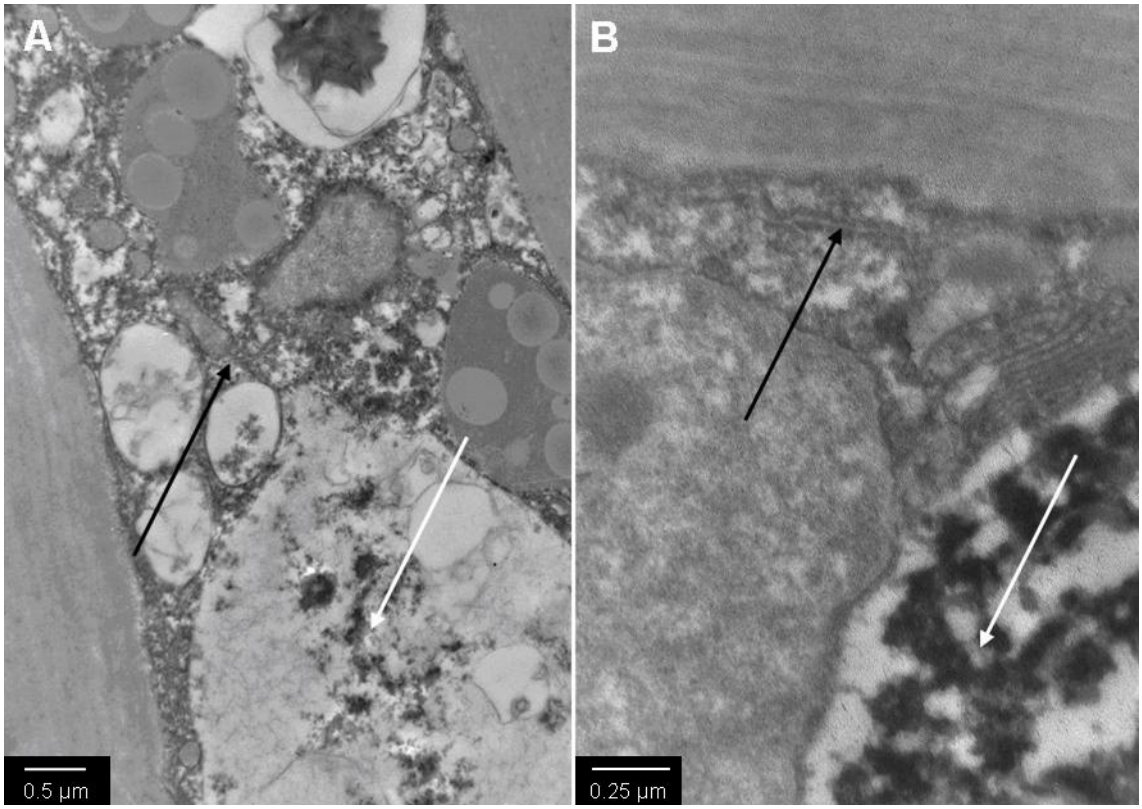

Figure 6

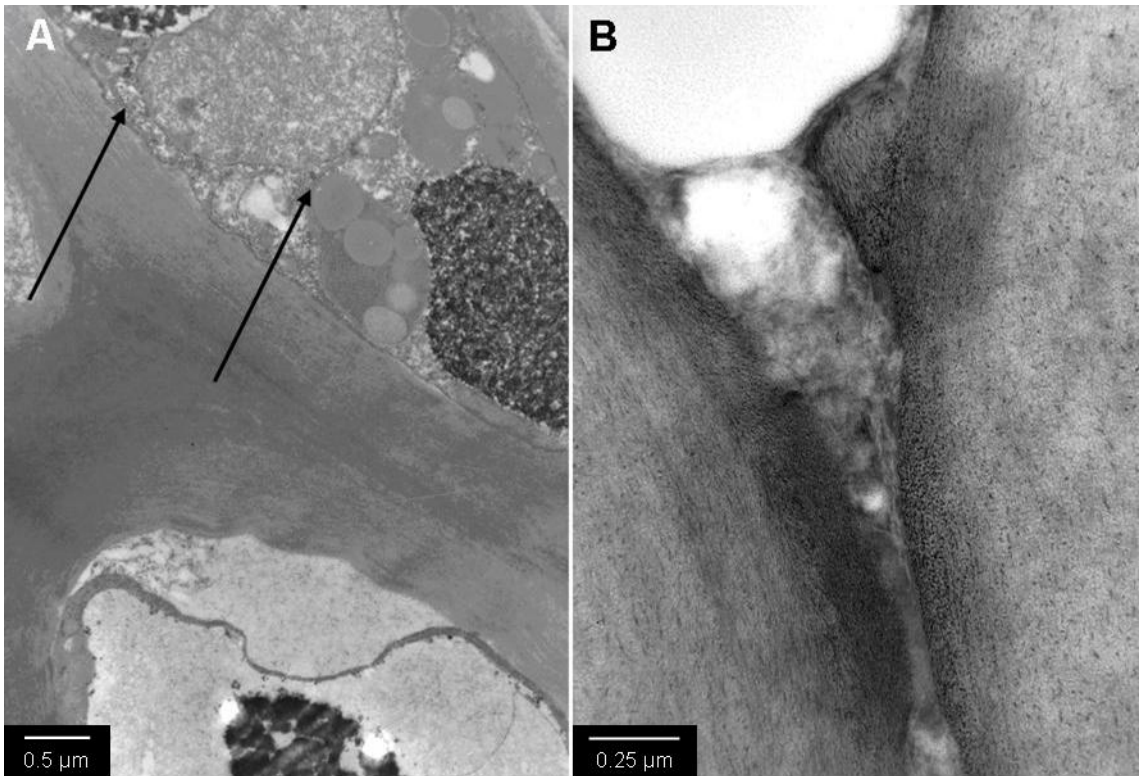

Figure 7

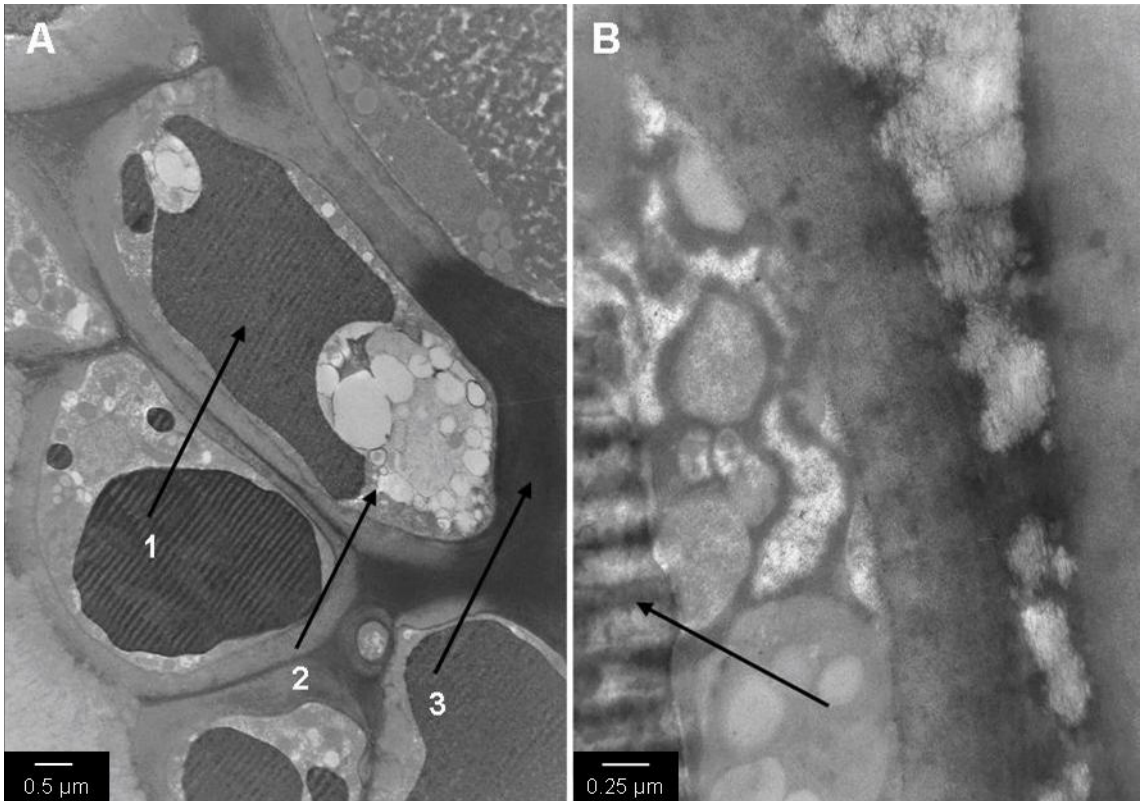

Figure 8

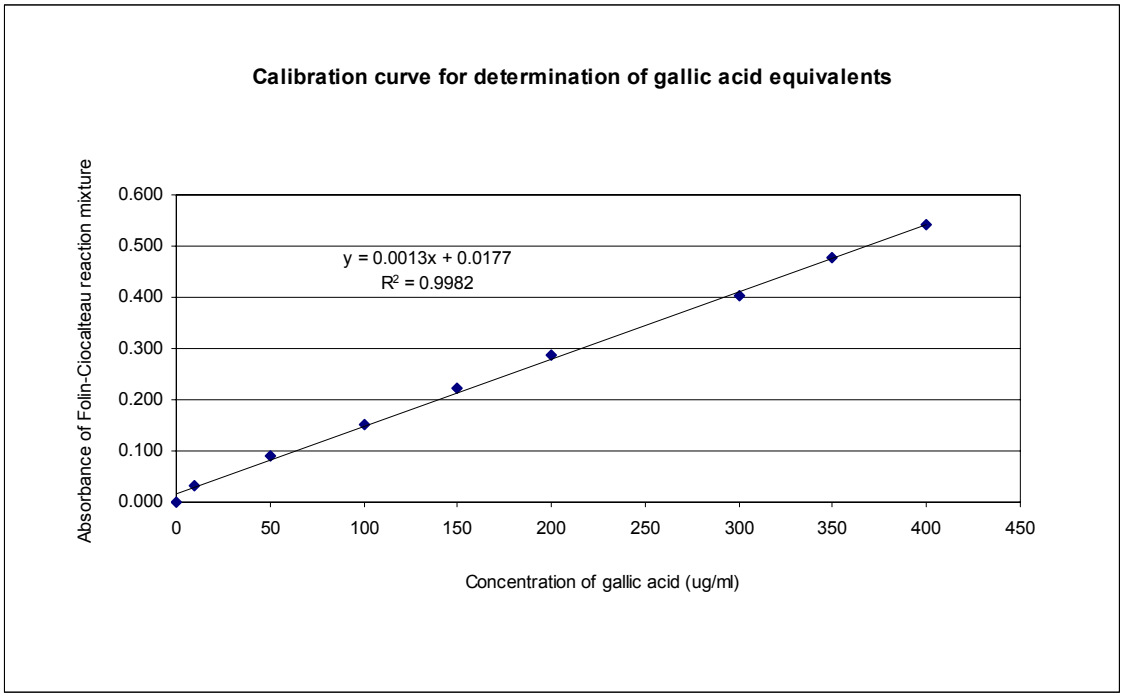

Figure 9

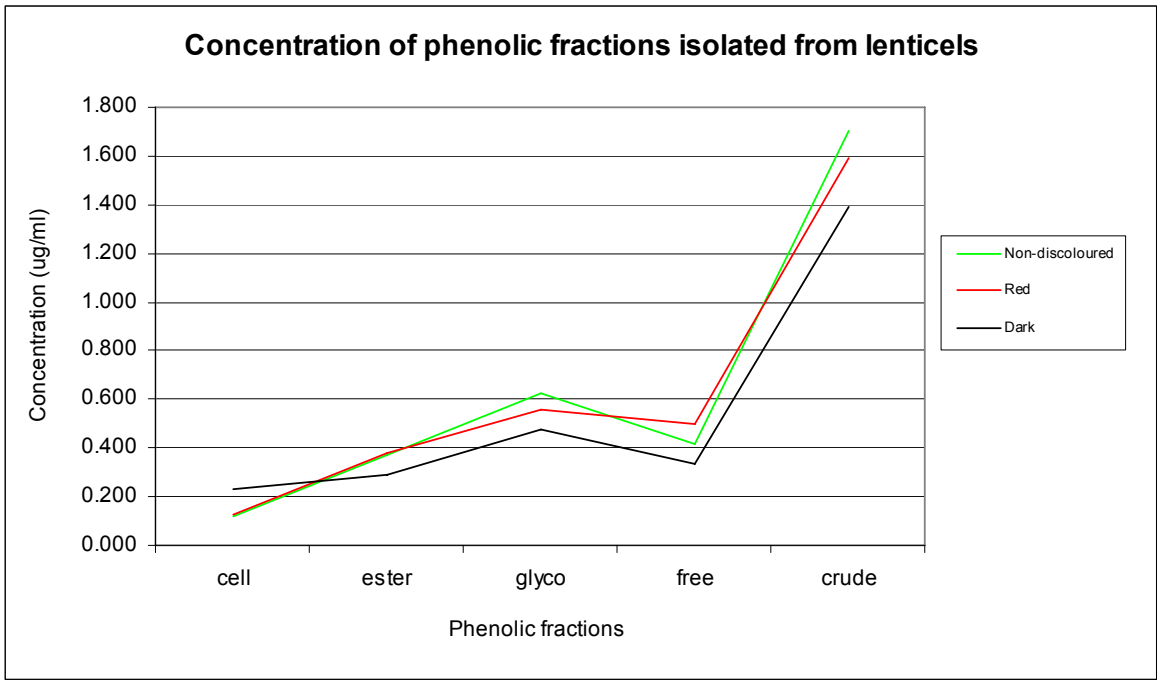

Figure 10

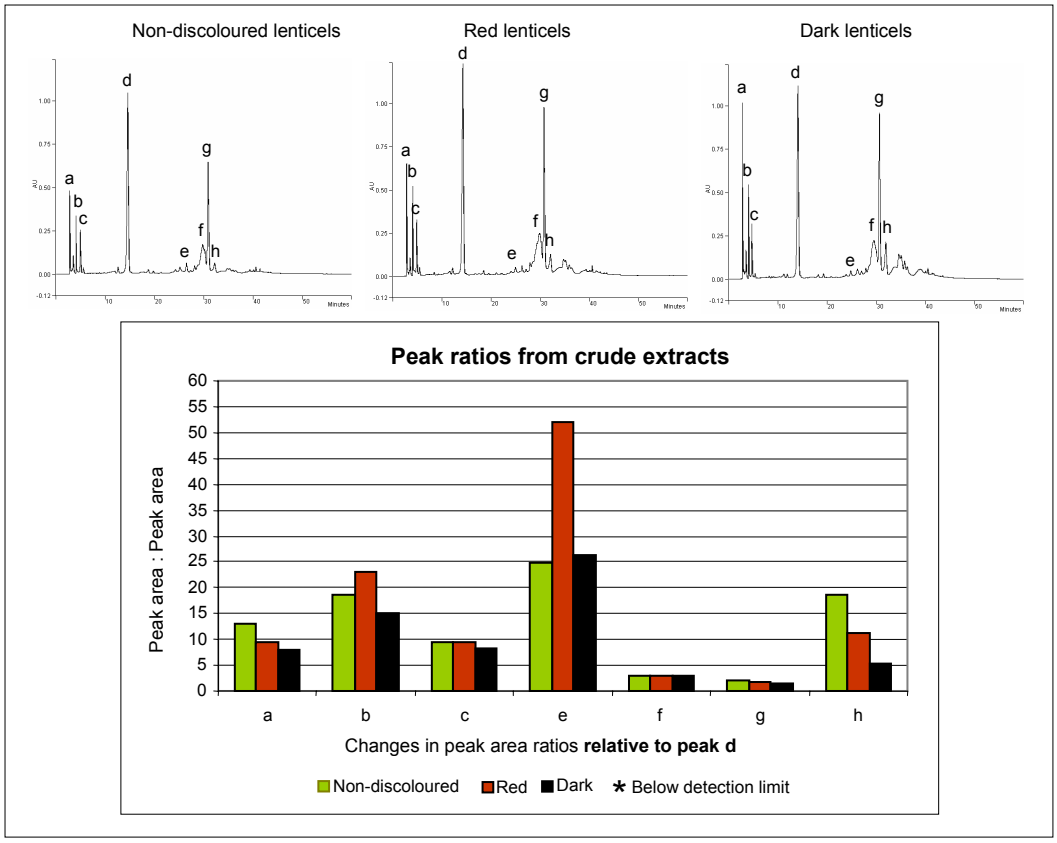

Figure 11

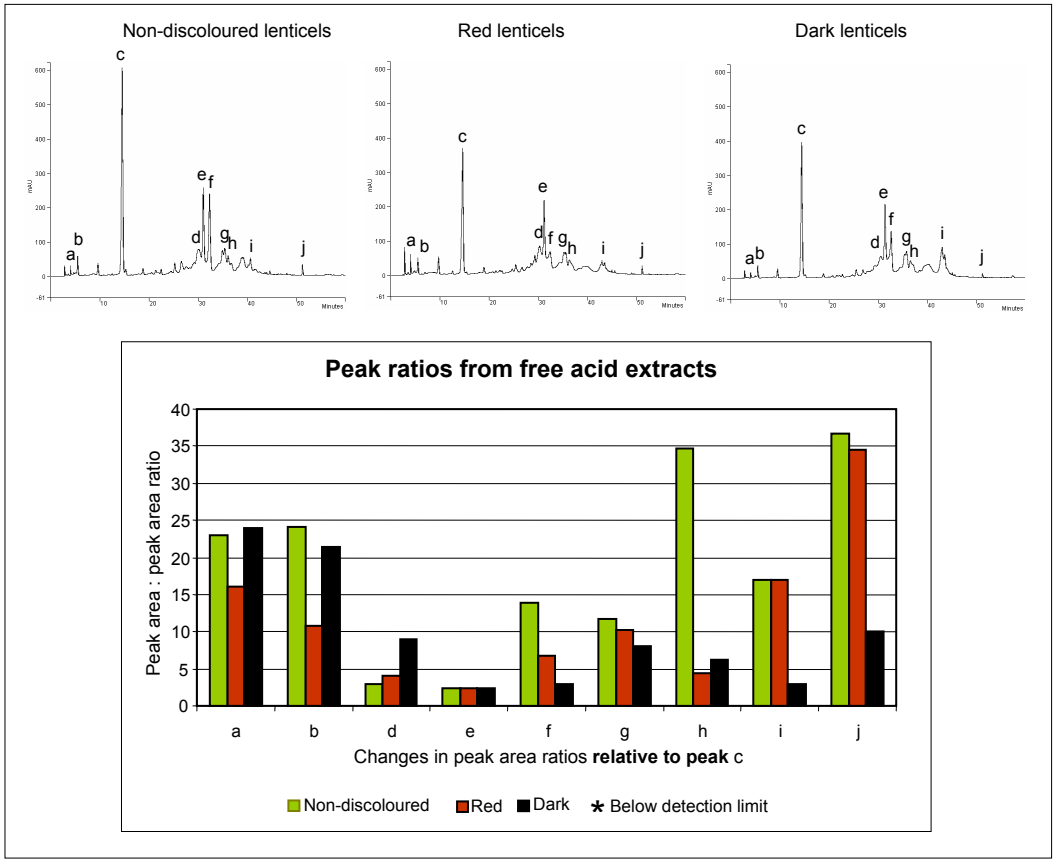

Figure 12

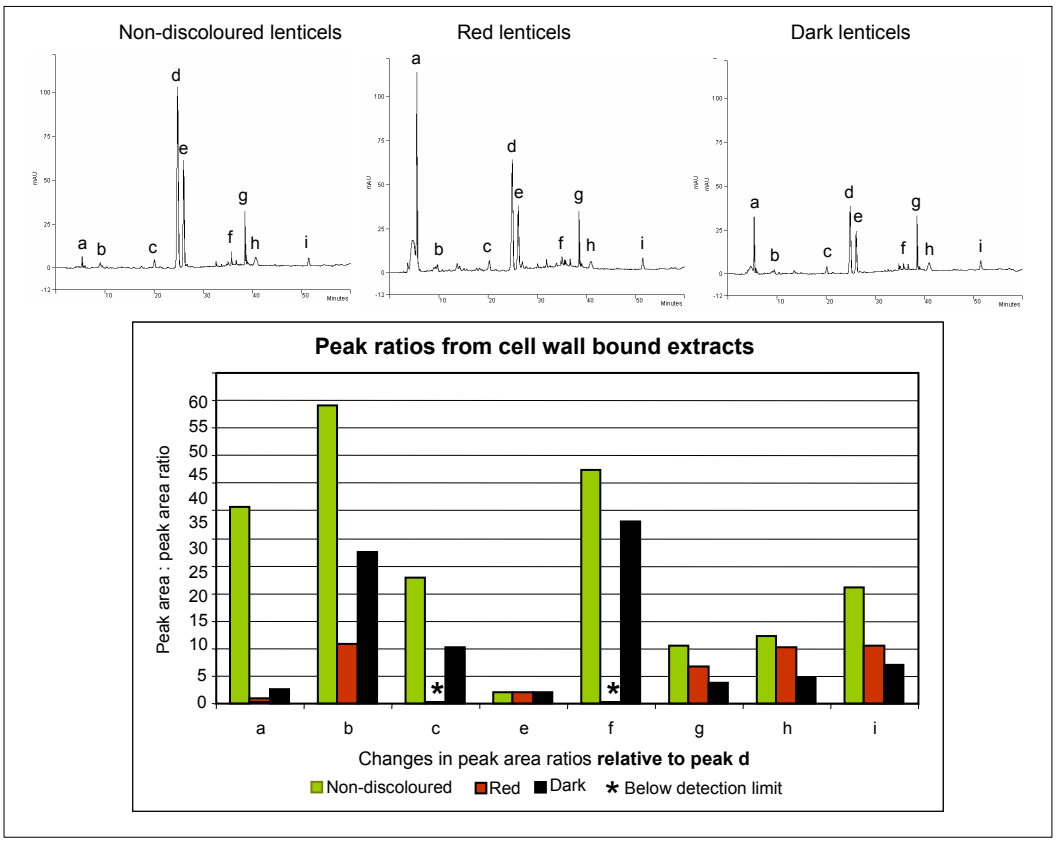

Figure 13

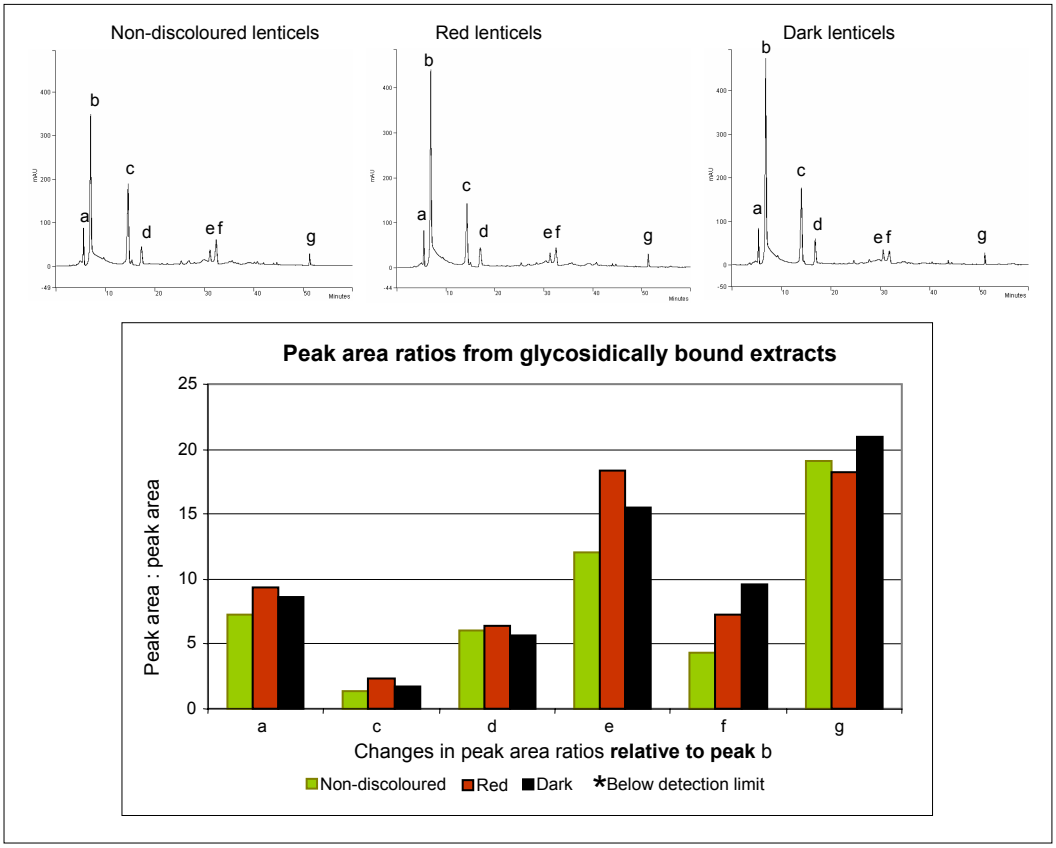

Figure 14

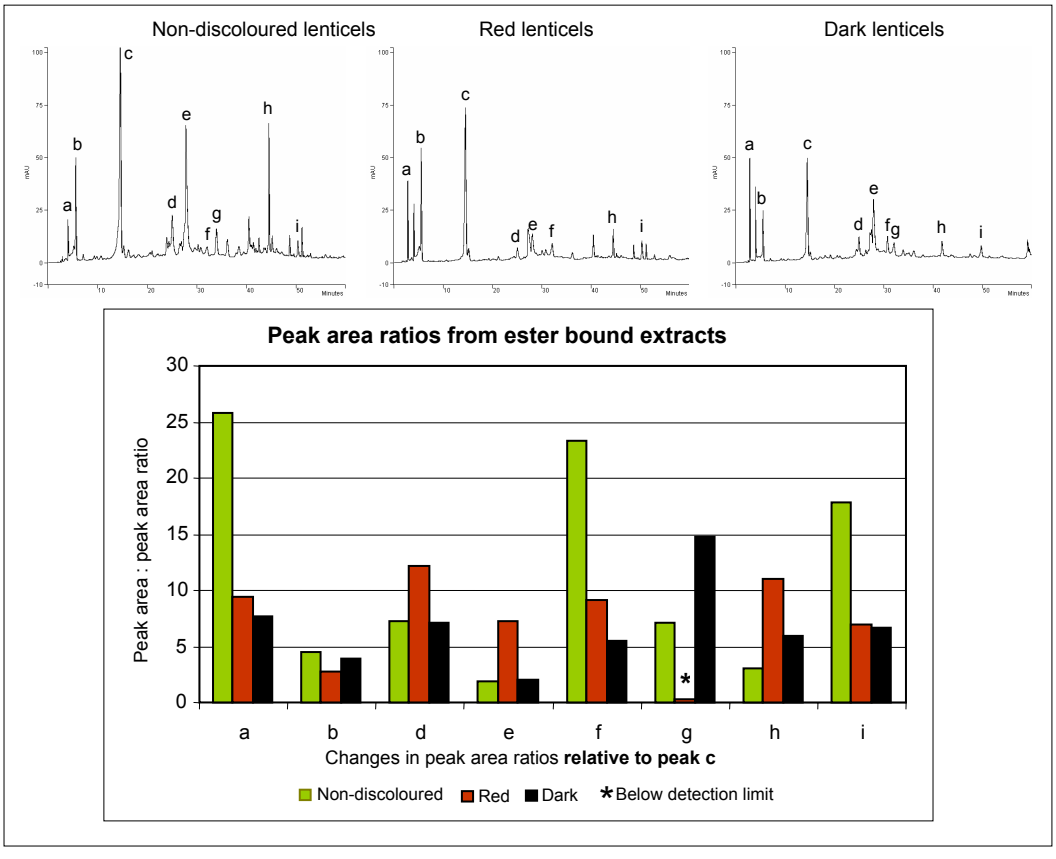

Figure 15

The great tragedy of science - the slaying of a beautiful hypothesis by an ugly fact.

Thomas Henry Huxley (1825-1895)

Evolutionary Biologist

Chapter 6

EFFECTS OF PREHARVEST TREATMENT WITH UNCALCINED KAOLIN ON POSTHARVEST APPLICATION OF COMMERCIAL WAX ON MANGO (*Mangifera Indica* L.) FRUIT

6.1 ABSTRACT

Mango cultivars have wide ranging developmental periods, with fruit reaching physiological ripeness during summer time when severe meteorological conditions prevail. Sunburn damage manifests as necrotic lesions, rendering fruit useless for both local and export markets. Mechanical measures currently employed against sunburn are expensive, time consuming and labour intensive. Alternative solutions must meet with the requirements posed by the target export markets in Europe. Kaolin is a lucrative proposition, but the physiological effect of the clay on mango fruit surfaces has not been investigated. Furthermore, the effect of the clay on the film formation of commercially applied wax formulations is also poorly understood. Results from this study indicate that kaolin affects epicuticular wax development. Persistent kaolin lamellae on fruit surfaces have a negative impact on commercial wax film formation.

6.2 INTRODUCTION

Mango cultivation is a lucrative industry in many parts of the world, being the second most popular tropical fruit crop (FAO, 2002). In South Africa, mangoes are primarily grown in the Eastern parts of the country, with some production in the Western and Northern Cape, in areas below 600 m. Main production farms are geographically situated between the 24° and 26°S latitudinal, and 30° and 31°E longitudinal lines. Environmental conditions in these areas are harsh during the periods of fruit set (Barkstrom, 2004). Typical early summer temperatures average 25 - 30 °C, often exceeding 38 °C in mid-summer, with solar insulation during this time reaching 6,35 kWh/m^2/day. The relative humidity in mid-summer can rise to 85 % with an average of 69.7 % cloud cover during daylight hours.

Continuous exposure to this intense light energy during fruit development annually causes up to 20 % loss of export quality material due to sunburn blemishes and physiological disorders (Le Lagadec, 2003). Huge investments in terms of both strategies for sunscald prevention and human resources are made during each growing season to reduce these losses. Currently, the most widely practiced sunscald prevention strategy in South Africa is the innovative practice of using caps made from aerothene on fruit exposed to afternoon sun (Silimela & Korsten, 2001). However, this practice is labour-intensive and expensive, and not always successful (Le Lagadec, 2003). The possibility of an alternative solution that may address these problems would be pursued enthusiastically, as was the case with kaolin.

Kaolin is a term that refers to rock consisting of at least 90 % kaolinite. It is also used as a general commercial term for material consisting of, amongst other minerals, kaolinite. Kaolin normally occurs in two geological environments (Newman, 1987):

- as a primary or residual ore where it formed *in situ* due to alteration or weathering of a parental rock which is often of granitic composition
- as secondary or sedimentary kaolin ores.

It usually forms a massive, white to light greyish or light yellowish rock in which kaolinite occurs with other more stable minerals such as quartz. A normal requirement is that kaolin ores are beneficiated, during which process impurities such as quartz and mica are removed from the product, and kaolinite is dry- or wet-milled, classified and bagged.

Kaolinite belongs to the phyllosilicate group of minerals because of a laminar physical nature, and simple 1:1 layered crystal structure containing the basic units from which all clay minerals are made (Newman, 1987). From this structure it is clear that kaolinite can be defined as a hydrated aluminium silicate mineral with the general formula $Al_4Si_4O_{10}(OH)_8$ with the following theoretical composition: SiO_2 = 46,5 %; Al_2O_3 = 39,5 %; H_2O = 14 %. From Figure 1 it can be seen that one Si coordinated tetrahedral layer is bound to an Al/Fe coordinated octahedral layer by the sharing of OH groups for apical oxygen atoms in the tetrahedral layer. Si in the tetrahedral layer can be substituted by limited numbers of Al ions while Al in the octahedral layer can be substituted by Fe. Microscopically, the clay often displays subhedral to euhedral, hexagonal grain shapes consisting of many thin platelets arranged face to face, which can be arranged in booklets (Huggett & Shaw, 1997). The length and breadth dimensions of a typical kaolinite platelet far exceed the thickness thereof, while the general dimensions vary greatly, depending on the mode of formation of the clay. Chemically, other elements which could be in solid solution in the kaolinite crystal structure, or which could be adsorbed onto the clay

platelets, include mostly Ti and Fe (Newman, 1987). Another hydrated aluminium silicate mineral that is mineralogically and chemically indistinguishable from kaolinite (except if special sample treatment tests are done), but which displays a typical tubular morphology, is halloysite. The presence of such alternative clay structures is typical of uncalcined kaolin.

Some of the most important characteristics of kaolin that makes it very useful in a wide variety of preharvest applications on tree crops are:

- its laminar or plate-like morphology and concomitant large surface area (Huggett & Shaw, 1997)
- high reflectivity (Glenn *et al*., 2002)
- ease of dispersion (Zaman & Mathur, 2004)
- chemical inertness (Newman, 1987).

The potential uses of kaolin on fruit surfaces gained prominence when it was applied as a particle film deterrent against ovipositioning by psylla on pears (Glenn *et al*., 1999; Puterka *et al*., 2000). This success was followed by application of kaolin against codling moth on apples and pears (Unruh *et al*., 2000). Since then, the use of kaolin against insect pests include the suppression of fruit fly infestations on nectarines, persimmons and apples (Mazor & Erez, 2004), and olives (Saour & Makee, 2004). The possibility of using kaolin as sunshield in a sunscald-prevention program was first investigated in studies on the preharvest physiology of apples (Glenn *et al*., 2001; Glenn *et al*., 2002). In 2003 it was applied for the same purpose on the surfaces of pomegranates in Spain (Malgarejo *et al*., 2003). The use of kaolin as a particle film barrier against sunscald on mango fruit were tested in field trials on locations in the Hoedspruit area (24.4°S, 31.0°E) of the Limpopo province of South Africa during the 2002/2003 season (Joubert *et al*., 2002; Le Lagadec & Ueckermann, 2002; Le Lagadec, 2003). Different concentrations of kaolin suspensions (3 % - 15 %) in water were applied by either spraying or dipping, and even the lowest concentration indicated acceptable results (Le Lagadec, 2003). The 2003/2004-production season saw the first commercial application of the clay on mangoes.

At the time of the first commercial application of kaolin on mangoes, limited quantified information existed on the preharvest sunblock properties of kaolin. No studies could be found on its possible effects on fruit physiology, with a single study pointing towards possible repercussions on the packline (Malgajero *et al*., 2004). During postharvest

processing of kaolin treated mangoes, immediately following washing and application of the commercial wax emulsion, it was noted that areas with receding wax developed while the wax was still wet. As the applied wax dried, uneven, dull spots created an unacceptable blotched surface appearance. Furthermore, this problem persisted when untreated fruit followed the batches of kaolin-treated fruit that had passed over the packline. Initially, the formulation of commercial wax emulsions was the suspected culprit, but this was dispelled after extensive quality checks were done by the manufacturer of the emulsions. Further investigations then indicated the presence of kaolin on fruit surfaces that experienced uneven wax coating.

The waxing problems exacerbated apprehension about a possible increase in the percentage of fruit developing lenticel damage after waxing. This apprehension was not based on any data or observations, but stemmed from the uncertainty about the nature of the initiators for development of a postharvest condition known as 'lenticel damage'. To determine the effects of kaolin on mango fruit surfaces, this two-fold investigation was undertaken. A study of the interaction of uncalcined kaolin and commercial wax emulsion was accompanied by an assessment of the threat of lenticel discolouration due to kaolin application.

6.3 MATERIALS AND METHODS

6.3.1 Plant material

During the 2003/2004 seasons, mango fruit (cultivar 'Tommy Atkins') produced and packed for export were obtained from Bavaria Fruit Packers, a commercial packhouse near Hoedspruit, Limpopo Province, South Africa. Two packlines were investigated, namely one used strictly for the packing of organically produced fruit, and the other for fruit that are produced and packed conventionally (non-organically). Two designated points on each packline were investigated, namely the elevated exit following the warm water bath, and the rollers after commercial wax application. From the conventional packline, two separate batches of treated samples were collected: one consisting of fruit that were dipped in 3% kaolin slurry and the other of fruit that were sprayed with 3% kaolin suspension. From the packline for organic production only fruit sprayed with 3% kaolin suspension was collected. Three fruit were collected from each sample point and a box with nine fruit from each batch collection point.

Triplicate samples, consisting of 5 fruit each, were also collected from two orchards where trees received preharvest treatments of kaolin that consisted of either dipping in or spraying with 3 % slurry. Fruit from untreated trees were collected as control samples. Table 1 is a summary of the sample collection schedule.

6.3.2 Application techniques of the kaolin-based product

A product consisting of uncalcined kaolin and copper oxychloride was made up to a 3 % suspension and applied as either a spray or slurry into which the fruit was dipped. Spray applications were done with commercial spray apparatus and delivered at 30l /tree, while dipped treatments were done manually by dipping individual fruit into buckets containing the slurry. Fruit that had not yet reached physiological ripeness were used for single-application spray and dip treatments. For the application of multiple spray-treatments in a study on insect control, sprays were delivered from the period when the peduncle to stamen end-length of the fruit was about 15 mm to physiological ripeness, totalling four applications per season.

6.3.3 Preparation of the fruit sample material

All the chemicals were obtained from SPI Supplies (SPI Supplies, West Chester, Pennsylvania). Fruit samples were collected from the orchards or commercial packline during normal operation and processed within 18 hours after collection. Three sections (each >25mm^2) of mango fruit rind were dissected from the shoulder area of each fruit, and processed by plunge freezing it in liquid propane at -180 °C (Reichert KF80, Vienna). Sections were then dried in a high vacuum freeze drier (custom built, Tshwane University of Technology, Pretoria, South Africa) at 1.39 x 10^{-7} mbar and -80 °C for 72 hours. All the samples were duplicated and prepared by standard fixation in 2.5 % glutaraldehyde, followed by OsO_4 post-fixation and critical point drying (Bio-Rad E3000, Watford, England). The two methods were used in parallel in order to eliminate the interpretation of any artefacts present due to the preparation technique. Sections from both preparation techniques were made conductive by exposing it to vapour from a 0.5 % solution of RuO_4 for 30 minutes (Trent *et al*., 1983; Van der Merwe & Peacock, 1999) before viewing in a JSM-6000F high-resolution field emission scanning electron microscope (FE-SEM) (JEOL, Tokyo, Japan).

6.3.4 Material and instrumentation for study of kaolin

Two sets of samples consisting of different types of commercially available kaolin were subjected to basic comparative analytical tests to determine differences in mineral and geochemical properties. The base of one set consisted of locally produced, uncalcined

kaolin. The base of the second set was a calcined kaolin product known as Surround®. Samples JH914, JH915 and JH916A consist of kaolin used as mango sunscreen, while JH916 is a carrier for copper oxychloride. JH914 and JH916 are samples of kaolin derived from a South African kaolin producer.

X-ray diffraction analysis (XRD) was done at room temperature, using a powder diffractometer (Siemens D500) (Siemens, Karlsruhe, Germany) in reflection mode with Cu-Kα radiation at generator settings of 40 kV and 35 mA, and at a speed of 0,02° step size per 1 sec. The powder diffractogrammes were run in a 2θ range from 5° to 65°. Phase concentrations are determined as semi-quantitative estimates, using relative peak heights/areas proportions (Brime, 1985). For the geochemical work, a Philips PW1480 X-ray fluorescence apparatus (Philips Analytical, Almelo, Netherlands) was used with a Sc anode for major-element analyses and a Rh anode for trace-element analyses.

Presence of uncalcined kaolin on the fruit surface and embedded in the commercial wax layer was confirmed by FE-SEM using backscatter mode at 20 kV, and a low vacuum JSM-5800LV SEM (JEOL, Tokyo, Japan) with an Energy Dispersive Spectroscopy (EDS) apparatus attached to it. For confirmation of the EDS analysis of kaolin inclusions on the fruit surface, kaolin samples were prepared by scattering a small amount of the powder onto double-sided carbon tape on an aluminium stub. The sample was sputter coated with gold to a thickness of approximately 8nm and viewed in EDS mode at P < 2.67 mBar.

6.4 RESULTS AND DISCUSSION

Results of the mineralogical analyses consisted of three parts, namely the semi-quantitative analyses of the mineralogy, including different types of clay minerals, by X-ray diffraction (XRD), analysis of the geochemical composition by X-ray fluorescence (XRF) and the geochemical composition of individual mineral grains such as kaolinite by electron dispersive spectroscopy (EDS). The latter studies confirmed the random presence of several elements in the clay, while features identified by XRD were used in diagnostic determination of the geochemistry of the clay samples investigated. Observations made with FE-SEM were related to the mineralogical results.

6.4.1 Results of X-ray diffraction analysis

Samples JH914 and JH916 consisted of 90 % kaolinite and approximately 6 % mica (Table 2). Mica is another member of the phyllosilicate group of minerals and closely

associated with the uncalcined kaolin. JH915 was a sample of imported kaolin (Engelhard, Surround®). The composition of this sample differed from that of JH914 and JH916 because of the presence of 55 % amorphous material. The amorphous mineral component was the result of heat treatment or calcining, during which time moisture in the clay was driven off (Table 2). Typical utilisation of the clay in JH915 included application as an insect repellant, sunscreen and temperature control product on ripening fruit. From the comparison of results in Table 2, it could be seen that, firstly, no amorphous material was present in the local product, the high crystallinity of JH914 implying that the clay had probably not been calcined. Secondly, the mineral component (except for the copper oxychloride, which is an additive and the trace minerals) was similar in both the South Africa derived kaolin and imported product.

6.4.2 Results of X-ray fluorescence

The presence and weight distribution of all the elements in the samples shown in Table 3 were determined by means XRF. The composition of JH914, uncalcined South African kaolin, compared well with the ideal theoretical kaolinite composition. JH915 was a sample of imported, calcined kaolin and the result of the compared mineralogical compositions was given in Table 3. Loss on ignition (LOI) of JH914 was approximately 17.4 %. Comparatively, JH915 had a LOI of only 2.27 %, similar to the mineralogical results in Table 2, indicating that this sample was calcined to less than 3 % total moisture. Silicon, titanium, aluminium, magnesium and manganese oxide concentrations had higher values in JH915 in comparison to JH914 due to normalisation to a calcined basis.

Chemically, other elements which could be in solid solution in the kaolinite crystal structure, or which could be adsorbed on the clay platelets, included mostly Ti and Fe.

6.4.3 Results of electron dispersive spectroscopy

Although EDS results were useful as both quantitative and qualitative data, EDS analysis of the JH-samples were only used as qualitative confirmation of observed kaolin particles inclusions of the wax film. This was because of the absence of standards against which to determine the mineralogy of each sample.

EDS data was used in combination with the visually determined results from the FE-SEM to confirm the texture of analysed samples. These results excluded the contribution of water, oxygen and carbon to the mass ratios represented in Table 4. The following were important in the consideration of the EDS results obtained:

- the chi-squared values of the scans varied between 1.09 and 1.46, which was well below the required value of ten, indicating an excellent fit of the graph (Fig. 2).
- all element weight percentages above 0.5 % were considered to be significant (standard EDS interpretation).
- qualitative results were indicative of the small component analysis of material investigated.

The presence and weight distribution of all the elements in the samples that were investigated were in agreement with results from the raw materials used in the formulation (Tables 2 & 3).

6.4.4 Scanning electron microscopy and other observations

The sun block agent/carrier was found to consist of a finely milled rock containing mostly (>90 %) a hydrated aluminium silicate (kaolinite). The length and breadth dimensions of each platelet far exceed the thickness thereof. Surface dimensions of the studied samples ranged between 0.2 – 0.5 µm, which meant it fell in the very fine category. Lamellae appeared both fragmented and still in the booklets (Fig. 3A & B). Halloysite was observed in the powder samples as well as on the plant surface (Fig. 3A - D). Quartz was also present in subordinate quantities in the sample material, and aggregates of kaolinite/mica/smectite showing rosebud textures, were observed (Fig. 3C). Copper oxychloride in the formulation was observed as fused, cubic crystals attached to the clay particles. Observed crystal sizes meant that these crystals could fit between mango fruit wax crystals comfortably (Fig. 3D, 4A-C).

Dip application of the clay caused irregular colour development (vein-like appearance of fruit colour pattern) (Fig. 5). The kaolin furthermore had serious repercussions on the packline, because apart from contact contamination of the lugs, bulk bins and other fruit, the outmost parts of the clay overload rinsed off in the different water baths (Fig. 6A). Dislodged clay particles from heavily encrusted fruit surfaces remained suspended in the water and could attach to fruit surfaces with available binding sites, creating a carry-over effect that eventually contaminated the complete packline.

Fruit subjected to spray application (Fig. 6B) were less negatively impacted in terms of colour development, while still being protected against sunburn, which included temperature control of developing fruit (Le Lagadec, 2003). However, the kaolin particle film could not be removed completely by any mechanical means and dispersing fractions

of the film contaminated water in dump and wash baths (Fig. 6C). Kaolin booklets (Fig. 2) delaminated and fragmented through mechanical wear. The kaolin debris fitted between the complex crystalline structures in the outermost (aliphatic) wax layer (Fig. 3D, 4A -C, and 6D) and would be trapped by mechanical compression of the wax crystals and cohesion forces.

Kaolin-free fruit passing through the wash processes at a later stage were shown to be affected by this particulate debris. Due to the size and pliability of epicuticular wax crystalloids, the fruit surface effectively captured some of the kaolin and associated fragments (Fig. 4 & 6D). Thus, although the fruit surfaces had a clean appearance at the exit of the packing line, a microscopic layer of clay lamellae still adhered to it, either by application or due to the attachment of suspended particles (Fig. 6C). Dynamic reconstitution disrupted plant wax over objects or inclusions on the fruit surface further contributed to the failure of the packline procedures to remove kaolin (Fig. 7A - C). The configuration of reconstituted wax was determined by the physical and chemical characteristics of the obstructions through which wax constituents have to migrate. Low absorption and good permeability of commercial wax polymers meant that the architecture of reconstituted wax crystals did not alter severely (Fig. 7A) after diffusion through this layer. Kaolin lamellae, on the other hands, have high absorptive properties that trap oily substances (Gu *et al*., 2003), which account for the atypical architecture of reconstituted fringing crystals (Fig. 7B & C).

This absorptive quality of kaolin caused particles attached to the fruit surface to form a physical barrier between the natural and commercial wax (Fig. 8A), preventing their integration and subsequent film formation. Although evidence could be found of the commercial wax flowing into spaces between epicuticular wax crystalloids, film formation was either incomplete or absent in most sampled material (Fig. 8B). The amount of commercial wax applied, as indicated by the thickness of its layer, did not affect film formation (Fig. 8C). Close scrutiny of set commercial wax emulsion showed that it did not set homogenously. The solidified emulsion had a granular appearance that was indicative of localized polymerization (Fig. 8D) due to loss of moisture and unsaturated oils to the kaolin lamellae. The loss of these fractions created incomplete coalescence of the emulsion constituents (Yan *et al*., 2003).

As seen from Table 5, kaolin present on the fruit surface also altered the surface tension of the wax substantially, causing insufficient adhesion between the commercial wax and plant (epicuticular) wax. This effect was found on both the organic line where a carnauba-

based wax was used and the normal packline where a polyethylene-based wax was used. Areas with preferential adherence corresponded with areas where the difference between the surface tension (dyne.cm^{-1}) of commercial wax and the fruit surface was the largest. Disturbance of the surface tension by any foreign inclusions, in either the commercial wax or the plant wax, would result in an irregular application of the wax emulsion (Fig. 9A - D). An uneven distribution of kaolin and the altered surface tension in coated areas therefore led to unsatisfactory wax application as encountered on the packline. Furthermore, the hygroscopic ability of the clay fractions also had a detrimental capillary effect on the available free water in the commercial wax (Brady *et al*., 1996; Zaman & Mathur, 2004). It is suspected that this withdrawal of capillary water from the particulate fraction of the commercial wax impeded film formation, which led to the wax film having a granular structure, and to disruption of coalescence of commercial wax polymers and plant wax crystalloids (Fig. 10A & B).

A problem that manifested only once the fruit had been packed in cartons, actually originated during processing on the line, when wet, swollen kaolin lamellae had formed inclusions during commercial wax application. Once these lamellae started to dry inside the wax film, they shrank, resulting in the formation of cavities in the wax film layer (Fig. 11A & B). These cavities, together with the kaolin inclusions changed the tensile properties of the wax film, and disrupted the mechanical integrity of the film, making it brittle (Fig. 12A).

Finally, kaolin debris that had entered into lenticels was observed in epicuticular membranes from the sample material studied. Such debris became trapped underneath the commercial wax emulsion that flowed into the lenticel (Fig. 12 B). However, it was not possible to unequivocally relate the discolouration of lenticels to the presence of kaolin debris, as discoloured lenticels without kaolin were also found.

6.5 CONCLUSION

Fruit intended for extended periods of transport are coated with commercial wax formulations. The ultimate goal of commercial wax application on fruit surfaces is to achieve a sound, homogenous wax film that gives an appealing gloss and adequate protection against desiccation and postharvest pathogens during export, without adding physiological stress to the fruit. During the 2003 - 2004 mango production season in South Africa, this objective was obstructed at a cooperative packhouse, when packed fruit

presented with an uneven and blotchy wax film. This investigation pointed out that it was due to the presence of uncalcined kaolin on the packlines and in the waterbaths. A number of producers were using a kaolin-rich product as a preharvest sunscreen and anti-fungal agent, and these fruit were part of the scheduled packing at the time that the problems were encountered.

In South Africa, uncalcined kaolin is available as a locally produced preharvest product which is sometimes regarded as being more cost efficient, but is applied more often. Calcined kaolin is imported and applied singularly as a sunscreen. Dip application of the mixture is not recommended, but this method was used by several growers in order to circumvent the overall misting effect and subsequent coating of complete trees by the spray. However, dipping resulted in a very heavy kaolin load on fruit surfaces and uneven colour development of the fruit rind. Nevertheless, regardless of application, persistent adherence of dispersed kaolin lamellae were confirmed, indicating that complete removal of the kaolin lamellae from the surface of the fruit during packing processes is impossible.

The interaction between commercial fruit wax and residual kaolin particles on the fruit surface is crucial, since it affects the integrity of the wax film formed. Persistent particles form a mechanical barrier to the commercial wax and affect the surface tension properties of both the fruit surface and commercial wax. During the period of the incident investigated, several batches of fruit received on the packline were covered extensively in kaolin. Subsequent batches of fruit not treated with kaolin were contaminated by carry-over of persistent clay fractions present on the packline. This resulted in all the fruit on the packlines involved being characterized by irregular application of the commercial wax. These irregularities were due to charge effects by the lamellae that interfered with the cohesion and adhesion properties of the various wax fractions involved (Brady *et al*., 1996). The resultant incompatibility of the surface tensions then manifested as visibly dull areas where the immiscible waxes separate. The phenomenon was more prevalent on organically grown fruit packed on a line dedicated to such fruit. This was due to the formula of the wax emulsion approved for organically produced and packed fruit being far less tolerant of surface tension deviations. Fruit from normal production lines similarly treated with uncalcined kaolin, also exhibited dull areas, but the presentation was far less severe.

Consumer preference has created a growing demand for organically produced crops. Kaolin meets the requirements for this type of produce, and the application of kaolin as a sunscreen may be effective in the orchard (Le Lagadec, 2003). However, the postharvest

repercussions for mangoes need to be investigated in more detail. A disruption of the adhesive qualities of the wax applied to organically produced fruit raised alarm to such repercussions. The synthetic wax used on the normal line had less severe adhesion problems, but uneven wax film formation associated with definite kaolin inclusions in the wax film were observed. The specific architecture and size of the wax crystals on the mango fruit surface is an efficient trap for both copper crystals and fractured kaolin lamellae, which can fit between the wax crystals comfortably, affecting its functionality. Epicuticular wax play a role in both light attenuation and light harvesting by the fruit, and therefore the effect of kaolin on the ultimate sugar and acid content of the fruit needs to be investigated.

In this study, no conclusive evidence was found to support or dismiss the anticipated increase in lenticel discolouration by kaolin. Lenticel morphology may determine the probability that kaolin can block up the entrance to the lenticel cavity, which could affect the susceptibility of a cultivar to the development of discoloured lenticels.

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6.7 TABLES

Table 1 Preharvest treatments of mango fruit (cultivar Tommy Atkins) with a locally obtained, uncalcined kaolin-based product

Application		***Point of Collection***
Number of replicate applications	***Method***	
3	None	Tree
1	Spray	Tree
4*	Spray	Tree
1	Dip	Tree
1	Dip	Packline (normal), after warm water bath
1	Dip	Packline (normal), after waxing
1	Spray	Packline (normal), after warm water bath
1	Spray	Packline (normal), after waxing
1	Spray	Packline (organic), after warm water bath
1	Spray	Packline (organic), after waxing

*: This was a experimental treatment to determine the feasibility of multiple kaolin applications to control insect pests during mango fruit development.

Table 2 Semi-quantitative analyses by X-ray diffraction, comparing three kaolin samples and copper oxychloride-bearing kaolin (% weight)

Analyte* / Sample	*Anatase*	*Jarosite*	*Crandalite/Fluorencite*	*Kaolinite*	*Mica*	*Cu Oxy-chloride*	*Amorphous matter*	*Total*
JH 914: Sample A (Uncalcined)	-	1	3	90	6	-	-	100
JH 915: Sample B (Calcined)	12	-	-	34	-	-	55	101†
JH 916: Uncalcined + Cu oxy-chloride	-	-	-	77	5	18	-	100
JH 916 A: Control (Uncalcined kaolin)	-	-	-	91	7	-	2	100

*: Chemical Composition of Analytes:

- Anatase = TiO_2;
- Jarosite = $KFe_3(SO_4)_2(OH)_6$;
- Crandalite / Fluorocite = $CaAl_3(PO_4)_2(OH) \cdot 5H_2O$;
- Mica = $KAl_2(Si_3Al)O_{10}(OH,F)_2$;
- Analcime = $NaAlSi_2O_6 \cdot H_2O$ - Not detected.

†: Decimal margins of error contributing to accrued value ≥100 %

Table 3 Results obtained from X-ray fluorescence analyses, depicting the chemical composition of locally produced kaolin versus imported kaolin (weight %), both of which are used in applications for insect repellent, sunscreen and temperature control on ripening fruit

Element	JH-914 Uncalcined kaolin	JH-915 Calcined kaolin
SiO_2	41.70	51.00
TiO_2	1.02	1.52
AL_2O_3	35.30	43.90
Fe_2O_3	0.86	0.66
MnO	0.01	0.02
MgO	0.40	0.50
CaO	0.42	0.16
K_2O	1.07	0.19
Na_2O	<0.1	<0.1
P_2O_5	0.22	0.11
Cr_2O_3	<0.01	<0.01
V_2O_5	0.01	0.02
Cl	0.02	0.01
S	0.30	0.10
H_2O	1.28	0.49
LOI	17.38	2.27
Total[†]	99.99	100.95

[†]: Decimal margins of error contributing to accrued values of Total ≠ 100 %

Table 4 Quantitation of spectroscopy results of uncalcined kaolin with added Cu-OCl

Accelerating Voltage: 20 KeV		Take Off Angle: 35°	
Live Time: 100 seconds		**Average Dead Time: 11.916**	
Chi-sqd * = 1.39			
Element	k-ratio (calc.)	Element Wt % †	Wt % Error (1-Sigma)
Al-K	0.2278	32.20	+/- 0.34
Si-K	0.2513	44.17	+/- 0.50
Cl-K	0.0262	4.20	+/- 0.15
K-K	0.0153	2.01	+/- 0.24
Ti-K	0.0158	1.89	+/- 0.16
Fe-K	0.0071	0.77	+/- 0.25
Cu-K	0.1289	14.57	+/- 1.07
Mg-K	0.0012	0.20	+/- 0.09
	Total	100.01§	

*: The chi-squared values of the scans varied between 1.09 and 1.46, which is well below the required value of ten, indicating an excellent fit of the graph.

†: All element weight percentages above 0.5 % are considered significant.

§: Decimal margins of error contributing to accrued values of Total > 100 %

Table 5 Surface tension values of commercially available wax and mango fruit collected from various sampling points on packline not dedicated to organic production

Sampling point	Surface tension * (dynes.cm^{-1})
Fruit, physiologically ripe, untreated	30-34
Fruit at various points along the line:	
After warm water bath	34-38
After Prochloraz application	30
After kaolin application	73
After commercial wax application	23-26†
Commercially available wax	20-21

* : Values were obtained by standard wettability tests conducted in cooperation with SASOL Wax Polymers. Fruit used to obtain the values were not treated or contaminated with kaolin.

† : The wax was applied by hand in these tests, therefore the variable values are due to differences in the thickness and consequently, moisture content of the integrated film of plant wax and commercial wax.

6.8 FIGURE CAPTIONS

Figure 1 Diagram of the relationship between Si and Al in the crystal lattice of kaolinite (after Newman, 1987).

Figure 2 Typical spectrograph obtained from EDS of a sample of uncalcined kaolin.

Figure 3 Micrograph of clay particles from a kaolin powder sample, showing lamellae arranged in a booklet (white arrow) and halloysite particles (tubular structures) (black arrows) (A). Close-up view of booklet structures (white arrows) and tubular halloysite structures (black arrows) (B). Micrograph of uncalcined kaolin containing kaolinite/mica/ smectite aggregates, showing rosebud textures (white arrow) and halloysite structures (black arrow) (C). Tubular halloysite structures (black arrows) were observed on fruit surfaces treated with uncalcined kaolin, while clay particles were sufficiently small to fit between epicuticular wax crystalloids (white arrow) (D).

Figure 4 Small crystals (between 0.2 - 0.5 µm) of Cu oxychloride (white arrow), attached to some clay lamellae (A). Similar Cu oxychloride crystals observed trapped underneath a layer of commercial wax (B). The presence of Cu oxychloride crystals was confirmed by the emission of secondary electrons in backscatter mode at 20 kV (C).

Figure 5 Fruit dipped in kaolin shows streaky and blotchy colouration due to the barrier effect of clay crusts on photosynthesis and colour pigment development.

Figure 6 Crust of kaolin (white arrow) on the surface of a fruit dipped in 3 % slurry. Epicuticular fruit wax crystalloids could be seen in areas where the slurry did not adhere to the fruit surface (black arrow) (A). Surface view of mango fruit sprayed with 3 % kaolin slurry (white arrow), with epicuticular wax crystalloids (black arrow) (B). Kaolin particles (white arrow) from the contaminated packline onto surfaces of fruit not exposed to preharvest treatment of kaolin. The black arrow indicates where brushes scarred the epicuticular wax (C). Transverse section of the epicuticular membrane, showing how particles of kaolin (large white arrow) and halloysite (small white arrow) could fit between the epicuticular wax crystalloids (black arrow) (D).

Figure 7 Epicuticular wax has a self-reconstructing ability, and tends to grow over objects or inclusions on the fruit surface (A - C). The configuration of the reconstituted wax (black arrow in all figures) is determined by the physical and chemical characteristics of the obstruction through which wax constituents have to migrate. Low absorption and good permeability of commercial wax polymers meant that the architecture of reconstituted wax crystals did not alter severely (A). Absorptive kaolin lamellae trap oily substances, leading to atypical reconstituted epicuticular crystalloids (B & C). Halloysite structures were visible (white arrows), confirming the presence of kaolin in the viewed area.

Figure 8 In micrographs A - D, a large white arrow indicates the commercial wax layer, epicuticular wax is pointed out by a small white arrow, a large black arrow shows the distribution of kaolin lamellae, and cutin is indicated by a small black arrow. Although evidence of the commercial wax penetrating between epicuticular wax crystalloids could be found, there was largely no integration of the two wax types during film formation (A & B). The barrier effect of kaolin lamellae on the integration of commercial wax and epicuticular wax did not depend on the amount of commercial wax applied(C). The commercial wax emulsion did not set homogenously, with localized polymerization possibly due to loss of moisture and unsaturated oils to the kaolin lamellae (D).

Figure 9 Features of poor adhesion and incomplete film formation between commercial wax and epicuticular wax was evident in surface views. (A) Poorly formed wax film, with inclusions of kaolin lamellae jutting from the wax layer (arrow) (A - C). A receding wax film (black arrow) left the epicuticular wax exposed (narrow white arrow) (B). The encircled area was enlarged in (C), detailing the commercial wax margin (black arrow), exposed fruit wax crystals (narrow white arrow) and kaolin lamellae (large white arrow). Side view of the commercial wax margin (black arrow), exposed fruit wax crystals (narrow white arrow) and kaolin lamellae (large white arrow) (D).

Figure 10 Micrographs of transverse sections through the wax film and epicuticular membrane, depicting typical separation of the wax emulsion during polymerization (white arrows) (A & B). This separation was due to failed integration of the waxes, as confirmed by the presence of epicuticular wax crystalloids (large black arrows). In micrograph (B), granular polymerization of the commercial wax, a result of water absorption from the wax emulsion by kaolin, was visible (narrow black arrow).

Figure 11 Micrographs of clay inclusions in the wax film, showing cavities left in the wax film after dehydration of the kaolin lamellae caused shrinkage (white arrows) (A & B).

Figure 12 Fruit from packed cartons, indicating damaged coverage of the fruit surface (A). Persistent kaolin fractions (white arrow) created an effective barrier preventing wax film formation between the plant wax (black arrow) and the commercial wax. The resultant brittle covering was ineffective and negates the objective of the fruit coating. In a transverse view of a lenticel (B), kaolin debris (white arrows) was visible below the flow of commercial wax that penetrated the lenticel cavity.

6.9 FIGURES

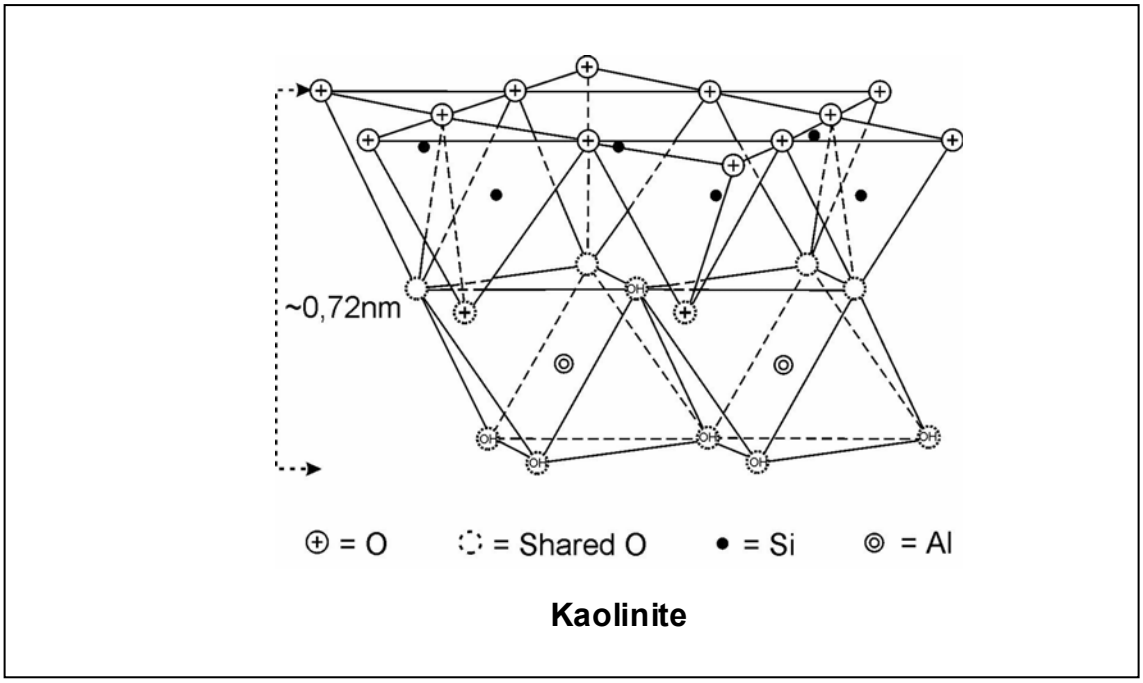
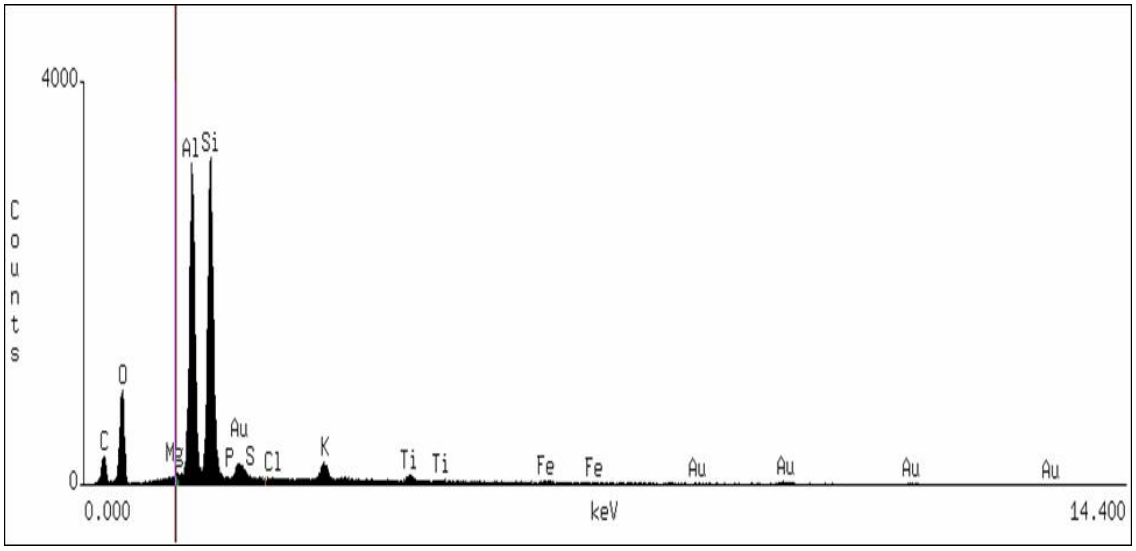


Figure 1



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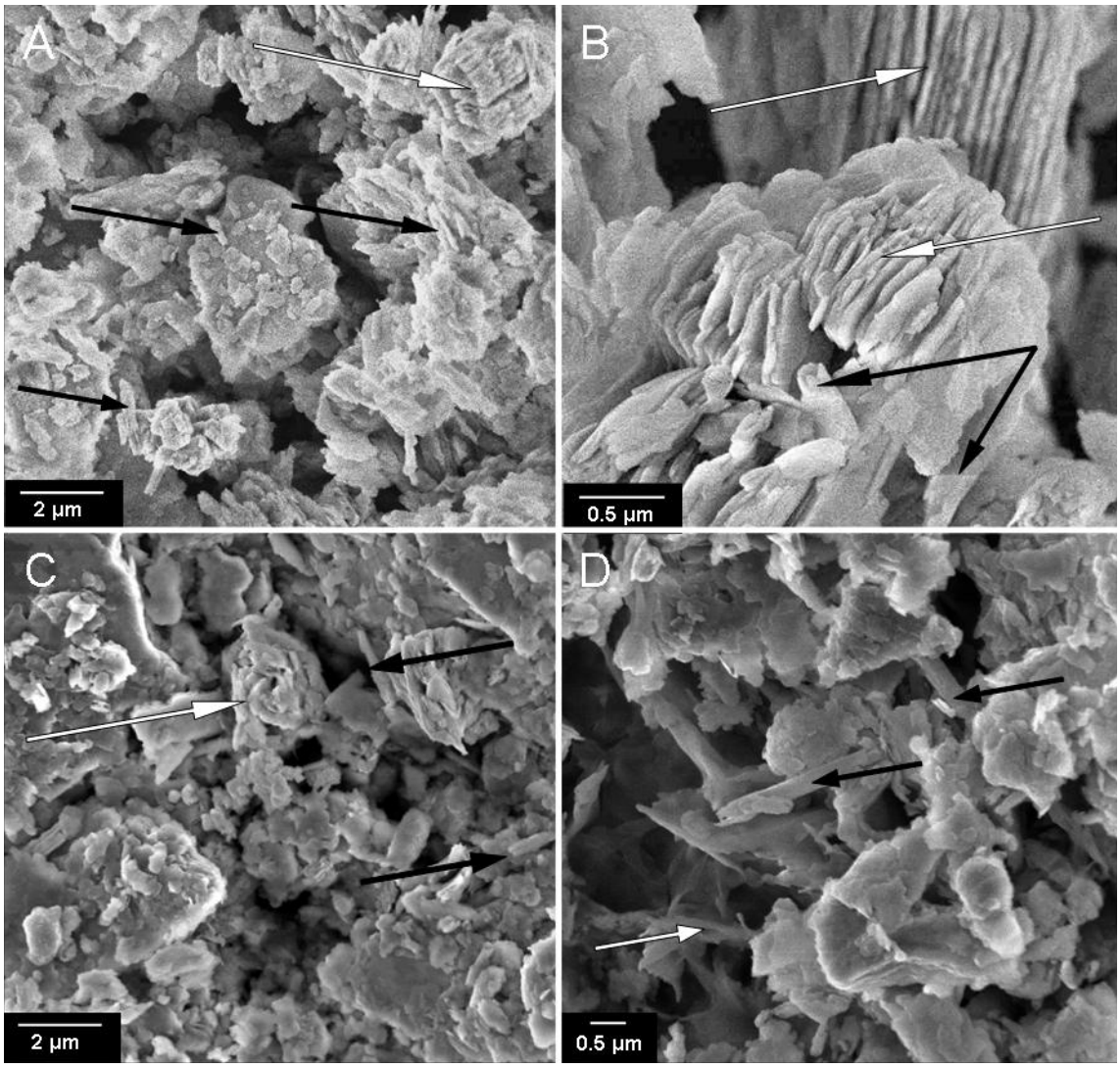

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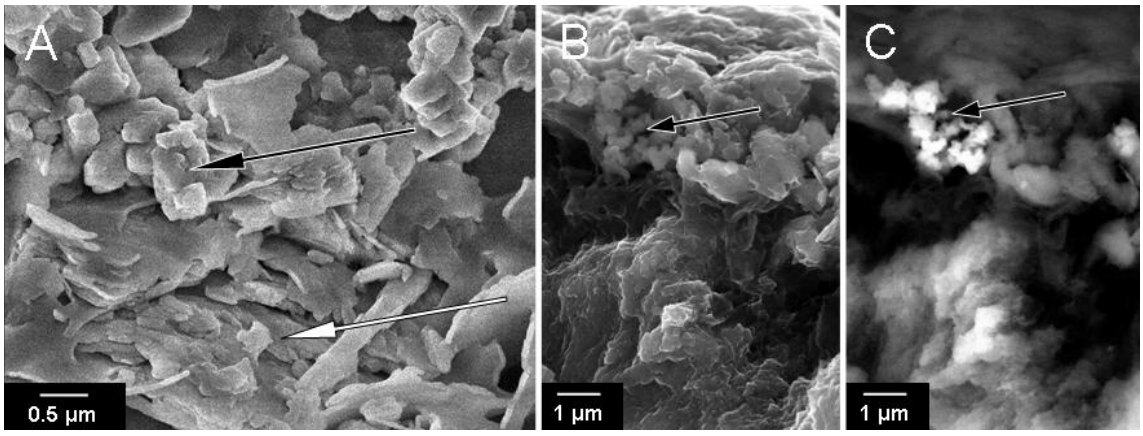
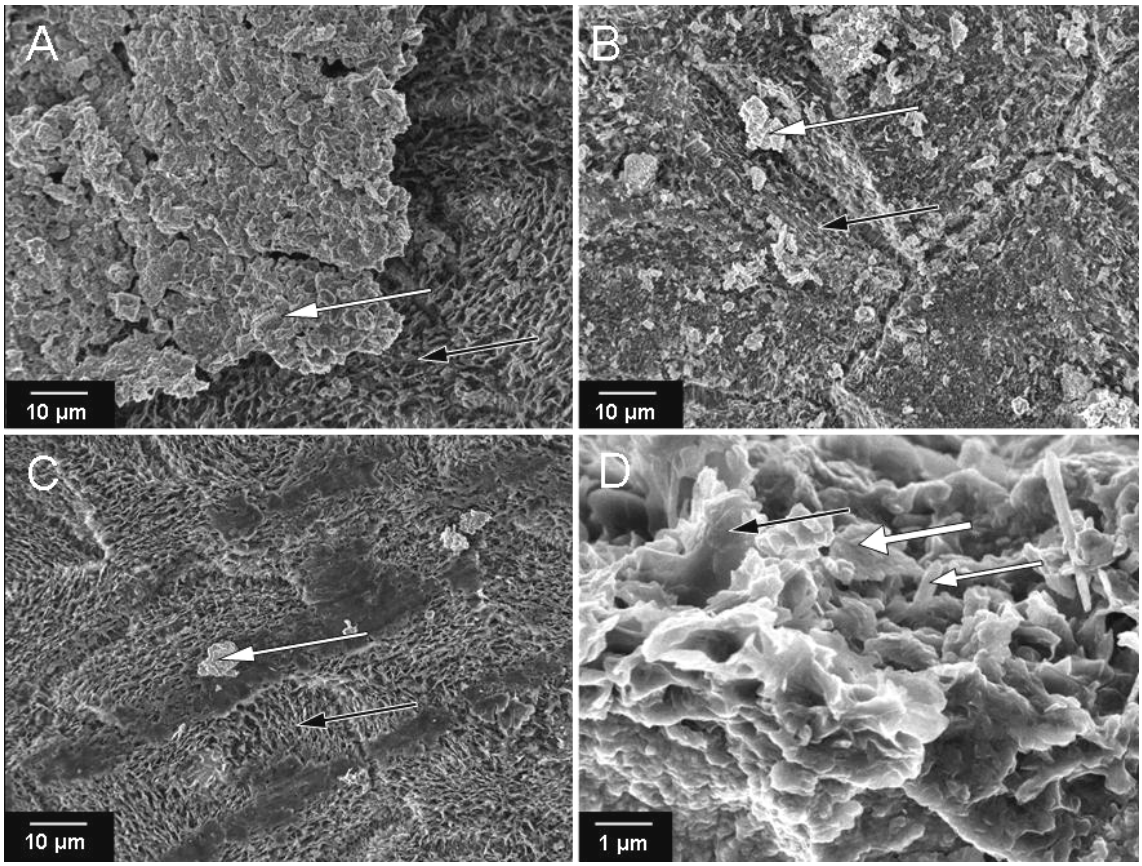


Figure 4

Figure 5



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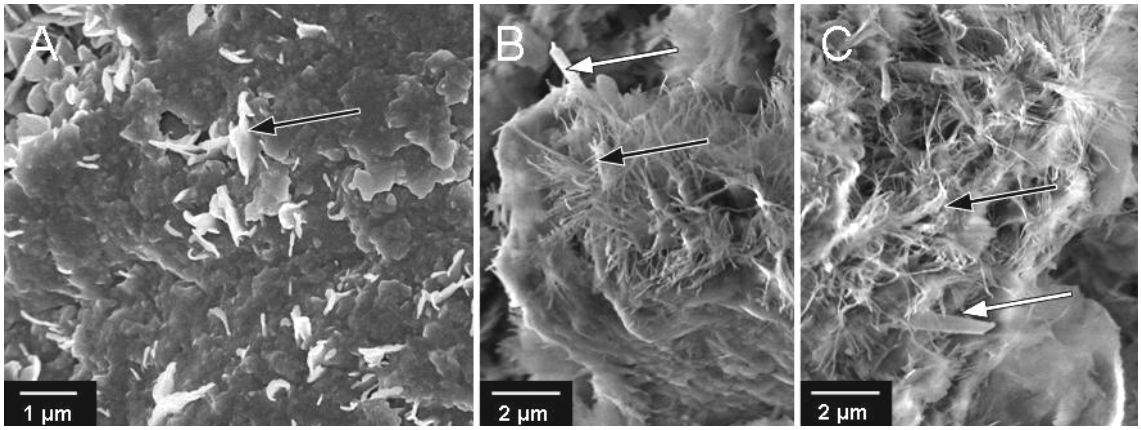

Figure 7

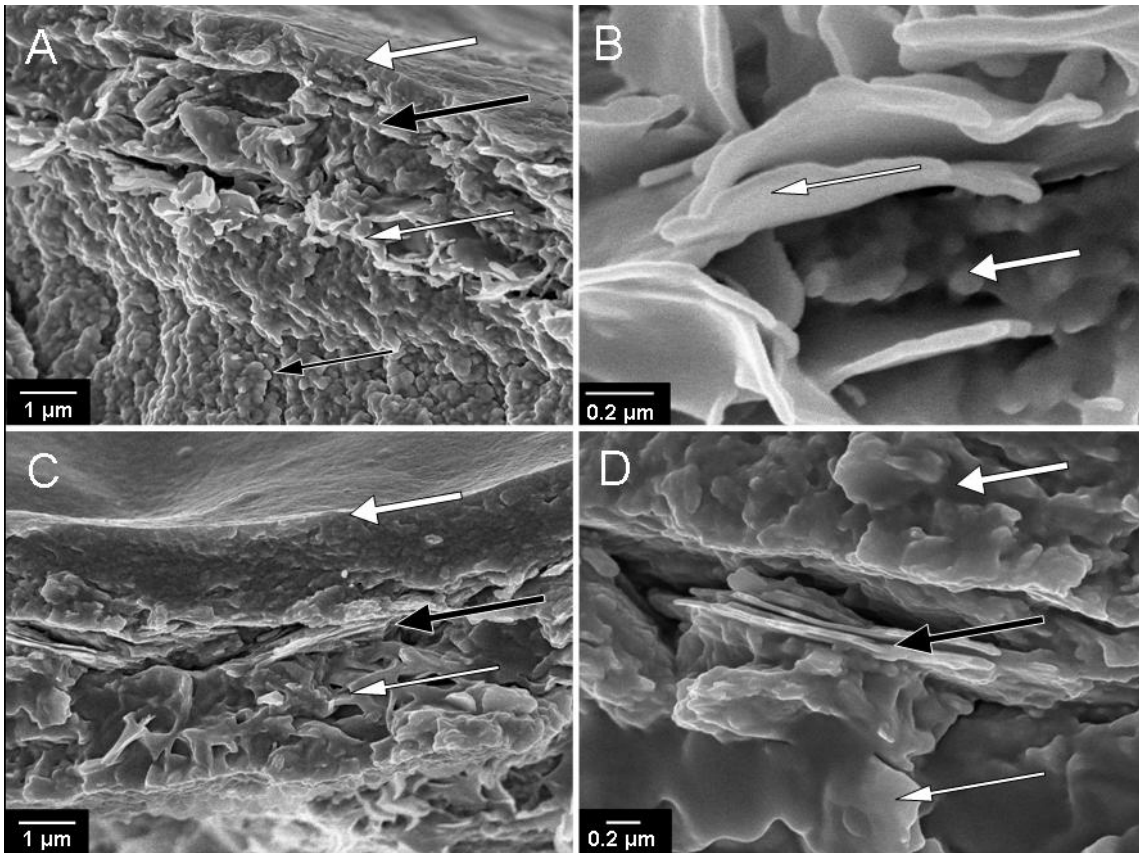

Figure 8

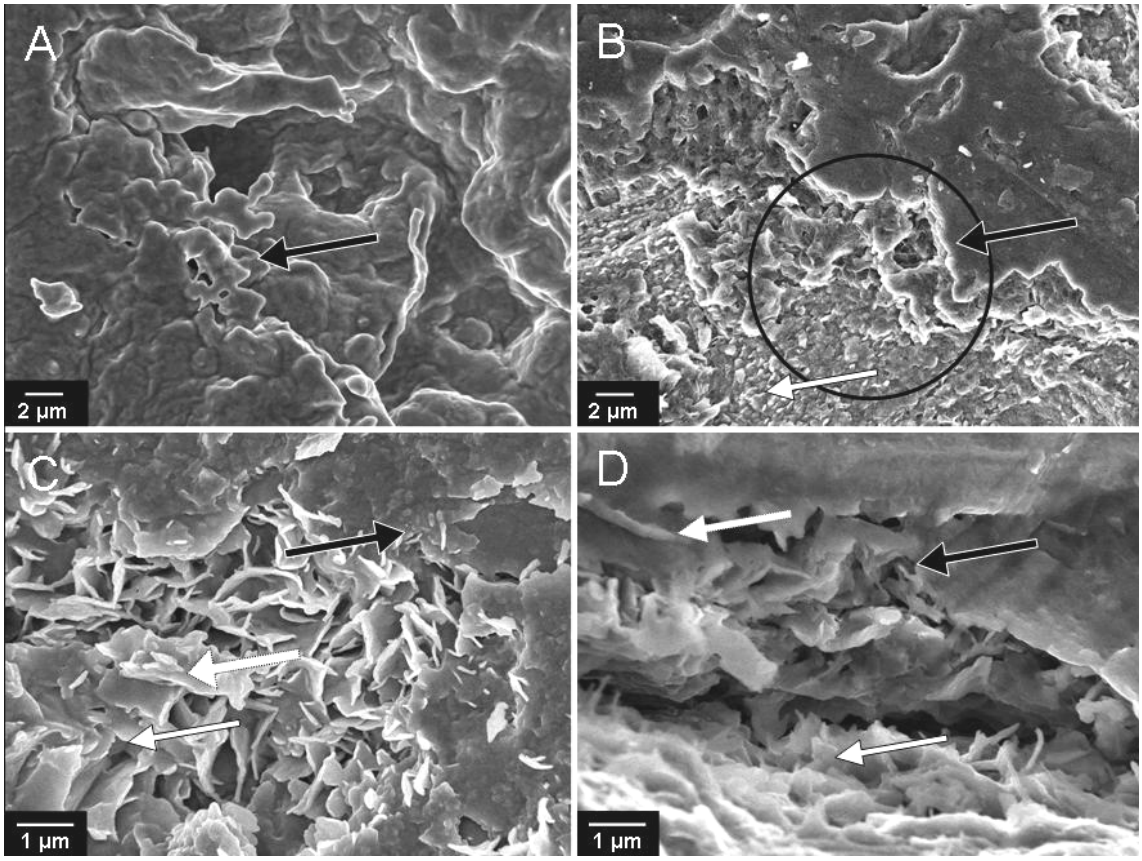

Figure 9

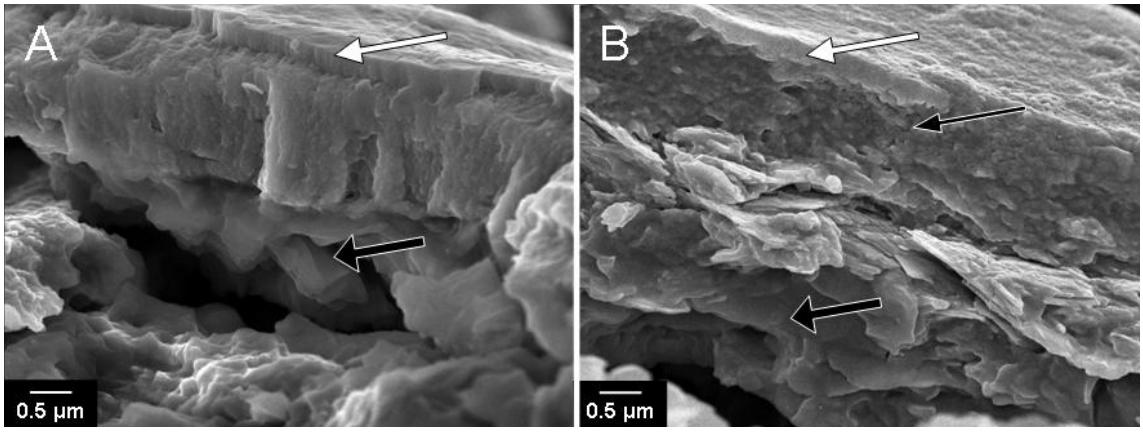

Figure 10

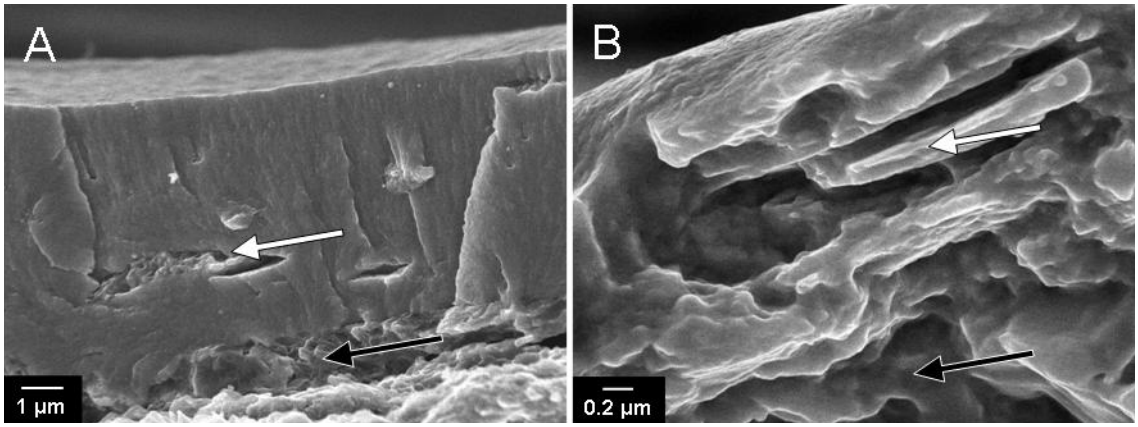

Figure 11

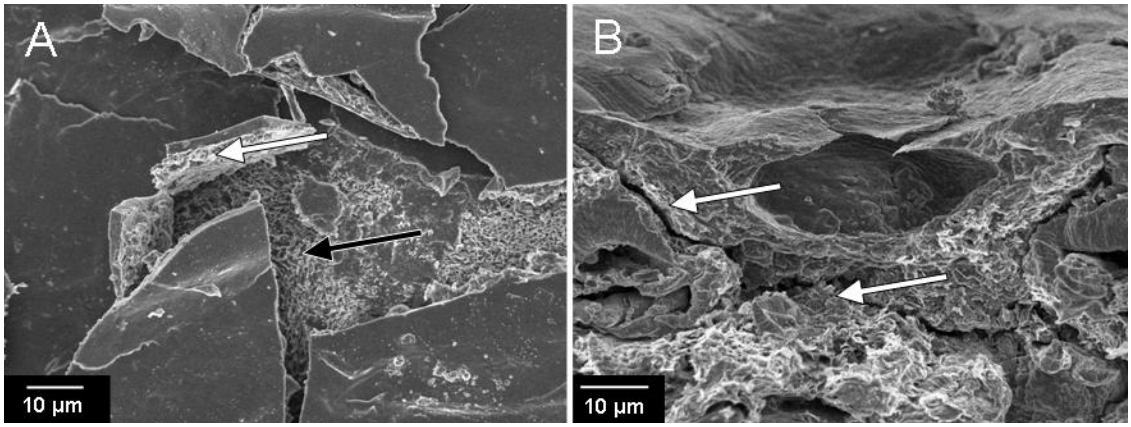

Figure 12

The important thing in science is not so much to obtain new facts as to discover new ways of thinking about them.

Sir William Bragg (1862 - 1942)

Physicist

Chapter 7

CHANGES TO EPICUTICULAR WAX OF MANGO (*Mangifera indica* L.) DUE TO HANDLING ON A COMMERCIAL PACKLINE

7.1 ABSTRACT

All plant surfaces are covered by an epicuticular membrane that plays an important role in plant defence mechanisms. This membrane consists of the epicuticular and cuticular wax, cutin and auxiliary, superficial structures such as trichomes and stomata. Each component of the epicuticular membrane of mango fruit contributes towards protection of the developing fruit. All packline practices and physical handling during the packing process will affect these structures. A problem that is often encountered and that decreases the quality of export fruit is the discolouration of lenticels associated with mango fruit rind. This is a study into the extent of changes imposed on the epicuticular wax during normal packline operations, including an investigation into the contribution of these changes to lenticel discolouration. Fruit samples from the cultivar 'Keitt' was collected from progressive points along a packline and studied using scanning electron microscopy. It was found that packline practices inflict substantial physical changes on the epicuticular structures of the fruit surface. No correlation between lenticel discolouration and epicuticular changes could be demonstrated.

7.2 INTRODUCTION

Increasing demand for mango (*Mangifera indica* L.) fruit has lead to worldwide attempts at improving the selection of commercially popular cultivars (Knight, 1997; Yadav, 1997). The fruit, a fleshy drupe, develops on sparsely bearing thyrses, and has variable metamorphic and sensorial characteristics. Consumers get their first impressions from visual aspects like colour, shape, size and aesthetics, with taste and smell only explored if the appearance is satisfying. Judgement of quality therefore relies largely on the external condition of the fruit, with external appearance correlated to physiological condition.

Display of sensorial characteristics depend primarily on combinations of genetic traits manipulated through cultivar selection (Judd *et al*., 2002, Human & Rheeder, 2004), but are also influenced by meteorological and geographic features of the cultivating area (Mercadante & Rodriguez-Amaya, 1998; Kruger *et al*., 2003). Apart from cultivar selection, other horticultural aspects under constant revision include preharvest pest control, picking and handling practices, and postharvest treatments (Macnish *et al*., 1997; Jacobi, *et al*., 2001, Reddy *et al*., 2003). All these different factors contribute towards difficulties experienced during production and selection of export quality fruit.

Mango fruit is climacteric and harvesting is done when fruit is considered to have reached physiological (or horticultural) ripeness. The wide-ranging variability of cultivar characteristics results in subjective determination of when picking should commence (Abbott, 1999). Parameters that are monitored include shape (the development of a fully rounded shoulder), internal and external colour development, and firmness (Knight, 1997; Kruger *et al*., 2003). Internal colour intensity is judged by means of an in-house colour chart developed by the South African Mango Growers Association (SAMGA, version 1997). Considerable quantities of fruit are either rejected for export, or downgraded, due to problems based on visual assessment of the produce.

Fruit is still very firm when it is picked, creating an erroneous perception of robustness. Picking practices are therefore quite forceful, aimed towards speed and bulk handling rather than physiological considerations. By the time fruit are packed and ready to be sold, it has been subjected to an array of physical practices. All of these have an impact on the outer layers of the fruit, notably the highly hydrophobic epicuticular wax layer that is the interface between the fruit and the environment (Barthlott & Neinhuis, 1997). This layer plays a decisive role in the attachment and colonisation potential of micro-organisms, providing innate fungal resistance (Comménil *et al.*, 1997; Mariani *et al.*, 2000). The fruit achieves this resistance by presenting a physical and chemical barrier that consists of wax crystalloids on the outside of the fruit. The morphology and composition of fruit wax is dependent on the species involved and often appear as complex, pliable structures (Kolattukudy, 1996).

Physical interference with the epicuticular wax layer may bring about permanent alteration of the crystalloid structures, but the plant can often rectify mechanical removal of the epicuticular wax by deposits on or over the altered area (Jackson & Danehower, 1996). Picking, handling and packline procedures cause physical and chemical changes to the epicuticular structures of fruit. The most noticeable changes are brought about by the

application of commercial wax coatings. The effect of these changes on the cuticular layers below the epicuticular wax and the postharvest quality of mango fruit is not known. One such effect is well demonstrated by a serious postharvest problem known as 'lenticel damage' (Tamjinda *et al*., 1992).

Annually causing a significant loss of return on investment in export fruit, this condition is characterised by a discolouration of the superficial area surrounding the lenticel. Lenticel discolouration does not manifest with the same severity in all commercial cultivars. 'Tommy Atkins' (early maturing) and 'Keitt' (late maturing) are both susceptible cultivars, with 'Tommy Atkins' exhibiting less severe symptoms than 'Keitt' (Willis & Duvenhage, 2002). Symptom expression can be attributed to the differences in fruit colouration, since 'Tommy Atkins' is a red cultivar that masks the discolouration more effectively. However, while 'Kent' and 'Keitt' are both more yellow cultivars, 'Kent' is not prone to the development of lenticel discolouration. Currently, there is no consensus about the environmental or horticultural initiators or triggers for the development of the lesions (Tamjinda, *et al*., 1992; Pesis *et al*., 2000; Willis & Duvenhage, 2002). The unpredictability of this condition results in consignments dispatched to export markets sometimes being downgraded by inspectors at the destination port due to late blemish development. It is also impractical and risky to make changes to commercial scale crop management systems based on experimental simulations of conditions that seem to induce the discolouration of lenticels (Willis & Duvenhage, 2002; Fallik, 2004).

The purpose of this study was to investigate the nature of physical changes of the epicuticular wax brought about by practices during the postharvest period, and to determine how these changes affects the lenticel structures.

7.3 MATERIALS AND METHODS

During the mango seasons of 2002, 2003 and 2004 samples were collected fortnightly from Bavaria Fruit Estates (S 24° 22' E 31° 02') in the Hoedspruit area of the Limpopo Province, South Africa. Cultivar 'Keitt' was used for the study and all fruit were regarded as physiologically mature, with three fruit replicates from each predetermined sample point. All samples were collected from the orchards and packhouse of this single location, thereby excluding any influence from geographical and meteorological factors. Sampling points were selected based on the severity of mechanical impact or where a change in the line environment occurred. Eight predetermined points were used for sampling, namely:

- Orchard (control fruit to determine the natural appearance of the surface, no visible interference or alteration)
- Picking bins
- Dumping bath
- Warm water bath
- Prochloraz application
- Immediately after commercial wax application
- After commercial wax drying (air knives)
- Packed cartons

Samples were processed within 18 hours of collection. Surfaces of collected fruit were protected at the time of sampling, whereafter each fruit was transported in confinement, so that no further alteration was possible. This ensured that the true surface condition during each stage of handling was reflected. Wet fruit were packed and allowed to air dry in the boxes used for transport; they were not exposed to windy or sunny conditions to accelerate the drying process.

Fresh material was sectioned and from each fruit, ten 5 x 5 mm squares were cut from the fruit shoulder area, which had been exposed towards the outside of the tree during growth. This side showed better colour development than the shaded side of the fruit. After sectioning, samples were immediately cryofixed in liquid propane at -180 °C, vacuum dried (Custom built, Tshwane University of Technology, Pretoria, South Africa) at -80 °C and 10^{-7} mBar (10^{-5} Pa) for 72 hours, and coated in 0.5 % Ruthenium vapour to obtain proper conductance. Scanning electron microscopy (SEM) (JSM 840, JEOL, Tokyo, Japan) at 5kV and a working distance of 12 mm was used to study the sample material.

7.4 RESULTS AND DISCUSSION

The fruit surface from control sample surfaces presented an undisturbed epicuticular covering (Fig. 1A) that was highly organised into two distinctive layers of wax crystalloids (Fig. 1B & C). The top (outermost) crystalloids were sepaline (Fig. 1D) and arranged in architecturally complex, funnel shaped lattices. The innermost layer adjacent to the cutin consisted of dense and anamorphous wax. Fruit surfaces from the picking bins were subjected to physical disruption and areas where chafing and scratching had taken place,

or where pressure had been applied, can be discerned (Fig. 2A & B, white arrows). From figure 2C and D it could be seen that the more pliable, delicate sepaline layer was affected by mechanical interference, with the more robust, amorphous layer still intact. Fruit entered the packline through a dumping bath, the first sampling point. There was no discernable difference in the nature of the surface disturbance between fruit collected from storage bins and fruit from the dump bath, although the amount of surface disruption had increased (Fig. 3A - D). Despite the fact that the first bath was intended to remove all dust and foreign matter from the fruit surface (Bally *et al*., 1996), loose debris positioned on top of the depressed wax layer was visible on some fruit surfaces, indicating that it had been deposited from the water in the bath (Fig. 3A). Overall, the surface topography became smoother due to the rolling and rubbing of fruit as they travel into and through the bath (Fig. 3B). Flattened crystalloids on the fruit surface trapped debris particles present in the affected area (arrows in Fig. 3C & D). There are several sources from where debris originate, one being the blank newspaper used to line the picking bins. The paper was dumped into the dumping bath along with the fruit and, by disintegrating, eventually contaminated the line, as did dust particles blown into the packhouse and onto the packline.

Exiting the dumping bath, the line passed over sorting tables into a warm water bath. Fruit was kept submerged in water at 47 °C (± 2 °C) for five minutes, so that the temperature of the fruit epicuticular membrane reached the ambient water temperature. Figure 4A show the radical change in the architecture of fruit wax crystalloids of the fruit surface at this point. Scarring by the brushes on the packline became visible (Fig. 4B). The warm water exposure caused the soft sepaline layer to collapse on the harder wax below (Fig. 4C & D). Entering the dumping bath containing a postharvest fungicide, the external fruit temperature was still 45 °C ± 2 °C, but matched the water temperature upon exit from this bath (34 °C ± 3 °C). Most of the fruit surface showed increased mechanical flattening of the epicuticular crystalloids (Fig. 5A - D), exacerbated by the softening of the wax in the warm water bath.

Immediately after the application of a commercial wax emulsion (17 % solids), but before passing through a jet of air, known as air knives, a set of fruit samples was collected. Left to dry naturally, the commercial wax coating had an uneven and lumpy appearance (Fig. 6A & B), which accentuated the importance of the air knives. In transverse sections, areas where the commercial wax emulsion had integrated poorly with the epicuticular wax were identified (Fig. 6C). In areas where some integration did take place, the incomplete process was visible as unevenly distributed densities in the wax layers (Fig. 6D). Another

set of samples were collected after the waxed fruit had passed through the air knives. (Fig. 7A - E). These focussed, forceful air jets levelled the wax film surface (Fig. 7A - C) and contributed to integration of the two wax fractions. The increased rate of integration resulted in a film with more consistent thickness and integrity (Fig. 7D & E). Lenticels from market-ready fruit are mostly flooded by the commercial wax (Fig. 6A & B, Fig. 7A & B), but the wax film has sufficient gas permeability not to have a suffocating effect (Louw, pers. comm., 2004). While not all fruit coated with commercial wax will develop discoloured lenticels, fruit trapped on the wax rollers are always blemished within a short time. Blemishes develop towards the tapered ends of the fruit, which is where excess wax accumulates on such trapped fruit. Although assurance of the gas permeability of the commercial wax was given, it must therefore be argued that when lenticels flooded or covered by a thick layer commercial wax, gaseous exchange will be impeded. This indicates the relevance of careful control over the thickness of the applied wax coating (López *et al*., 1995; Manzano *et al*., 1997) and the uninterrupted flow of fruit on the packline.

Application of commercial wax eventually alters the fruit surface into a smooth, featureless capsule. However, the protective wax film itself was exposed to physical damage, with scratches and cracks visible on fruit from collected packed cartons (Fig. 8A). Such damage could have been inflicted anytime after waxing and drying, since the fruit rolled along the packline, was handled by packers and rubbed against each other as they were placed into boxes. Damage was presented in the form of cracking and tearing of the wax film, and the fruit surface was visible where the combined layer of plant wax/commercial wax was defaced (Fig. 8A & B). Fungal spores trapped in the wax film (Fig. 8C & D) could have originated from a number of sources. These include inefficient prior washing of the fruit surface in the various baths, contamination from the baths or packline, or introduced into the packhouse by wind. The commercial wax emulsions were stored in sealed containers up to the point of application by spray nozzles; therefore this was not a likely source of contamination.

The fruit cuticle from all the samples from the various sampling points seemed unaffected by the physical impacts. No changes in layer thickness, intracuticular wax distribution or cutin integrity were observed in any of the material. In Figure 9A, recrystallised fruit wax was observed on the exterior surface of the commercial wax coating fruit samples from the cartons packed for shipping. These crystals differed morphologically from the natural wax crystalloids (Fig. 9B) with the most likely reason the loss of wax constituents during the migration of these fractions through the integrated wax film. The origin of wax

constituents is still debated but it is accepted that cuticular waxes accumulate in the cutin (Lui & Post-Beittenmiller, 1995; Kolattukudy, 1996). Cuticular waxes play a role in cuticular transport and act as the source of precursors for epicuticular crystalloids (Merk *et al*., 1998; Riederer & Schreiber, 2001). Environmentally induced changes in the morphology and chemical properties of the epicuticular wax layers may, however, influence the functionality of these as well as successive layers (Kolattukudy, 1996; Batzli & Dawson, 1999; Haas *et al*, 2001). This will impact on the physiology of the total fruit and ultimately, its postharvest appearance (López *et al*., 1995).

Fruit sampling from the packline was done at random, and most samples had no lenticel discolouration. Despite the existence of structural variance in terms of morphology, internal lenticel structures did not alter due to the amount of physical impact as it travelled down the packline. This is illustrated by Figure 10A, which shows a lenticel from a sample taken from the dumping bath and Figure 10B, which shows a lenticel from a sample taken from the warm water bath. The external fruit wax in the vicinity of lenticels showed equal scarring to the rest of the surface at every sampling point (Fig. 10C). Using SEM, it was impossible to distinguish between the external morphology of a discoloured lenticel (Fig. 10C) and a non-discoloured lenticel (Fig. 10D).

7.5 CONCLUSION

Epicuticular wax crystalloids undergo morphological changes during the progression of fruit along the packline due to the impact of commercial postharvest practices on these structures. The protective function of the crystalloids during fruit development is indicated by the angular alignment and denseness, as this relates to light scattering abilities (Juniper & Cox, 1973; Grant *et al*., 2003). Epicuticular wax therefore has an impact on the temperature regulation and subsequent water management by the plant during fruit development (Toivonen, 2002).

Mango fruit receives rigorous postharvest treatment on commercial packlines aimed at prolongling shelf life, for postharvest disease control measure and to improve fruit appearance (Srinivasa *et al*., 2004). It was shown that debris scattered around lenticels could enter these epicuticular structures from the packline environment during postharvest handling. Such foreign objects will injure the soft internal mesophyll tissue, triggering plant defence systems (Beckman, 2000). Evidence of commercial wax flowing into lenticels confirmed that blockage by commercial wax was possible. According to López *et al*.

(1995) and Manzano *et al*. (1997) blocked lenticels would experience altered gaseous exchange, which, in turn, would affect the rate of metabolism, and influence physiological reactions in the fruit tissue. Despite the physical impact of temperature changes, mechanical packline procedures and waxing on the epicuticular structures, cutin had an unaffected appearance, confirming the resilience of the biopolymer structure.

Uneven spread of the fungicide applied immediately after the warm water bath indicated the possible removal or chemical change of heat labile fractions in the fruit wax. In a study on the effect of hot water treatments, Fallik (2004) described heat induced biochemical changes in the fruit rind involving polyamines and proteins. However, no information on the chemical interaction (or absence of such reactions) between the natural fruit wax and the chemicals applied on the packline was available. Plant pathogens and phytophagous insects have evolved chemical targeting mechanisms based on the alkanes, lipids, fatty alcohols, sterols and wax esters of the epicuticular layer (Eigenbrode, 1996; Espelie, 1996; Jackson & Danehower, 1996; Comménil *et al*, 1997). Determining the exact nature and composition of the fruit wax constituents will enable more precise tracking of the induced chemical changes in the epicuticular layer. This will give credence to toxicity studies of the mango fruit wax layers towards micro-organisms, contributing to a closer understanding of the function of the different wax layers (Schwab *et al*., 1995; Jackson, & Danehower, 1996; Comménil *et al*., 1997). Identifying biologically active compounds will also advance beneficial characteristics of commercial fruit coatings.

This study has shown that a threat posed by the application of commercial coating wax is the embedding of micro-organisms. Furthermore, damage to the integrity of the integrated wax film may serve as entry points for postharvest organisms (Schwab *et al.*, 1995, Comménil *et al.*, 1997). Mango fruit is climacteric and continuing physiological changes during further ripening will lead to cracking of the commercial wax coating. Maintenance of metabolic stasis through suitable postharvest storage is therefore imperative.

Fruit that are trapped on the packline often exhibit the worst signs of discolouration and physiological stress. The primary role of lenticels is gaseous exchange and its development is synchronised with the development and maturation of the epicuticular layers of the fruit rind. Although it is a harmless and natural phenomenon, development of excessively visible lenticels are indicative of physiological stress (Larson *et al*., 1991; Batzli & Dawson, 1999). Trapped fruit is subjected to prolonged submersion (in a dumping bath) or excessive wax application (wax rollers). The lenticels will eventually become flooded, inhibiting gaseous exchange. Gaseous exchange within the fruit rind and in the

particular vicinity of the lenticel cavity takes place in micro atmospheres. These micro atmospheres are modified by physical additions to the epicuticular layer, creating a point of physiological stress. Affected fruit tissue will perceive this induced stress as injurious, triggering an oxidative burst in localised cells, and resulting in a cascade of reactions to form self-defence phenolics (Grace & Logan, 2000).

The build-up of phenolics, combined with the layers of cutin and epicuticular wax lining the lenticel lumen, ensure that the lenticel area is delimited by chemical as well as physical measures against infection (Beckman, 2000). It has been found that a discernable relationship between lenticel discolouration, lenticel morphology and cultivar exists.

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7.7 FIGURE CAPTIONS

Figure 1 Scanning electron micrograph of mango fruit surface with pristine wax (A). Transversal section of the epicuticular membrane, showing the cutin and wax bilayer (B). Close-up of the wax bilayer, with an amorphous layer separating the cutin and the layer of sepaline crystalloids (C). Micrograph of a typical sepaline crystalloid from the outer wax layer (D).

Figure 2 Fruit from picking bins with marks where chafing and scratching had taken place, or where pressure had been applied can be discerned (A & B, white arrows). The more pliable, delicate top layer was affected by mechanical interference, with the more robust, amorphous layer still intact (C & D).

Figure 3 Fruit from the dump bath, with increased surface disruption (A & B). Debris trapped in the flattened wax is indicated by arrows in C and D.

Figure 4 The change in the architecture of the epicuticular wax crystalloids on surfaces of fruit emerging from the warm water bath (A). A scar produced by the brushes on the packline can be seen in (B). The soft, pliable top layer of epicuticular wax had collapsed on the harder wax below and debris became trapped in the flattened wax (arrows, C & D).

Figure 5 Progressively increased flattening of the epicuticular wax crystalloids from fruit surfaces after Prochloraz application (A - D), exacerbated by softening of the wax in the previous warm water bath. Crystalloids lining the grooves in between epicuticular plates (white arrows, B & C) were less exposed to both mechanical abrasion and the effect of heat than the planar surfaces (black arrows, C & D).

Figure 6 Without forced air drying, commercial wax emulsion had an uneven and lumpy appearance immediately after application (A & B). Transverse section showing poor integration of the commercial wax with epicuticular wax (C). Incomplete polymerisation of the commercial wax (black arrow), with destructured fruit wax visible as a dark layer adjacent to the cutin (white arrow, D).

Figure 7 Lenticels flooded with commercial wax emulsion (A & B). Focussed jets of forced air smooth the commercial wax after application (C). Forced air drying effects close union of commercial and epicuticular wax, resulting in good film formation (D) and more uniform integration (E).

Figure 8 Compromised surfaces from fruit in commercial sales cartons with defaced wax film and the exposed cutin underneath (A & B). Contaminating fungal spores trapped by the wax film (C & D).

Figure 9 Recrystallisation on top of the commercial wax film (A). Reconstituted crystalloids showed architectural differences in comparison to normal crystalloids (B).

Figure 10 Structural variance of lenticels were not due to impact from the various sample points (A & B). No distinguishing morphology between a discoloured lenticel (C) and non-discoloured lenticel (D) was detectable by scanning electron microscopy.

7.8 FIGURES

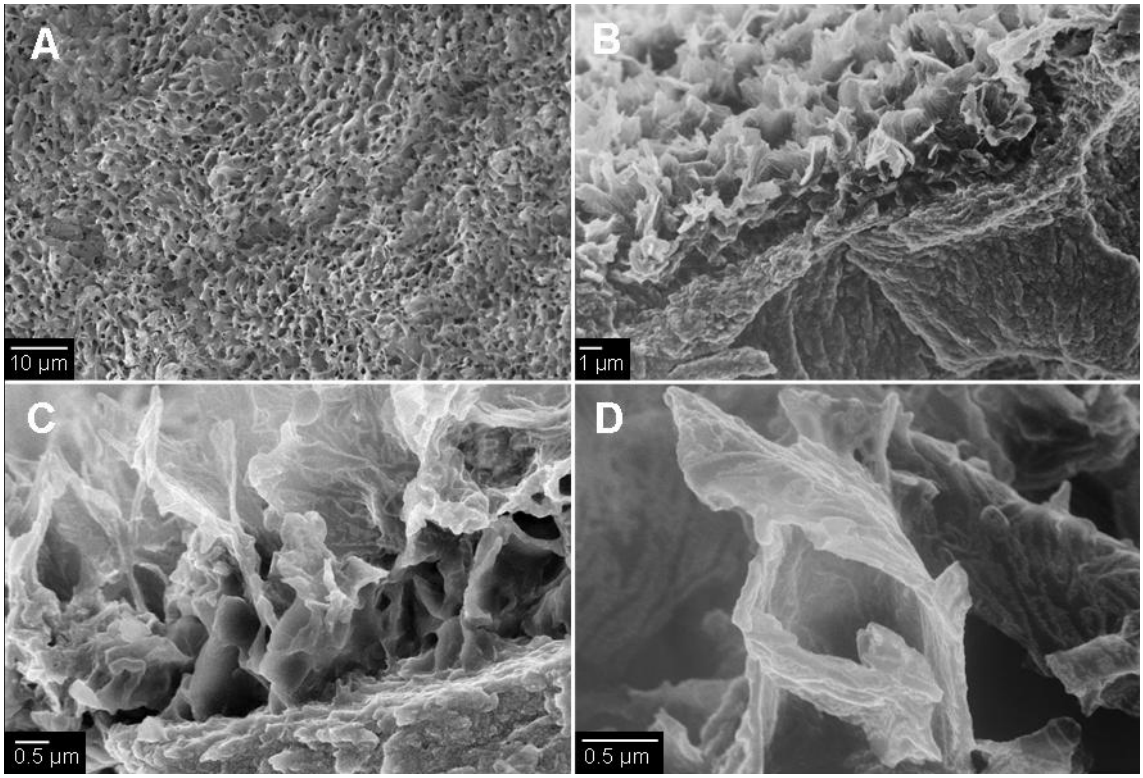
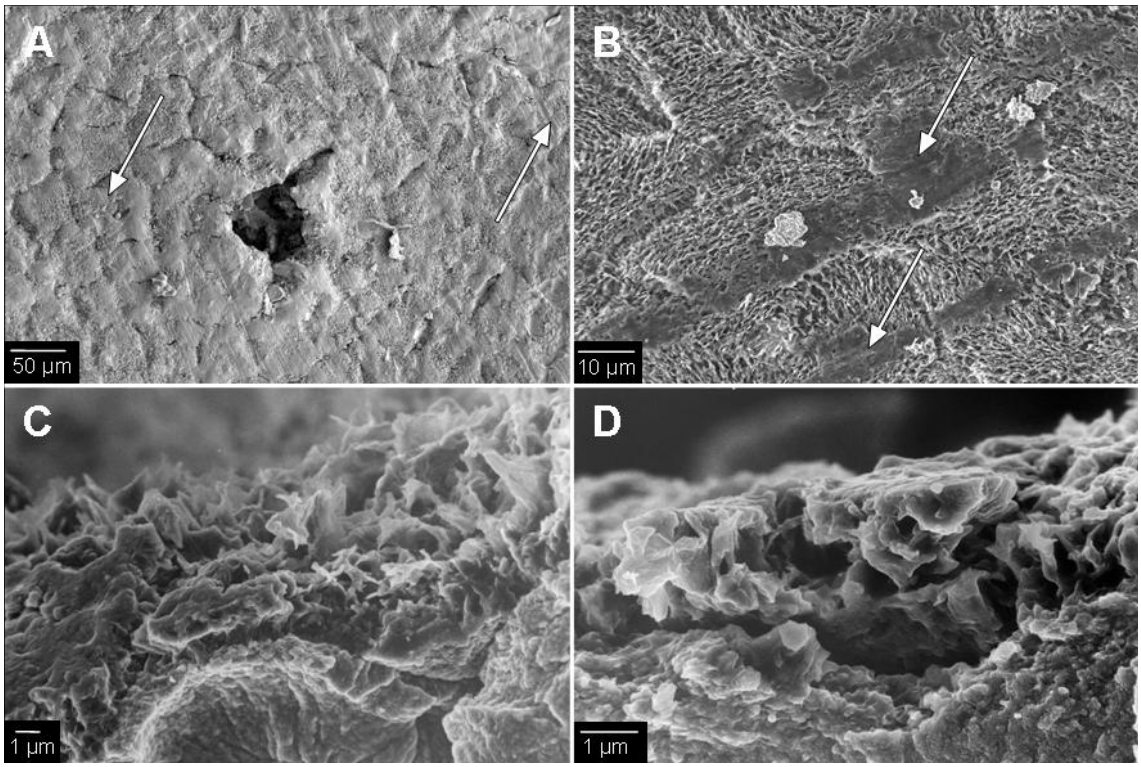


Figure 1



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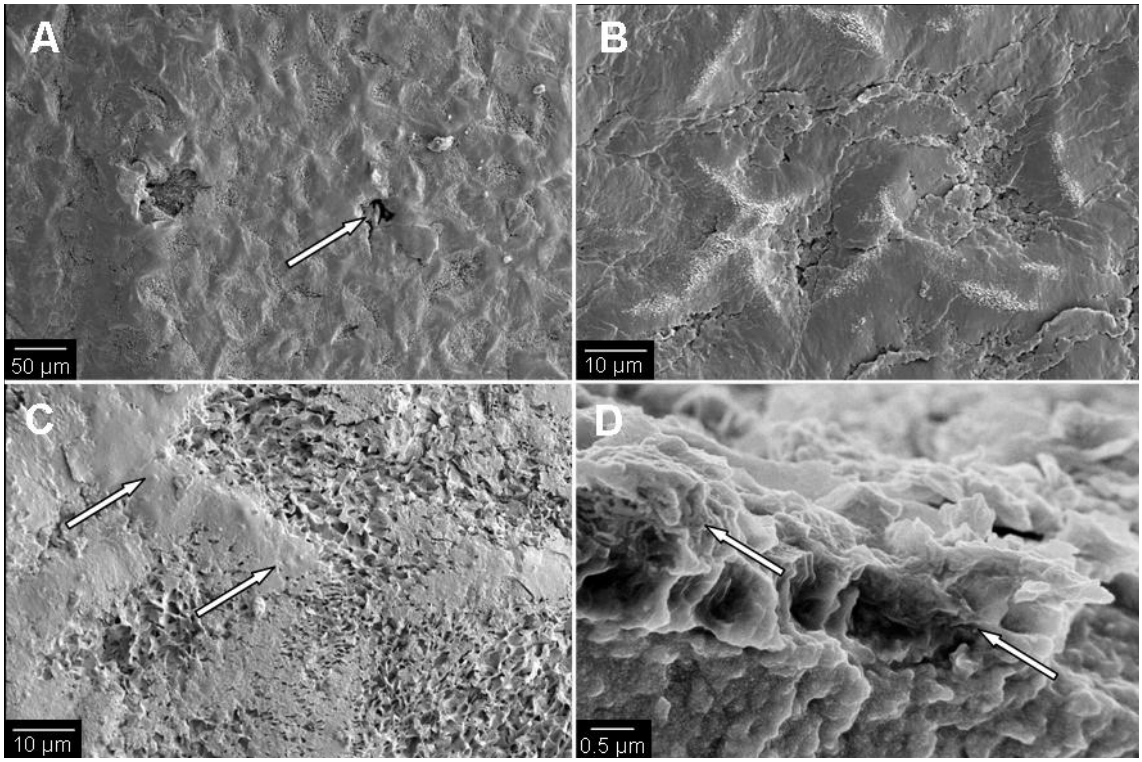

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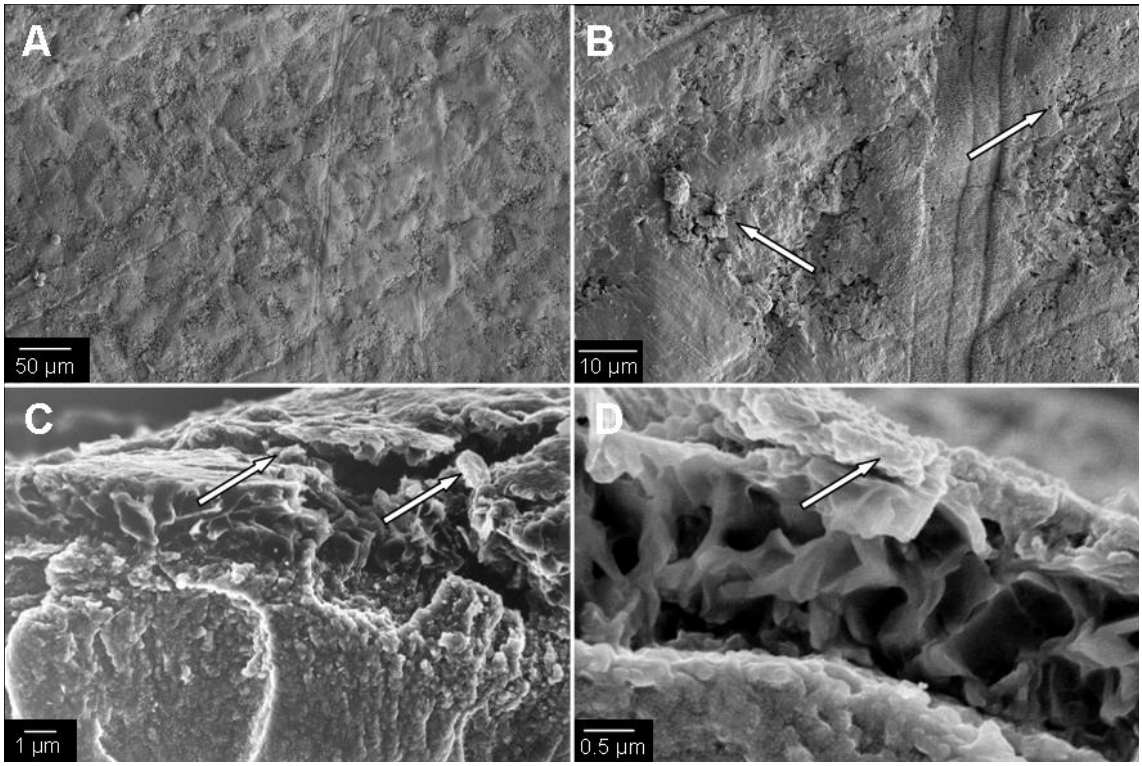

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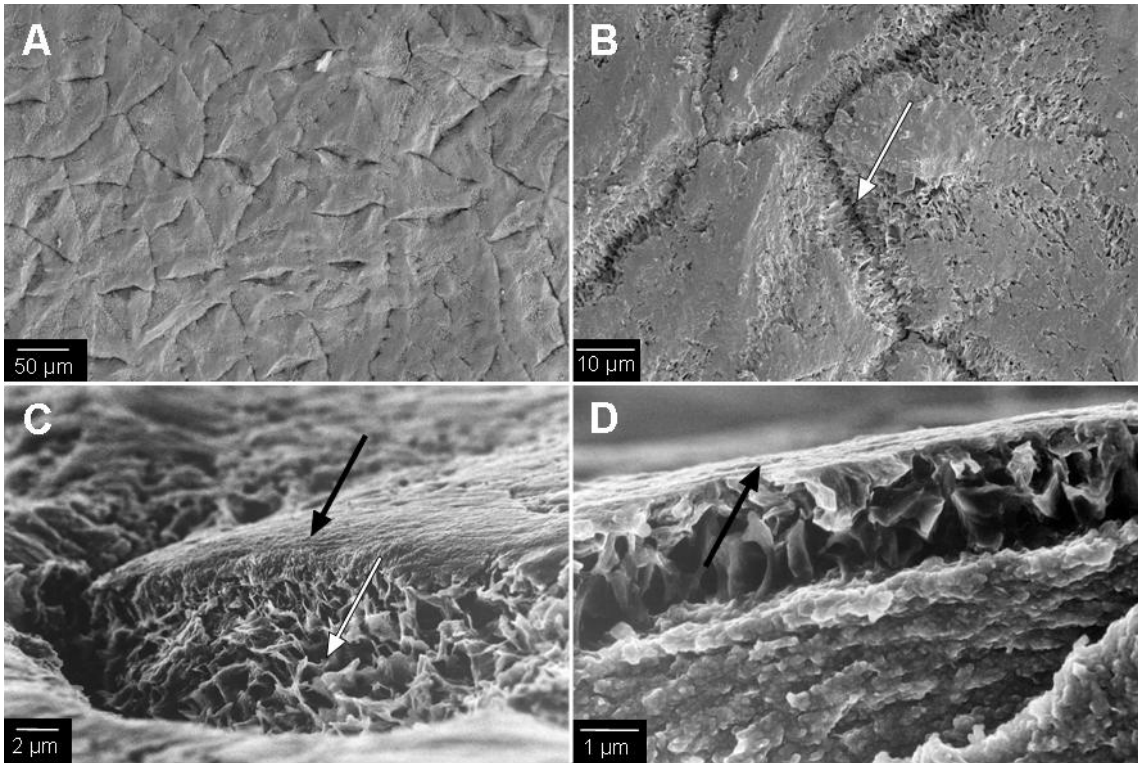

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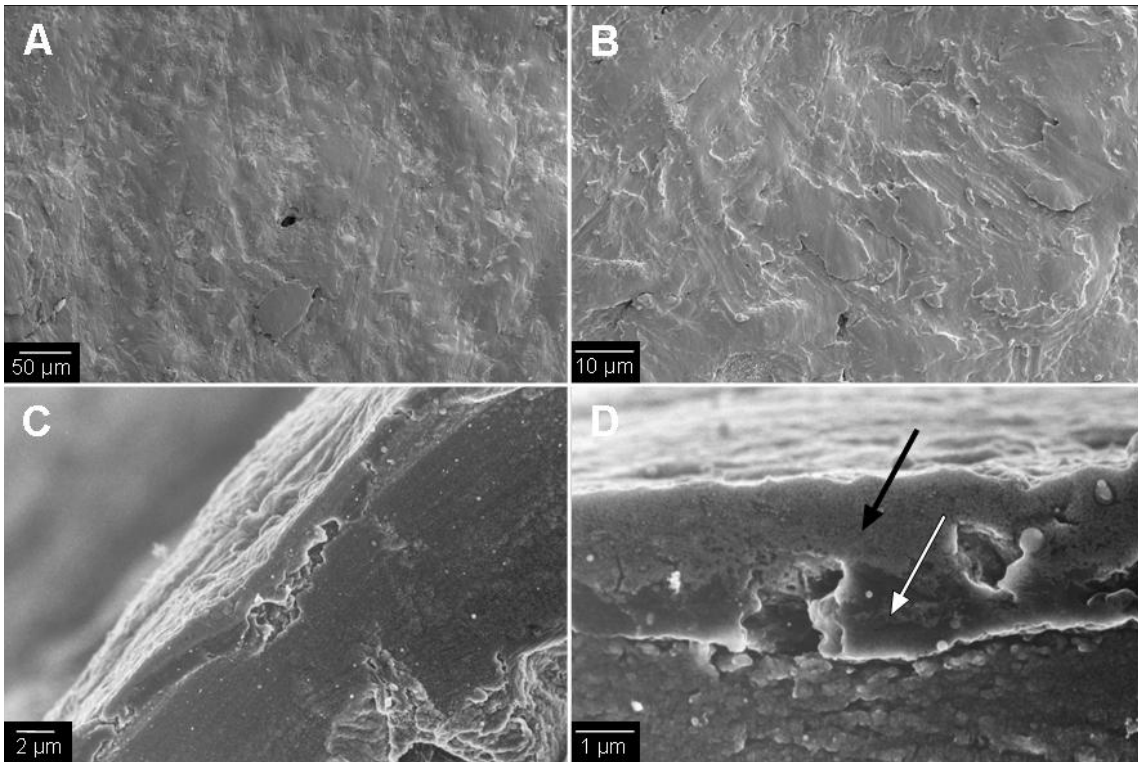

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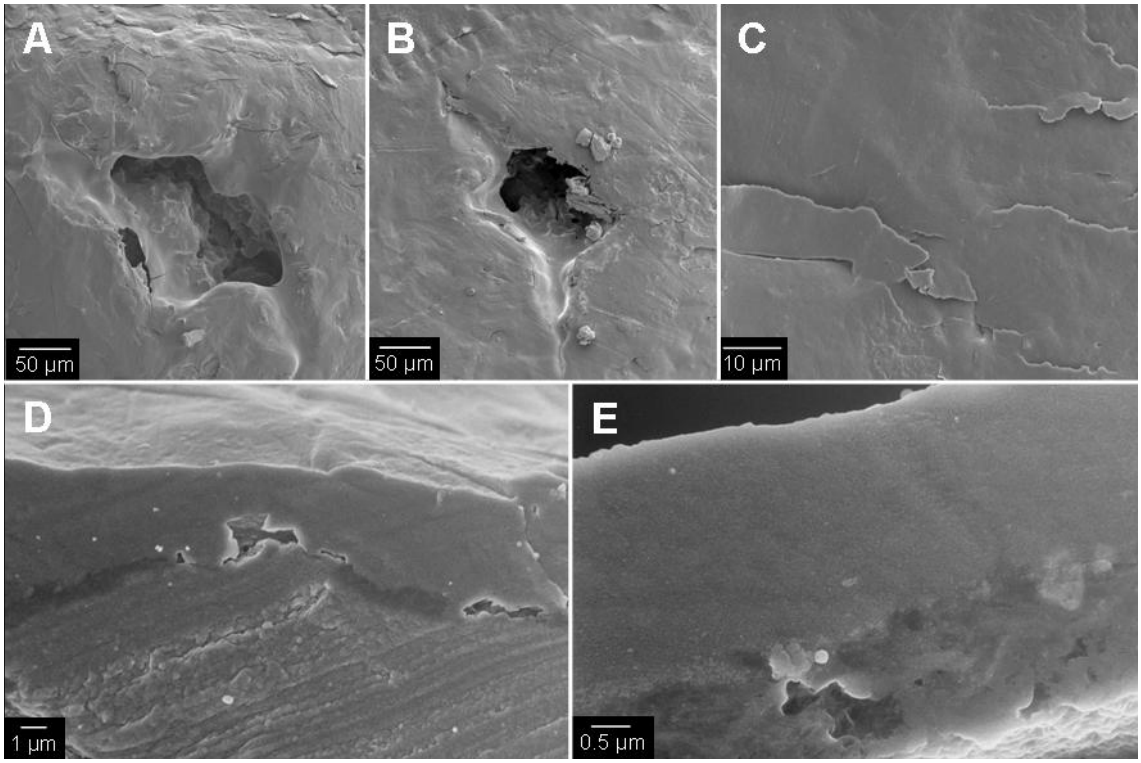

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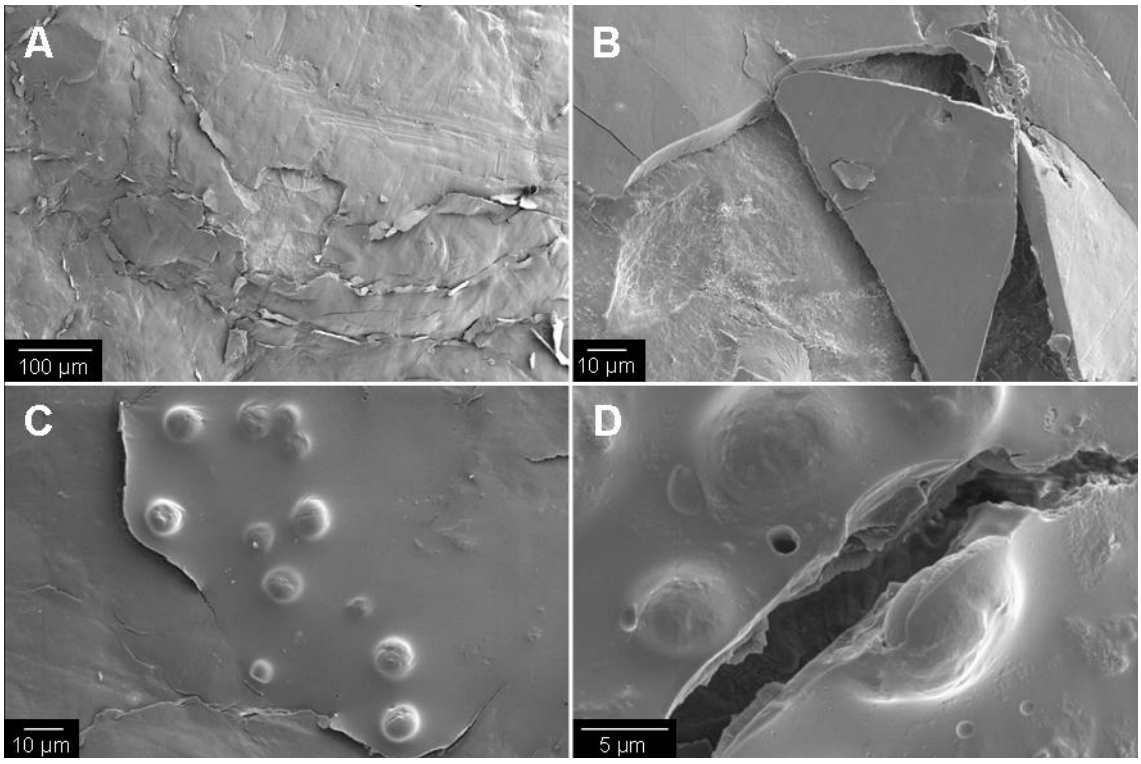

Figure 8

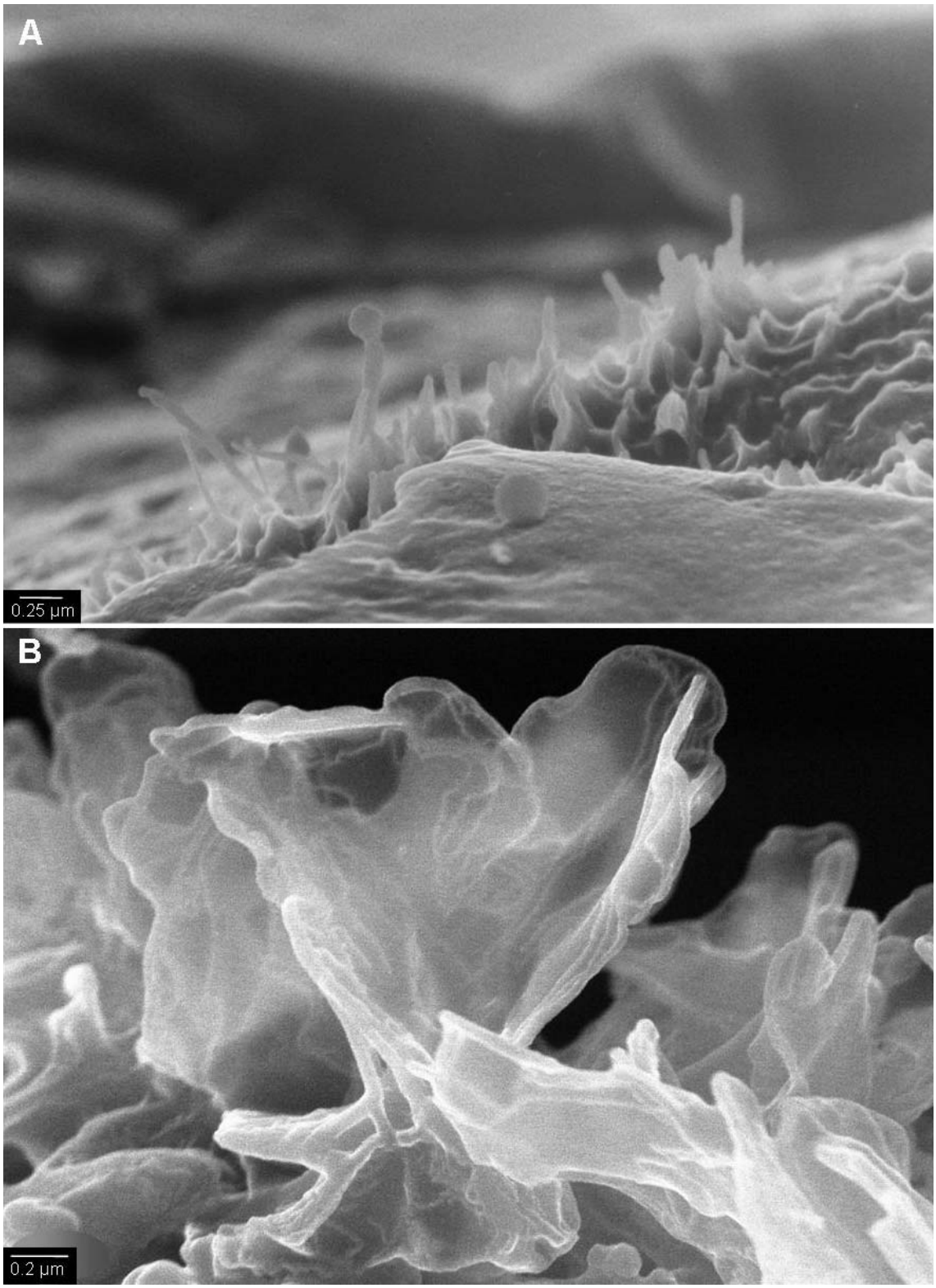

Figure 9

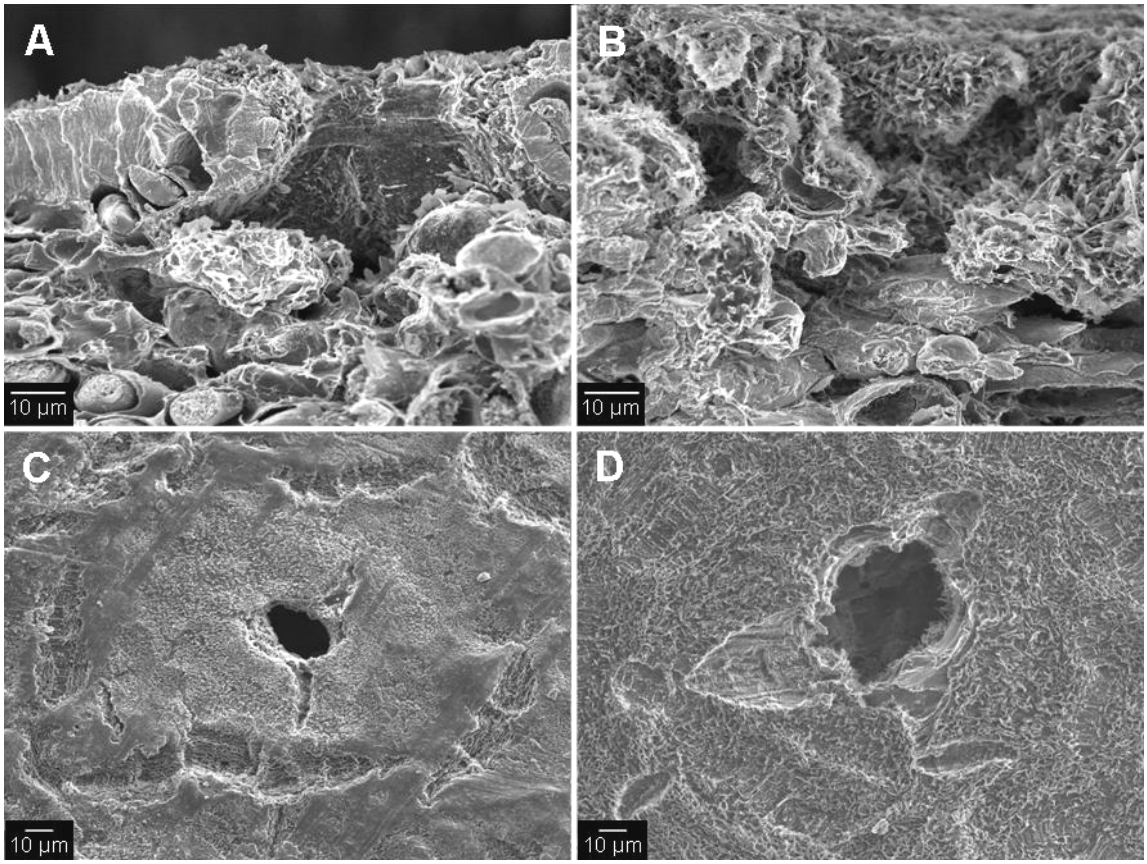

Figure 10

I am among those who think that science has great beauty. A scientist in his laboratory is not only a technician: he is also a child placed before natural phenomena which impress him like a fairy tale.

Marie Curie (1867 - 1934)

Chemist

Chapter 8

DISCUSSION

Aerial plant parts are structured and presented in such a way as to ensure survival and procreation. They are also exposed to all possible forms of meteorological and environmental effects. Therefore, plant surfaces and immediate subcuticular tissue in particular, must ensure continued existence through the evolution of an array of mechanisms and morphological adaptations (Jeffree, 1996; Barthlott & Neinhuis, 1997). These adaptations all contribute to protection of plant tissue against extreme levels of light, debilitating temperatures, water stress (due to flooding or drought), wind induced strain, chilling and desiccation, weight stress in snow and rain, and salt spray (Kolattukudy, 1996). Biotic interactions such as microbial attacks, predation and competition also contribute to morphological adaptations (Kays, 1999). Fruit, with the single most important function of dispersing subsequent generations, are particularly vulnerable to all the aforementioned calamities.

Management of fruit crops therefore rely on successful intervention of these potentially detrimental factors. Crop management can take the form of manipulation of both nutritional and water status, disease control by biological and chemical means, physical alteration through horticultural practices, and genetically predetermined breeding strategies. Mango fruit cultivar development exemplifies the expansion of characteristics like shape, colour, texture, taste and smell, while simultaneously facilitating commercial production of cultivars suited to export markets dictated by consumer and cultural influences (Majumder *et al*., 1972; Saúco, 2004). In this way, custom, diet and trade provide the impetus for the growth of fruit production in general. Benefits in fruit trade lead to concurrent growth and development of applicable skills and knowledge bases, secondary industries, and community infrastructures.

Less obvious, yet as important, is the particular role of the fruit surface in all phases of crop management and cultivar development. It not only is the interface between fruit contents and environment, but it also is the primary sensory input for consumers. Lack of

knowledge of the fruit surface and its importance as environmental interface invariably leads to costly and time consuming inefficiencies (Joubert, 2001). The complexity of mango fruit surfaces revealed by the ontogenetical study of the epicuticular wax, and subsequent study of lenticel morphology, illustrates this point. Exhaustive attempts have been made to manage the manifestation of lenticel discolouration (O'Hare *et al*., 1999), with the cosmetic nature of the condition established by this study. It was found that wax crystalloid structures were not cultivar dependant, but that morphological characteristics of lenticels play an important role. Although unclear what evolutionary pressures lead to the development of chronologically and structurally intricate layers, their chemical and morphological distinctiveness would suggest functionalities not yet fully understood. However, the relevance of epicuticular wax in the propensity of some cultivars towards lenticel discolouration has been proven (Donkin & Oosthuyse, 1996). Lenticel discolouration is not only associated with lenticels of a particular size and shape, but also with the density and complexity of the wax crystalloids inside the lenticels. With known contribution of environmental volatiles to epicuticular wax (Riederer & Markstädter, 1996), the role of volatiles in the chemistry of mango wax must be established. These volatiles determine cultivar aroma (Beaulieu & Lea, 2003), and may contribute to lenticel discolouration. If this should be the case, such information may assist in the formulation of postharvest management practices aimed at controlling the condition.

However, both postharvest and preharvest management can benefit from better understanding of the dynamics in the epicuticular membrane, since all crop management practices impact on the fruit surface. The vulnerability of mango fruit during its development, and continually more stringent requirements for non-chemical crop management, necessitates continued consideration of alternative measures for preharvest crop protection (De Oliveira Fonseca & Salomão, 2004). Such measures, however, must be based on complete information that takes fruit physiology as well as morphology into account. To this extent, understanding fruit epicuticular wax and its role as part of the fruit-atmosphere interface is important, even more so in monoculture crops. Cultivar development must take innate physiological and morphological qualities of different commodities into account. Wax crystalloids act both as light attenuators to screen against harmful ultraviolet light (Grant *et al*., 2003), and assisting in light harvesting during fruit set (Kolb *et al*., 2001). With the high levels of solarisation in South Africa, sunburn is a very serious problem. Application of kaolin was found to be a promising alternative measure for both sunburn and insect control. Nonetheless, repercussions and interference with the epicuticular interface during fruit development are uncertain and warrant further study. This includes not only the chemical interaction of the kaolin and the fruit wax, but also the

effect of the kaolin on the morphological development of the fruit surface, since kaolin may impact on the fluidity of the cuticular wax and cutin integrity.

Both mechanical and chemical interference with epicuticular fruit structures cannot be avoided in commercially cultivated fruit such as mango. Application of commercial wax on the packline is the final and most severe interference with epicuticular structures. Wherever plausible, such interferences should augment the physiological resilience of the crop, as is the objective in commercial wax formulation development (López *et al.,* 1995; Manzano *et al.,* 1997). These applications completely negate the architecture of the fruit wax, integrating it with the commercial wax. Still, commercially applied formulations are important in ensuring an appealing appearance and prolonged shelf life of marketable fruit. Furthermore, despite this structural disruption, antimicrobial properties of fruit wax (Moyna & Heinzen, 2001) may continue to be imbued in the aggregate film. However, this was not established in the present study and, together with more detailed chemical analyses of the fruit wax, should be the topic of future research.

Although many new questions have arisen and are still unanswered, this study of the development, and structural and chemical complexity of the mango fruit epicuticular membrane has contributed to current understanding of the mango fruit surface.

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Courage is the thing. All goes if courage goes.

J.M Barrie (1935 -)

Author

Resumè

Title: Aspects of mango (*Mangifera indica* L) fruit rind morphology and chemistry and their implications for postharvest quality

Supervisor: Professor Lisa Korsten

Department: Microbiology and Plant Pathology

As the second most popular fruit world-wide, cultivation of mangoes in South Africa is of strategic economic importance. Competitive export markets require horticultural practices that meet international regulations concerning cultivation, pest control and maintenance of fruit physiology during export. The implementation of such practices, however, cannot be cost effective and successful without detailed and scientific-based knowledge of the commodity concerned. This study of the epicuticular surface of mango fruit has described the ontogeny, morphology and some chemical aspects thereof. Contributions from this study include the following findings:

- Ontogeny and morphology of mango fruit wax: This study found that the highly intricate wax crystalloid structures were not strictly cultivar dependant. A complex series of events constitutes epicuticular wax development. The development of epicuticular crystalloids is accompanied by considerable changes in cutin and epidermal cell morphology.
- Morphology of mango lenticels: An unusual morphology with some cultivar dependent lenticel characteristics was described. Exhaustive past attempts at management of the manifestation of the economically important lenticel discolouration were placed in perspective by establishing the cosmetic nature of the condition. It was found that the density and distribution of epicuticular wax contribute to morphological characteristics of lenticels of individual cultivars.
- Chemical characterisation of mango fruit wax: The chemical complexity of the dual layered epicuticular wax of mango fruit was established by this study, and the validity of interchanging Raman spectroscopy and Fourier transform infrared spectroscopy as investigative techniques established.

- Chemical profiles of discolouring lenticels: The development of lenticel discolouration as a stress-related self-defence mechanism was shown through use of combined chemical and visualisation techniques. This study confirmed the superficial nature and self-defence role of discoloured lenticels.
- Impact of some pre- and postharvest practices on mango fruit wax:
  Not only postharvest, but also preharvest management of mango fruit must consider the epicuticular membrane as part of the fruit-atmosphere interface. Maintaining a balance between them depends on a better understanding of the interdependence of management and fructosphere dynamics. Both a preharvest and a postharvest practice were studied:
  - Preharvest treatment of mangoes with uncalcined kaolin.
    Sunburn is an economically important problem in all fruit and vegetable crops. This study has showed that solutions to contain the problem can, however, not be transferred between crops without scientific knowledge of the physiological impacts and long term repercussions thereof.
  - Effect of mechanical handling on the packline and commercial wax coating.
    Physical and chemical impacts from the packline bring about progressive, irreversible changes to the fruit epicuticular wax. To benefit from these changes, strict management and process control must be practiced.

Research outputs

PRESENTATIONS AND POSTERS

Du Plooy, W. & Korsten, L. 2001. Total quality management systems for food production in South Africa. 39th Annual Congress, *Southern African Society for Plant Pathologists*, Poster, Greenway Woods, Witrivier, 21 - 24 January.

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