Cuticle deposition in apple fruit: A radial gradient in cuticle age

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Yiru Si, M.Sc.

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Referent: Prof. Dr. agr. Moritz Knoche

Korreferentin: Prof. Dr. rer. hort. Traud Winkelmann

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Abstract

The skins of most fruitcrop species are subject to marked strain due to the continuing expansion of the fruit surface throughout the long developmental period. A mechanical failure of the cuticle during fruit expansion leads to fruit skin disorders. Fruit skin disorders compromise fruit appearance and decrease market value. The cuticular membrane (CM) of apple fruit copes with the ongoing skin strain by continuing to deposit cutin and wax. Recent evidence suggests a radial gradient in strain between the outer (more strain) and the inner surfaces (less strain) of the apple fruit CM. The strain gradient is consistent with the observation that microcracks being initially limited on the outer surface of the CM. It is hypothesized that an ongoing deposition of cutin on the inner surface of the CM would account for this gradient in strain. Unfortunately, direct experimental evidence is lacking. This dissertation is dedicated: (1) to establish a system using the radioactive carbon isotope (^{14}C) and the stable carbon isotope (^{13}C) to quantify CM deposition in developing apple fruit; (2) to identify factors affecting CM deposition in the field and (3) to provide conclusive experimental evidence for a radial gradient in cutin deposition such that the inner surface of the CM is 'younger' than the outer surface.

Labelled oleic acid or palmitic acid was applied as supposed precursors for cutin monomers to the surface of 'Idared' fruit using 'infinite dose', 'finite dose' or 'injection' methods. Of these methods, infinite dose feeding resulted in more consistent and higher precursor incorporation. Incorporation of oleic acid was significantly higher than of palmitic acid. Oleic acid was incorporated in the cutin fraction, but not in the wax fraction. There was no difference in the incorporation of ^{13}C or of ^{14}C labelled oleic acid. Potential gradients in cuticle age were investigated following feeding developing apples with ^{13}C labelled oleic acid. The ^{13}C content was highest in non-ablated CM isolated immediately after the feeding and incorporation period. The ^{13}C content decreased as the CM was progressively ablated from the inner surface. When the ^{13}C content of CMs was quantified in fruit harvested at maturity, the ^{13}C-enriched layer was found deep in the CM following the continued deposition of un-labelled cutin occurring after ^{13}C feeding period. The results recorded the presence of an age gradient in the apple fruit CM between the younger inner surface and the older outer surface. This gradient is the direct result of the deposition of cutin being on the CM's inner surface.

Keywords: cuticle, cutin, deposition, cuticular age, CAPP, wax, microcracks

Zusammenfassung

Die Fruchthäute der meisten Obstarten werden während des Fruchtwachstums durch permanente Zunahme der Fruchtoberfläche während der langen Entwicklungsperiode einer starken Dehnung ausgesetzt. Das Versagen der Kutikula (CM) während des Wachstums führt zu Störungen in der Fruchthaut. Die Fruchthäute des Apfels bewältigen die permanente Dehnung durch kontinuierliche Deposition von Cutin und Wachsen. Kürzlich wurde ein radialer Dehnungsgradient zwischen der äußeren (hohe Dehnung) und der inneren Oberfläche (weniger Dehnung) der Apfel-Kutikula demonstriert. Dieser Gradient stimmt mit der Beobachtung überein, dass die Bildung von Mikrorissen an der äußeren Oberfläche der Kutikula beginnt. Daher wird vermutet, dass eine fortwährende Deposition von Cutin an der inneren Oberfläche der Kutikula diesen Dehnungsgradient erklärt. Dazu gibt es bisher allerdings noch keine experimentellen Beweise. Die Ziele dieser Arbeit sind (1) die Etablierung eines Systems unter Einsatz radioaktiver (^{14}C) und stabiler Isotope (^{13}C) zur Quantifizierung der Kutikula-Deposition in sich entwickelnden Äpfeln, (2) die Identifikation von Faktoren, die die Kutikula-Deposition im Feld beeinflussen und (3) die Lieferung eines experimentellen Belegs für die Existenz eines radialen Gradienten im Alter der Kutikula. Ein radialer Gradient im Alter belegt, dass die innere Oberfläche der Kutikula jünger ist als die äußere Oberfläche. Markierte Öl- und Palimitinsäure wurden als Vorstufen für Cutinmonomere mittels ‚Infinite dose Methode', ‚Finite dose Methode' und mittels ‚Injektion' auf bzw. unter die Fruchthaut von ‚Idared' Äpfeln appliziert. Hierbei hat das Füttern mittels Infinite dose Methode zu konsistenter und hoher Inkorporation geführt. Die Inkorporation von Ölsäure war signifikant höher als die von Palmitinsäure. Ölsäure wurde in die Cutinfraktion, nicht aber in die Wachsfraktion inkorporiert. Potentielle Gradienten im Alter der Kutikula wurden nach Füttern von Äpfeln mit ^{13}C-markierter Ölsäure mit der ‚Infinite dose Methode' untersucht. Der ^{13}C-Gehalt war am höchsten in solchen CMs, die direkt nach der Fütterungs- und Inkubationsperiode isoliert wurden. Trug man die Kutikula von der Innenseite her sukzessive ab, so nahm der ^{13}C-Gehalt mit fortschreitendem Abtragen von der inneren Oberfläche ab. Verblieben die Früchte nach der Fütterung bis zur Erntereife am Baum, befand sich die ^{13}C-markierte Schicht tiefer in der CM. Die Ergebnisse beweisen die Existenz eines radialen Gradienten im Alter der Kutikula zwischen der physiologisch jungen inneren Oberfläche und der älteren, äußeren Oberfläche. Dieser Gradient ist die Folge der Deposition von Cutin an der inneren Oberfläche der Kutikula.

Schlüsselwörter: Kutikula, Cutin, Deposition, Alter der Kutikula, CAPP, Wachs, Mikrorisse

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Abbreviations

%C	carbon content of the respective sample
^{12}C	stable isotope of carbon consisting of six protons and six neutrons
^{13}C	stable isotope of carbon consisting of six protons and seven neutrons
^{14}C	radioactive isotope of carbon consisting of six protons and eight neutrons
ai	active ingredient
at%	atomic percentage value of applied ^{13}C
BF_3	boron trifluoride
C	unlabelled control sample
$CaCl_2$	Calcium chloride
CAPP	cold atmospheric pressure plasma
cd 1	cutin deficient 1
CL	cuticular layer
CM	cuticular membrane
CO_2	carbon dioxide
CoA	Coenzyme A
CP	cuticle proper
d	day
DAFB	days after full bloom
DCM	dewaxed CM
DGAT	diacylglicerol acyltransferase
E	east
ER	endoplasmic reticulum
ES	epidermal skin segment
GPAT	glycerol-3-phosphate acyltransferase
h	hour
HCl	hydrogen chloride
IAEA	International Atomic Energy Agency
IRMS	isotopic ratio mass spectrometer
$K_{cut/w}$	cuticle/water partition coefficient
$K_{oct/w}$	octanol/water partition coefficient

L	labelled sample
LACS	long-chain acyl-CoA synthetase
lat.	latitude
$LiAlH_4$	lithium aluminium hydride
long.	longitude
LSC	liquid scintillation counting
m_{sample}	molar mass of carbon in the sample
M_{sample}	total mass of sample
M_{Tracer}	total amount of new carbon
N	north
n	number of replicates
NaN_3	sodium azide
NaOH	sodium hydroxide
O_2	oxygen
P (statistics)	probability level
r^2/R^2 (statistics)	coefficient of determination
R_{Tracer}	the relative contribution of tracer derived carbon to total carbon pool
SE	standard error
SEM	scanning eletron microscopy
T	tracer
VLCFAs	very-long-chain fatty acids
VPDB	Vienna Pee Dee Belemnite

1. General Introduction

Skin appearance determines the market value of most fruits, particularly of the table-fruit species. Fruit-skin disorders impair fruit appearance and have severe economic consequences for growers. Examples of skin disorders in apple are russeting (Faust and Shear, 1972; Khanal et al., 2013a; Knoche et al., 2011) and skin spot (Grimm et al., 2012; Hampson and Kemp, 2003). Both disorders result from microscopic failures of the cuticle (cuticular membrane; CM). Failure of the cuticle also impairs its barrier functions to the passage of water and to the respiratory gasses. Hence cuticular failure compromises the pre- and postharvest performance of the fruit, both factors further reduce the fruit's market value. Maintenance of cuticular integrity is, therefore, of high commercial importance.

A fruit cuticle undergoes ongoing strain as fruit size and fruit surface area increase throughout development – for an apple fruit development is over about a five-month period (Lai et al., 2016). The skin's cellular structures, including the epidermis and hypodermis, cope with this expansion growth by cell division, cell expansion and a change in cell aspect ratio. In contrast, the cuticle is a non-living polymer that is simply dragged along and strained by the ongoing growth of the underlying cell layers (Knoche et al., 2011; Knoche and Lang, 2017). Microscopic cracks ('microcracks') appear when the limit of cuticular extensibility is exceeded. To cope with this challenge, some fruit species, such as apple, continuously deposit new cuticle material to the inner surface of the cuticle during the entire developmental period (Knoche et al., 2011; Lai et al., 2016). This ongoing cuticle deposition 'fixes' the cuticular strain as the deposited wax is incorporated into the strained cutin matrix to 'fix' the elastic strain (Khanal et al., 2013b). This process generates a radial gradient in the strain of the CM, with the outer CM layers being more strained than the inner CM layers (Khanal, et al., 2014). Microcracks are initially limited to the more strained outer layers of the CM and do not traverse (Maguire, 1998). From a fruit quality point of view, this process is of great interest and potential commercial value. The most likely explanation for the gradient in strain across the CM is that cuticle deposition occurs on the inner surface of the CM resulting in a gradient in 'age' between the inner (younger) surface and the outer (older) surface. Unfortunately, direct experimental evidence for this hypothesis is lacking.

Investigation of cuticle deposition in apple requires an experimental system that allows quantification of cuticle deposition in the developing apple fruit under field conditions. The objectives of this study are therefore: 1) to develop a system that allows quantification of

cuticle deposition in the field and 2) to use this system to establish the presence of a radial gradient in cuticle age between the outer and the inner surfaces of the CM.

The subsequent literature overview is not intended to provide a comprehensive review of all aspects of the physiology of the cuticle, but to provide the necessary background information to identify the current knowledge gaps in our understanding of cuticle physiology and so better define the objectives of this dissertation.

1.1 Major functions of cuticle

The outer primary surfaces of most leaves, fruits and other aerial parts of terrestrial plants are covered by a cuticle. It is a non-living, hydrophobic extracellular polymer deposited on the surfaces of all 'outer' cell walls of epidermal cells. As the critical interface between the internal (aqueous) environment of a plant and the external (dry) environment of the atmosphere, the cuticle is involved in numerous functions during plant growth. Thus the cuticle acts dynamically, together with the stomata and lenticels, to control transpirational water loss (Martin and Rose, 2014; Riederer and Schreiber, 2001, Yeats and Rose, 2013) and to allow the regulated exchange of the respiratory gases (Jeffrey, 1996). The barrier function of the cuticle to water loss is attributed to the cuticular waxes (Schönherr, 1976). Extraction of the waxes with organic solvents significantly increases the cuticle's permeability (Schönherr and Riederer, 1989). Epicuticular wax crystals can also mitigate cell damage from excessive light and heat by enhancing the reflection of solar radiation (Jeffrey, 1996). These wax crystals also allow self-cleaning (the lotus effect) with their very high water repellency (Barthlott and Neinhuis, 1997). The cuticle also serves as a primary interface for substance transport such as of lipophilic compounds (Riederer and Friedmann, 2006; Riederer and Schönherr, 1984). Lastly, the cuticle presents a significant physical barrier against invasion by pathogens (Huang, 2001; Köller, 1991; Serrano et al., 2014). To fulfil these functions, the cuticle must remain intact throughout fruit expansion.

1.2 Composition and structure of cuticle

Plant cuticle comprises an insoluble polymeric cutin matrix, solvent-soluble wax and polysaccharides. Cutin is a natural polyester composed of long-chain hydroxy fatty acids, cross-linked by ester bonds (Heredia, 2003). Cutin contains two major and distinct classes of monomers: one class is derived from a C16 family of acids consisting of 9- or 10,16-dihydroxyhexadecanoic acid and 16-hydroxyhexadecanoic acid. The other class is derived from a C18 family of acids consisting of 9,10,18-trihydroxyoctadecanoic acid and 18-

hydroxy-9,10-epoxyoctadecanoic acid (Fich et al., 2016; Heredia, 2003; Kolattukudy, 2001; Yeats and Rose, 2013). The cutin monomers can vary depending on plant species, plant organ and developmental stage (Martin and Rose, 2014). Thus, for example, the C18 acids are dominant in the cutin of angiosperms but are a minor component in gymnosperms (Holloway, 1982a; Fich et al., 2016). The cutin of an apple fruit (*Malus domestica*) has less of the C16 compounds than an apple leaf (Holloway, 1973). Higher amounts of the C16 family of monomers are to be found in young and fast-growing plant organs, than in mature ones (Kolattukudy and Walton, 1972; Kolattukudy, 1980, 2005).

Cuticular wax is deposited in the cutin matrix as intracuticular wax and deposited on the surface of matrix as epicuticular wax. The major components of cuticular wax are derived from very-long-chain fatty acids (VLCFAs), including alkanes, ketones, aldehydes, primary and secondary alcohols (Kunst and Samuels, 2003; Yeats and Rose, 2013).

Polysaccharides from epidermal cell walls comprise pectin and cellulose that are embedded in the inner surface of the cuticle (Jeffrey, 1996).

Cutan, another polymeric constituent of unknown chemistry, is also reported to occur in the cuticles of some plants. Cutan is a highly aliphatic biopolymer identified in *Agave americana* and *Clivia miniata* (Boom et al., 2005). There is no information on the possible presence of cutan in the apple fruit cuticle.

Finally, secondary metabolites including triterpenoids and flavonoids are also found in cuticles (Samuels et al., 2008).

The CM in most plants is not chemically or structurally homogenous but lamellate (Holloway, 1982b). It is spatially divided into two domains: • the external domain with epicuticular and intracuticular waxes referred to as the cuticle proper (CP) and • the cutin-rich domain with embedded polysaccharides underlying the CP referred to as the cuticular layer (CL). Polysaccharide microfibrils extend into the CL which are therefore interpreted as extensions of the pectin lamella, and these bond the CL to the epidermal cell walls (Jeffrey, 1996; Popp, 2005). The pectin lamella enables the isolation of CM by enzymatic hydrolysis (Buchholz, 2006).

1.3 Synthesis of cuticle

The synthetic pathway of cuticle has been established in model systems (Kunst and Samuels, 2003; Pollard et al., 2008; Yeats and Rose, 2013). Cuticle synthesis begins with *de novo* fatty

acid synthesis in the plastids of epidermal cells. The plastid-derived fatty acids are then modified for cutin monomers and wax components in the endoplasmic reticulum (ER). Three steps are involved in the synthesis of cutin monomers: 1) producing acyl-CoA by esterification of CoA to a fatty acid involving long-chain acyl-CoA synthetase (LACS) proteins, 2) oxidizing the terminal and/or mid-chain carbon involving cytochrome P450 enzymes, 3) generating the mature monoacylglycerol cutin monomers by transferring the fatty acid from CoA to glycerol involving glycerol-3-phosphate acyltransferase (GPAT) enzymes (Fich et al., 2016).

To synthesize wax, C16 and C18 fatty acid are elongated to yield VLCFAs having C20 – C34 chains. The VLCFAs are modified through two pathways into major wax classes: 1) the acyl reduction pathway to produce wax esters and primary alcohols and 2) the decarboxylation pathway to produce alkanes, ketones, aldehydes and secondary alcohols (Samuels et al., 2008).

Cutin monomers and wax components are transported from the ER to the plasma membrane, across the polysaccharide cell wall and thence to the nascent CM (Fich et al., 2016; Samuels et al., 2008; Yeats and Rose, 2013). Studies on the mechanism and site of cutin polymerization upon arrival at the growing cuticle are restricted to: 1) the identification of *cutin deficient 1* (*cd1*) in tomato fruit (Yeats et al., 2012) – an extracellular enzyme localizing in the cuticle and involved in cutin synthesis, 2) the hypothesis that the self-assembled polyhydroxy fatty acids vesicles, termed cutinsomes, may be an additional tool for transporting enzymes by observing the cuticle enzymes GPAT6 and DGAT2 moving together with cutinsomes through cytoplasm to the cuticle in the ovary epidermis of *Ornithogalum umbellatum* (Heredia-Guerrero et al., 2008; Stepinski et al., 2016).

The synthesis and the deposition of cuticle have been investigated in the laboratory several decades ago using radiolabelled (^{14}C or ^{3}H) precursors of cutin monomers, fed to leaf discs (Heredia, 2003; Kolattukudy, 1980; Lendzian and Schönherr, 1983). The synthesis of wax was studied by applying Na-salts of ^{14}C-acetate, ^{14}C-pentanoate or ^{14}C-hexanoate to leaves of *Brassica oleracea* (Kolattukudy, 1965). The leaves were excised from 4- to 6-week-old seedlings and the petioles were immersed in feeding solution containing the precursors. The wax was then extracted by dipping leaves in chloroform. The extracts were separated by thin layer chromatography. In the 1970s, ^{14}C labelled fatty acid precursors, mainly palmitic or oleic acid, were applied to leaves of *Vicia faba* (Kolattukudy, 1970a, 1970b; Kolattukudy and

Walton, 1972) as well as to fruit of apple and grape (Kolattukudy et al., 1973). Discs were excised from leaves or fruits, and incubated in the donor solutions containing ^{14}C labelled precursor. At the termination of incubation, the wax was extracted with methanol/chloroform and the remaining cutin depolymerized with $LiAlH_4$ and diluted HCl. The constituents were separated by thin layer chromatography and the amount of ^{14}C determined in a liquid scintillation counter. Similarly, Lendzian and Schönherrr (1983) applied ^{3}H labelled palmitic acid as discrete droplets to leaves of *Clivia miniata* Reg.. The incorporation of ^{3}H labelled palmitic acid into the cutin fraction was quantified after depolymerisation of cutin with BF_3-methanol.

1.4 Role of cuticle deposition in fruit skin disorders

Skin disorders such as neck shrivel in plum (Stösser and Neubeller, 1985), shrivel in litchi (Underhill and Simons, 1993), maturity bronzing in banana (Williams et al., 1990), russeting in apple and pear (Faust and Shear, 1972; Khanal et al., 2013a) and skin spot in apple (Grimm et al., 2012; Hampson and Kemp, 2003) are all associated with extensive microcracking of the fruit cuticle. Excessive strain resulting from rapid growth without a concomitant increase in cuticle deposition is considered causal.

During growth, the maintenance of cuticular integrity presents a particular challenge for fruit. Plant organs experience continuous surface area extension during organ development, for leaves and shoots this period is usually only about two or three weeks long. However, for most fruits the duration of surface area extension is usually much longer – generally the period from flowering to harvest (Khanal et al., 2014). For apple fruits, surface area extension occurs over about a 20-week period (Knoche et al., 2011). Throughout this long period, as flesh volume steadily increases, so skin area must increase accordingly. The epidermal and hypodermal cells of the skin accommodate this ongoing area strain by cell division (increased cell numbers), cell extension (increased cell size) and change in cell shape (the anticlinal:periclinal aspect ratio increases)(Maguire, 1998). Meanwhile, the non-living polymeric cuticle is subjected to ongoing bidirectional tangential strain (Skene, 1980) as it is dragged along and stretched out by the moving epidermal cell surfaces just beneath (Knoche et al., 2011; Knoche and Lang, 2017). If either the rate or the extent of the stretching of this polymeric layer exceeds some critical mechanical limit, the cuticle fails and a microcrack forms.

A positive correlation has been reported between cuticle thickness and microcrack resistance in tomato (Matas et al., 2004). By continuous cuticle deposition, the cuticle thickness increases, and cuticle failure can be reduced (Lai et al., 2016). The pattern of cuticle deposition during fruit development varies between species. Cuticle deposition in sweet cherries, grape berries and *Ribes* berries (Becker and Knoche, 2012; Khanal et al., 2011; Knoche et al., 2004) follows a discontinuous pattern. It does not keep pace with the surface area expansion but only occurs in the early stages of fruit growth. The lack of cuticular deposition in the later stages results in a decrease in cuticle thickness and an increase in elastic strain. This increases the risk that microcracking will occur. However, in apple fruit, cuticle deposition continues throughout fruit growth (Knoche et al., 2011; Lai et al., 2016). The cutin matrix is markedly strained during the entire development and the strain is progressively 'fixed' by the additions of cutin, together with the incorporation of intracuticular wax (Khanal et al., 2013b). Both processes convert a reversible elastic strain into an irreversible plastic strain.

The deposition of cutin on the inner surface of the CM is a possible explanation for the radial gradient in elastic strain across it – with the outer CM surface being more strained than the inner CM surface (Khanal et al., 2014). This fits with the observation that microcracks are initially limited to the outer surface of a CM. The deposition of cutin on the inner surface could also tend to prevent a microcrack from traversing the entire cuticle thickness. This outcome, in turn, is likely to reduce or prevent russeting. Thus, cuticle deposition on the inner surface may be viewed as a repair process that prevents the catastrophic consequences of an impairment of the barrier properties of the cuticle.

1.5 Gap of knowledge

(1) Cuticle deposition on the inner surface of the CM has been hypothesized to be the most likely explanation for the radial gradient in strain reported previously (Khanal et al., 2014). Direct experimental evidence for deposition on the inner surface is however lacking.

(2) To investigate the site of cuticle deposition, and so identify the basis of the gradient in cuticle strain, an experimental system is needed that allows quantification of cuticle deposition in an attached and growing apple fruit in the field. Most previous studies were conducted under *in vitro* conditions and using radiolabelled precursors. These are, however, prohibited under today's much stricter safety regulations. To our knowledge,

there are no published studies that investigate the incorporation of precursors labelled with the stable carbon isotope ^{13}C.

(3) It is important that such a study should distinguish between a simple partitioning of the precursor due to its lipophilicity into the lipophilic cuticle (Schönherr and Riederer, 1989) and a polymerization of the precursor that would indicate incorporation. This distinction was not made in many earlier studies.

(4) There is no information on factors that may affect cuticle deposition in developing apple fruit. Such information would be useful to identify: • periods of development during which cuticle deposition may not match surface area expansion and • environmental conditions under which cuticle deposition may be depressed, so also causing a mismatch. Either factor has the potential to increase the level of elastic strain and thus the risk and severity of microcracking and thus russeting.

1.6 Objectives

The objectives of this study therefore were:

(1) To establish a system that would allow quantification of cuticle deposition, using a stable isotope, and under field conditions.

(2) To identify factors that affect cuticle deposition, in a developing fruit, under field conditions.

(3) To identify whether a radial gradient exists in cuticle age, that would result from cuticle deposition occurring only on the inner CM surface.

2. Publications and Manuscripts

2.1 Cutin synthesis in developing, field-grown apple fruit examined by external feeding of labelled precursors

Yiru Si[1], Bishnu P. Khanal[1], Leopold Sauheitl [2] and Moritz Knoche[1]

[1]Institute of Horticultural Production Systems, Fruit Science Section, Leibniz University Hannover, Herrenhäuser Straße 2, 30419 Hannover, Germany

[2]Institute of Soil Science, Leibniz University Hannover, Herrenhäuser Straße 2, 30419 Hannover, Germany

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Cutin Synthesis in Developing, Field-Grown Apple Fruit Examined by External Feeding of Labelled Precursors

Yiru Si [1], Bishnu P. Khanal [1], Leopold Sauheitl [2] and Moritz Knoche [1,*]

[1] Institute of Horticultural Production Systems, Fruit Science Section, Leibniz University Hannover, Herrenhäuser Straße 2, 30419 Hannover, Germany; yiru.si@obst.uni-hannover.de (Y.S.); khanal@obst.uni-hannover.de (B.P.K.)
[2] Institute of Soil Science, Leibniz University Hannover, Herrenhäuser Straße 2, 30419 Hannover, Germany; sauheitl@ifbk.uni-hannover.de
* Correspondence: moritz.knoche@obst.uni-hannover.de; Tel.: +49-511-762-9020

Abstract: An intact skin is essential in high-quality apples. Ongoing deposition of cuticular material during fruit development may decrease microcracking. Our objective was to establish a system for quantifying cutin and wax deposition in developing apple fruit. Oleic acid (^{13}C and ^{14}C labelled) and palmitic acid (^{14}C labelled) were fed to developing apples and the amounts incorporated in the cutin and wax fractions were quantified. The incorporation of ^{14}C oleic acid (C18) was significantly higher than that of ^{14}C palmitic acid (C16) and the incorporation in the cutin fraction exceeded that in the wax fraction. The amount of precursor incorporated in the cutin increased asymptotically with time, but the amount in the wax fraction remained about constant. Increasing the concentration of the precursor applied generally increased incorporation. Incorporation in the cutin fraction was high during early development (43 days after full bloom) and decreased towards maturity. Incorporation was higher from a dilute donor solution (infinite dose feeding) than from a donor solution subjected to drying (finite dose feeding) or from perfusion of the precursor by injection. Feeding the skin of a developing apple with oleic acid resulted in significant incorporation in the cutin fraction under both laboratory and field conditions.

Keywords: cutin; wax; oleic acid; palmitic acid; *Malus* × *domestica*; cuticle; epidermis; hypodermis

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1. Introduction

Maintaining an intact fruit skin is essential in the production of high-quality fruit of all species. Skin defects impair the cuticle's barrier properties [1]. They allow uncontrolled passage of substances including water into and out of the fruit, leading to excessive water loss (in the dry) and shrivel or to water uptake (in the wet), cracking and russeting [2–7]. In addition, the cuticle's function as a barrier against invasion by pathogens is also impaired [8,9]. Thus, skin defects compromise both the pre- and postharvest performance of many fruit crop species. A fruit with multiple or severe skin defects is rejected by consumers, so excluded from the marketing chain and so of value only for processing. Hence, skin defects cause serious economic loss to growers.

Microscopic cracks ('microcracks') in the cuticle are the first visible sign of many fruit skin defects including the cracking of grapes or sweet cherries [10,11], skin spot in apple [12,13], russeting in apple [2,14,15] or neck shrivel in plum [16].

Microcracking of the cuticle results from excessive skin strain caused by a mismatch between skin surface area growth and cuticular deposition [17–19]. During fruit growth, the epidermal and hypodermal cell layers accommodate the increases in skin surface area by cell division (more cells), by cell expansion (larger cells) and by changes in the anticlinal aspect ratio (cell deformation)—they change from portrait to landscape [20]. In contrast, the polymeric cuticle is dragged along and strained by the growth of the un-

derlying cell walls [1]. A concurrent deposition of cutin and an incorporation of wax in the strained cuticle during growth 'fixes' the elastic portion of this growth strain [21]. This process leads to a gradient in elastic strain between the outer and inner layers of the cuticle [22] and to a reduced incidence of microcracking. From a fruit-quality perspective, both effects are beneficial, since the likelihood of failure is decreased. Despite their economic importance, not much is known about the factors affecting cuticular deposition in developing fruit, in the field.

Several decades ago, the synthesis and deposition of cuticle was studied in laboratory assays, where radiolabelled fatty acids (^{14}C or ^{3}H) were applied as precursors to growing leaves and fruit [23–25]. Kolattukudy [26] was the first to publish a study on the incorporation of Na-salts of ^{14}C-acetate, ^{14}C-pentanoate or ^{14}C-hexanoate in the wax of leaves of *Brassica oleracea*. These compounds were fed to excised leaves by immersing their petioles in a labelled donor solution. Following an incubation period, the leaf wax was extracted, the extract separated by thin layer chromatography and the amounts of radioactivity associated with the various wax fractions quantified by liquid scintillation spectrometry (LSC) [26]. In the 1970s, ^{14}C labelled longer-chain fatty acid precursors, mostly palmitic and oleic acid, were fed to leaves of *Vicia faba* [27–29] and to the fruits of apple and grape [30]. Discs were excised from leaves or fruits and were incubated in donor solutions containing the ^{14}C labelled precursor. Because the volume of the donor solution was large compared to the volume of the discs, the concentration of the precursor remained essentially constant during an experiment. We therefore refer to this technique as 'infinite dose feeding'. At the end of the incubation period, the discs were extracted and the wax was removed using a chloroform/methanol solution. The remaining cutin was depolymerized, the constituents obtained separated by thin-layer chromatography. The amount of ^{14}C precursor incorporated was determined by LSC. A slightly different approach was taken by Lendzian and Schönherr [25]. In their study ^{3}H-labelled palmitic acid was applied as discrete droplets to leaves of *Clivia miniata* Reg. [25]. This system is similar to a finite dose system, where the precursor concentration in the donor droplet changes as penetration of the precursor into the leaves proceeds [31,32]. The incorporation of ^{3}H-labelled palmitic acid was quantified after depolymerization of the cutin with BF_3-methanol. To our knowledge, no studies have been published on cuticle formation under field conditions. Here, the use of ^{14}C-labelled precursors is prohibited, so other methods such as ^{13}C-labelled precursors must be used instead.

The objective of this study was to develop a system that permits quantification of cuticle, that is, cutin and wax, deposition in intact, growing fruit. We choose developing apple fruit as a model, since (1) apple is an economically very important fruit crop, (2) the apple cuticle is well characterized [21,22,33] and (3) apples continue to deposit cutin and wax throughout their development [17].

2. Results

The supernatant of donor solutions containing ^{14}C oleic acid decreased in concentration with time (Figure 1). The decrease followed an exponential pattern. For a typical run, the duration between the final vortexing of the donor solution and the end of the application averaged about 10 min. During this time, the relative decrease in radioactive concentration in the supernatant of the donor solution was about 3%.

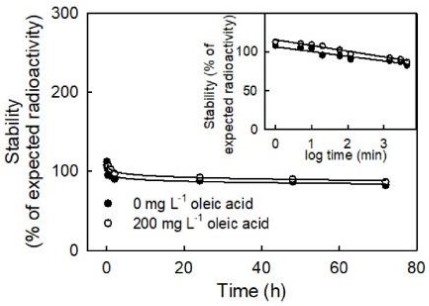

Figure 1. Stability of fatty acid solutions. Time course of change in radioactivity in the lower layers of an oleic acid solution in a scintillation vial without shaking (solution height 12 mm, solution volume 5 mL). Both solutions had 19,269 (±160) dpm total radioactivity and 0 or 200 mg L^{-1} of un-labelled (cold) oleic acid. Time on the *x*-axis is log-transformed and re-plotted in the inset. The values are means ± SEs of five replications.

Incorporation in the cuticular membrane (CM) and the cutin fractions was significantly higher following application of ^{14}C oleic acid (C18) as compared to ^{14}C palmitic acid (C16) (Figure 2). Infinite and finite dose feeding resulted in higher incorporations than perfusion. In contrast to the cutin fraction, there was little difference between the two precursors for the wax fraction. Based on the higher incorporation, oleic acid was selected as the precursor for all subsequent experiments.

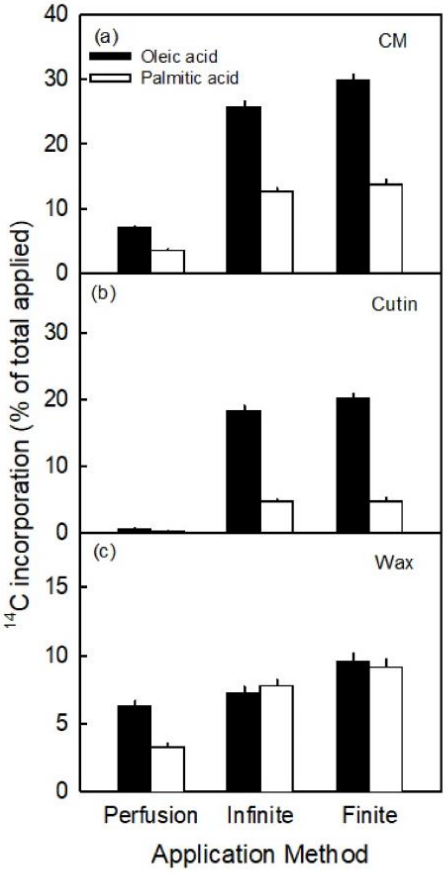

Figure 2. Incorporation of ^{14}C (% of total applied) of oleic acid and palmitic acid in cuticular membrane (CM) (**a**), cutin (**b**) and wax (**c**) as affected by application method. ^{14}C labelled oleic or palmitic acid was applied to the excised exocarp segments (ES) of young fruit of 'Idared' apples sampled at 43 days after full bloom (DAFB). The values are means ± SEs of 15 replications.

The amount of ^{14}C oleic acid incorporated in the CM and the cutin fraction following infinite dose feeding increased asymptotically with time. The radioactivity associated with the wax fraction was nearly constant and essentially independent of time (Figure 3). Incorporation following finite dose feeding was rapid into the CM and the cutin fractions during the drying of the donor solution (within 1 d). Thereafter, incorporation slowed down and proceeded at a low rate. Incorporation in wax decreased slowly after 2 d of finite dose feeding. There was higher incorporation in wax after finite dose feeding than after infinite dose feeding. Following perfusion, incorporation in the CM and the wax fractions decreased with time. The incorporation in the cutin fraction was only marginal. After 7 d, incorporation into the CM and the cutin fraction was highest in infinite dose feeding, followed by finite dose feeding. The lowest incorporation was measured following perfusion.

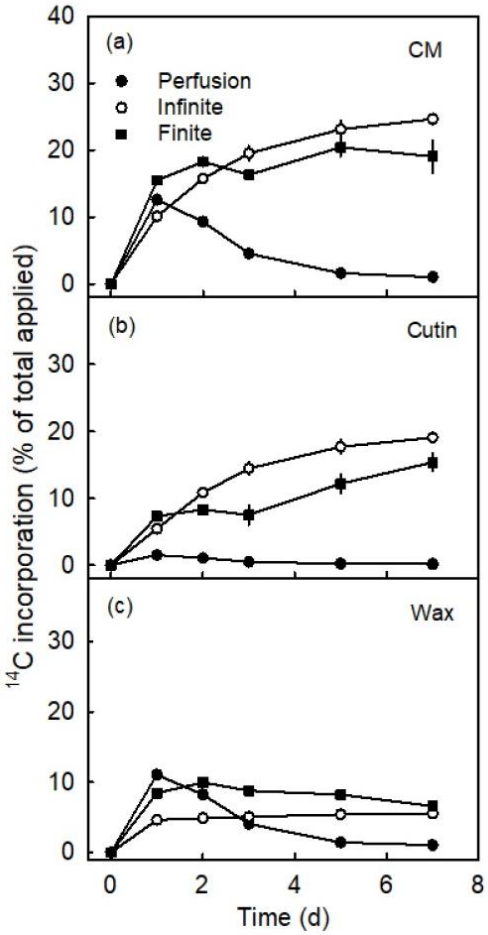

Figure 3. Time course of ^{14}C incorporation (% of total applied) in cuticular membrane (CM) (**a**), cutin (**b**) and wax (**c**). ^{14}C labelled oleic acid was applied to the excised exocarp segments (ES) of young 'Idared' apples, sampled at 62 days after full bloom (DAFB), using three different application methods. The values are means ± SEs of 15 replications.

Increasing the concentration of oleic acid in the feeding solution increased the amount incorporated in the cuticle, the cutin and the wax fractions (Figure 4). Incorporation was consistently higher after infinite dose feeding, followed by finite dose feeding and by perfusion. On the basis of the percent of mass applied, incorporation into the cuticle and the cutin fractions, but not into the wax fraction, was generally higher following infinite dose feeding.

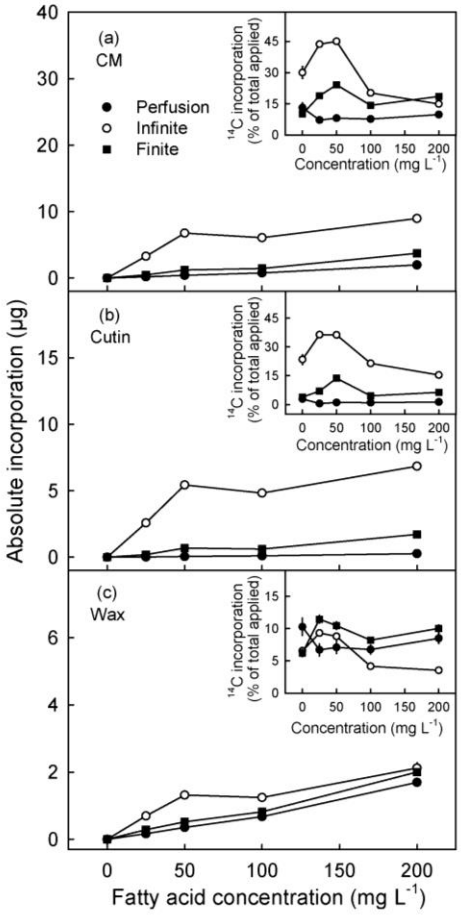

Figure 4. Effect of fatty acid concentration of feeding solution in the incorporation of ^{14}C in absolute amount (ng; main figures) and in relative amount (% of total applied; inset figures) in cuticular membrane (CM) (**a**), cutin (**b**) and wax (**c**). ^{14}C labelled oleic acid was applied to the excised exocarp segments (ES) of young 'Idared' apples, sampled at 83 days after full bloom (DAFB), using three different application methods. The concentrations of fatty acid in the feeding solutions were adjusted using un-labelled (cold) oleic acid. The values are means ± SEs of 15 replications.

The developmental time course revealed high incorporation in the cuticle and the cutin fractions during early development and following infinite and finite dose feeding (Figure 5). Incorporation in both fractions then decreased between 43 and 83 days after full bloom (DAFB). For the CM, incorporation increased again towards maturity. There was no consistent change in the cutin fraction after 83 DAFB. This increase in the CM fraction was primarily due to a consistent increase of incorporation in the wax fraction. There was essentially no incorporation following perfusion feeding in the cutin, but incorporation in the CM and the wax fraction significantly increased towards maturity. Incorporation was lowest following perfusion feeding.

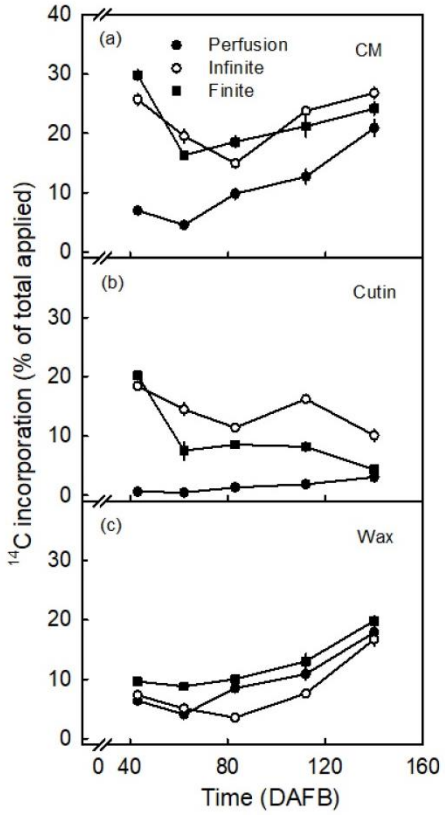

Figure 5. Effect of fruit development stage on ^{14}C incorporation (% of total applied) in cuticular membrane (CM) (**a**), cutin (**b**) and wax (**c**). ^{14}C labelled oleic acid was applied to the excised exocarp segments (ES) of 'Idared' apples, sampled at various stages of development between 43 days after full bloom (DAFB) and maturity (140 DAFB), using three different application methods. The values are means ± SEs of 15 replications.

The results obtained when feeding developing attached fruit in the field using ^{13}C oleic acid and detached fruit from the same trees in the lab using ^{14}C oleic acid were generally similar. Incorporation was highest in the CM and the cutin fractions and following infinite dose and finite dose feeding (Table 1). The lowest incorporation was observed using perfusion feeding.

Table 1. Incorporation of ^{13}C or ^{14}C (% of total applied) in the cuticular membrane (CM), cutin and wax of young (62 or 65 days after full bloom; DAFB) 'Idared' apple fruit. In vivo: Three alternative methods were used to apply ^{13}C labelled oleic acid to the surface of growing fruit in the field. Incorporation was quantified by mass spectrometry. *In vitro*: ^{14}C labelled oleic acid was fed to excised exocarp segments (ES) and incorporation quantified by radioactivity counting. For details see Materials and Methods. The values are means ± SEs of 8–15 replications.

			Incorporation (% of Total Applied)		
Precursor	**Stage (DAFB)**	**Method**	**CM**	**Cutin**	**Wax**
^{13}C	65	Perfusion	4.0 ± 0.6	1.9 ± 0.3	2.1 ± 0.5
		Infinite	17.0 ± 2.0	10.1 ± 1.7	6.9 ± 1.1
		Finite	15.4 ± 2.6	10.6 ± 1.7	4.9 ± 2.0
^{14}C	62	Perfusion	4.5 ± 0.3	0.5 ± 0.0	4.1 ± 0.3
		Infinite	19.5 ± 1.1	14.5 ± 1.0	5.1 ± 0.2
		Finite	16.3 ± 0.7	7.5 ± 0.5	8.8 ± 0.3

3. Discussion

The major findings are: (1) Patterns of incorporation of precursors by the CM reflected patterns of synthesis, deposition and incorporation by the cutin fraction. For the wax fraction we observed what was probably a simple partitioning phenomenon; (2) Incorporation from infinite dose feeding was slightly greater than that from the finite dose feeding. Incorporation from perfusion was consistently the least; (3) Incorporation of oleic acid was greater than that of palmitic acid.

3.1. Incorporation into the Cutin Fraction Reflects Synthesis, but not a Simple Partitioning

It can be argued that the amount of radioactivity associated with the cuticle, in this and earlier studies, resulted from a simple partitioning of the precursors into the cuticle. Indeed, the cuticle is a lipophilic sorption compartment and both precursors are fatty acids [34]. However, several arguments suggest that simple partitioning did not play a significant role.

(I) The incorporation of the precursors in the cutin fraction was highly selective. It consistently favored oleic acid over palmitic acid, despite their very similar octanol/water partition coefficients (Koct/w) (i.e., for oleic acid logarithm of Koct/w is 7.64 and for palmitic acid it is 7.17) [35]. The value of log Koct/w is closely related to the logarithm of the cuticle/water partition coefficient (log Kcut/w) [36]. The two calculated log Kcut/w values are 7.47 for oleic acid and 7.01 for palmitic acid. The measured selectivity of incorporation in the cutin fraction is consistent with the composition of apple fruit cutin where C18 compounds dominate over C16 ones, with ratios ranging from 2.2:1 to 3.4:1 [37–39]. This ratio is close to that obtained in our study (range 3.8 and 4.2 for the infinite and finite dose feeding, respectively; Figure 2).

(II) When the cuticle was extracted using a chloroform/methanol solution, the radiolabel associated with the cuticle remained in the cutin polymer. If the two precursors were only partitioned into the cutin, we would have expected both precursors to be extracted by the chloroform/methanol solution and, hence, to end up in the supernatant as part of the wax fraction. However, this was clearly not the case for the cutin fraction.

(III) Kolattukudy and Walton [29] and Kolattukudy et al. [38] reported that externally applied oleic acid converts into 18-hydroxy-C18 or 10,18-dihydroxy-C18 or 9,10,18-trihydroxy-C18 acid and externally applied palmitic acid into 16-hydroxy-C16 or 10,16-dihydroxy-C16 acid before incorporation into the cutin.

The results obtained for the wax fraction differed from those for the cutin fraction indicating that a clear preference and selectivity for one or other of the two precursors was absent. After feeding the two fatty acids, the ratio of the radioactivity in the wax fraction was close to 1 (range 0.9 and 1.0 for the infinite and finite dose feeding, respectively; Figure 2). This is not surprising given: (1) the similarity in lipophilicity between the two precursors as indexed by their similar log Koct/w and log Kcut/w values, (2) the diversity of pathways for the syntheses of individual constituents that contribute to the so-called wax and (3) the monomeric character of the wax. In contrast to the polymeric cutin, the wax fraction is monomeric. Hence, it is technically impossible to distinguish a radioactive fraction of precursors that resulted from simple partitioning of the fatty acid into a lipoidal wax fraction, from a fraction that resulted from metabolic modifications of the precursors and their incorporation into newly-synthesized constituents of the apple fruit wax. Because of these restrictions, our subsequent discussion is focused on the cutin fraction.

3.2. Incorporation Was Higher Following Infinite Dose Feeding Than Finite Dose Feeding—Both Methods Were Superior to Perfusion

Incorporation following infinite dose feeding was slightly higher than that following finite dose feeding. Two factors may be involved. First, in finite dose feeding, a dry deposit forms after evaporation of the water solvent. While the concentration of the pre-

cursor in the dry deposit is expected to be high, the mobility of the precursor may be limiting after drying due to the absence of a solvent. This would not be the case in infinite dose feeding, where the concentration of the precursor is markedly lower and probably decreases slightly due to penetration and incorporation. The continuing presence of the solvent ensures the precursor remains available for uptake in infinite dose feeding. Second, in finite dose feeding, during the drying of the donor solution the deposit formed does not form a uniform layer over the interface area originally wetted. Instead, as the donor solution dries, it contracts to form a deposit of non-uniform thickness. Hence, the interface area covered by the dried deposit is markedly smaller than the initial contact area between donor solution and fruit surface at the time of application. These two factors are likely to contribute to the slightly lower uptake and incorporation of precursor from the finite, as compared to the infinite dose system.

Incorporation following perfusion was consistently the least. In perfusion, the donor solution containing the precursor is injected into the fruit. The tissue in contact with the precursor is largely sub-epidermal and sub-hypodermal parenchyma, rather than epidermal, whereas the precursors are metabolized within the epidermal layer [7,40,41]. These cell layers contain plastids [42] which are the sites of fatty acid synthesis and, hence, of the precursors of the cutin monomer [41,43–45]. This explanation most likely accounts for the low incorporation following perfusion by injection, as compared to either infinite or finite dose feeding where the precursors penetrate directly into the cell layers responsible for the synthesis of cutin, namely the epidermal and hypodermal layers.

3.3. Incorporation of Oleic Acid Exceeds That of Palmitic Acid

The proportion of the C16 and C18 monomers in cutin varies between plant species, between organ types, and with organ age. The 9- or 10,16-dihydroxyhexadecanoic acid and the 16-hydroxyhexadecanoic acid of the C16 family and the 18-hydroxy-9,10-epoxyoctadecanoic acid and the 9,10,18-trihydroxyoctadecanoic acid of the C18 family are the dominating monomers of the cutins of various plant species [23]. It is reported that the C16 family of monomers predominates in the cuticles of young and rapidly-expanding plant organs. The proportion of the C18 monomers increases during organ development. Thus, the cutin of a mature organ with a thicker cuticle, contains a larger portion of the C18 monomers [24,29,46]. Also, in apple fruit cutin, higher proportions of C18 monomers than C16 monomer have been reported [37,39]. Our findings are consistent with these reports. According to Kolattukudy and Walton [29] and Walton and Kolattukudy [47], the conversion of exogenously applied palmitic acid into 10,16-dihydroxy-C16 was lower in old leaves than in young leaves of *Vicia faba*. This observation further helps explain the lower incorporation of palmitic acid as compared to oleic acid in apple fruit cutin, since our experiments were all carried out on older fruit, sampled at or later than 40 DAFB.

3.4. Conclusions

The results demonstrate that feeding the skin of developing apple fruit with oleic acid results in the incorporation of this precursor into the cutin fraction. Thus, the procedure described herein allows quantification of the deposition of cutin on developing apple fruit using labelled (radioactive or stable isotope) precursors. The precursor of choice for field experimentation is ^{13}C-labelled oleic acid. The amount of ^{13}C-incorporation is analyzed by mass spectrometry. Our results offer proof that infinite or finite dose feeding is superior to perfusion, which results in a low rate of incorporation. The procedure described herein can be used in future studies to monitor cuticle deposition in the developing fruit of apple and, most probably, other fruit crops under field conditions.

4. Materials and Methods

4.1. Plant Materials

Developing 'Idared' apples (*Malus* × *domestica* Borkh.) were sampled in an experimental orchard of the Leibniz University at Ruthe, Germany (lat. 52°14′ N, long. 9°49′ E). Trees were grafted on M9 rootstocks and cultivated according to current regulations for integrated fruit production.

For the in vitro laboratory assays, ^{14}C-labelled precursor fruit were sampled randomly from 150 trees in two adjacent rows. Fruits were immediately transferred to the laboratory where all experiments involving radiolabelled compounds were conducted. For the in vivo assays involving ^{13}C-labelled precursor fruit remained attached to the tree and the whole experiment was conducted in the field.

4.2. In Vitro Laboratory Assay Using ^{14}C

4.2.1. Preparation of Donor Solutions

Donor solutions were prepared usually containing 200 mg L^{-1} of unlabelled (cold) oleic acid (≥99%; Sigma-Aldrich, Deisenhofen, Germany) or palmitic acid (≥99%; Sigma-Aldrich). A surfactant was added at a final concentration of 0.05% (Glucopon 215 UP/MB; BASF SE, Ludwigshafen, Germany). The solution was vortexed for at least 3 min. Thereafter, donor solutions were spiked using ^{14}C labelled oleic acid (specific activity 2.2 GBq $mmol^{-1}$, radiochemical purity > 97%; PerkinElmer, Boston, MA, USA) or palmitic acid (specific activity 2.1 GBq $mmol^{-1}$, radiochemical purity ≥ 98%; Moravek Biochemicals, Brea, CA, USA). Both fatty acids were carboxyl labelled. Subsequently, solutions were again vortexed for 3 min. The stability of the donor solutions was checked by sampling the donor solutions repeatedly over time. Aliquots (10 µL) were taken and 3 mL of scintillation liquid (Ultima Gold XR; PerkinElmer, Boston, MA, USA) was added. Samples were radioassayed by liquid scintillation counting (LS 6500; Beckman Coulter Inc., Brea, CA, USA).

4.2.2. Feeding Epidermal Skin Sample with ^{14}C-Oleic and ^{14}C-Palmitic Acid

Epidermal skin samples (ES) were excised from the equatorial region of the fruit using a cork borer (17 mm inside diameter). The ES were cut to a uniform thickness of about 2–3 mm.

For both the infinite and finite dose feeding, dosing vials were prepared and mounted on the fruit surface (see Figure S1). Briefly, the dosing vials were cut to a 14 mm length from the central portion of a 10 mL polyethylene centrifuge tube (14 mm diameter; Carl Roth, Karlsruhe, Germany). These sections were mounted on the surface of the ES using a non-phytotoxic silicon rubber (Dowsil™ SE 9186 Clear Sealant; Dow Toray, Tokyo, Japan). The ES with tubes attached were placed individually in glass jars that provided a high humidity environment to prevent desiccation during feeding. For the dosing by perfusion, the ES remained without tube and were placed cuticle down in the glass jars.

The ^{14}C labelled donor solution was fed to the ES in three different ways:

(1) In the finite dose method, 100 µL of donor solution containing 3×10^6 dpm mL^{-1} radioactivity was applied to the tube on the ES. After feeding, the glass jar was left open to allow evaporation of the water from the feeding solution. Within 15 h of feeding, the donor solution had formed a macroscopically visible dry deposit on the ES. Thereafter, the glass jar was closed.

(2) In the infinite dose method, 300 µL of donor solution containing 1×10^6 dpm mL^{-1} radioactivity was applied to the tube on the ES. Immediately after feeding the glass jar was closed.

(3) In the perfusion method, 100 µL of donor solution containing 3×10^6 dpm mL^{-1} radioactivity was applied directly to the cut surface of the ES. This application mimicked

a perfusion of donor solution by injection as performed in experiments using intact fruit attached to a tree in the field.

For all application methods, a water vapor saturated atmosphere was maintained inside the glass jar by adding a piece of moist tissue paper and by closing the jar using a lid. The feeding experiments were carried out at 22 °C. Unless otherwise indicated, the donor deposit (finite dose) or solution (infinite dose) remained visible on the surface of the ES for 72 h.

4.2.3. Sample Preparation and Analysis of Radioactivity

At the end of the feeding period, the donor solution remaining on the ES surface in the infinite setup was collected in a scintillation vial using a pipette. This fraction is referred to as the 'donor' (Fraction 'Donor').

Thereafter, the outer surface of the ES of the finite and infinite dose setup was rinsed six times with 1% glucopon surfactant solution (300 µL per rinse). The tube was removed from the ES. Subsequently, the tube was rinsed with surfactant solution. All rinse solutions were combined in a scintillation vial. Any residue that may have remained on the surface following the rinsing procedure and that may have been trapped between crystals of epicuticular wax on the surface of the ES was stripped using cellulose acetate stripping [48]. Viscous solutions of cellulose acetate were prepared in acetone and painted on the outer surface of the ES. After drying for 30 min the cellulose acetate solution had hardened and formed a continuous film. The film was peeled off from the surface and dissolved in 1.5 mL acetone in a scintillation vial. The radioactivity in the cellulose acetate strip plus that in the rinse solutions was combined in a 'rinse' fraction (Fraction 'Rinse').

To separate the cuticular membrane (CM) from adhering tissue of the ES, the discs were incubated in 50 mM citric acid buffer solution (pH 4.0) containing pectinase (90 mL L^{-1}, Panzym Super E flüssig; Novozymes A/S, Krogshoejvej, Bagsvaerd, Denmark), cellulose (5 mL L^{-1}, Cellubrix L; Novozymes A/S, Krogshoejvej, Bagsvaerd, Denmark). Sodium azide (NaN_3) was added at a final concentration of 30 mM to avoid microbial growth. Following isolation, CMs were rinsed with deionized water, dried and dewaxed by incubating in 2.0 mL chloroform/methanol (1:1, v/v) for 16 h at room temperature. Dewaxed CMs (DCMs/cutin) were rinsed with 0.5 mL of chloroform/methanol solution. The chloroform/methanol extract represents the wax fraction (Fraction 'Wax'). The DCM was depolymerized in tissue solubilizer (Biolutes S; Zinsser Analytic, Frankfurt, Germany) in a scintillation vial for 16 h (Fraction 'Cutin').

The isolation solution containing the digested tissue was filtered through cellulose filter paper (Whatman 595; GE Healthcare, Buckinghamshire, UK) and the filtrate collected in a scintillation vial. The residue plus filter paper were dried and subsequently oxidized (OX300; Zinsser Analytic von Gardner Denver, Eschborn, Germany). The radioactivity present in the filtrate plus that in the residue on the filter paper represents the radioactivity in the tissue fraction (Fraction 'Tissue').

The radioactivity in the different fractions (Donor, Rinse, Wax, Tissue, and Cutin) was quantified by liquid scintillation counting. A fraction 'CM' was calculated as the sum of the cutin plus the wax fractions. Corrections were made for quenching and oxidizer efficiency where appropriate. Recovery of radioactivity was calculated by dividing the sum of radioactivity in all fractions by the amount of radioactivity applied. For a representative data set the recovery of radioactivity averaged 89.1 ± 1.3%, 85.6 ± 1.1% and 47.7 ± 0.9% for the finite, infinite and perfusion feeding, respectively. The number of replicates for each method was 15.

4.3. Experiments

The time course of the uptake and incorporation of ^{14}C labelled oleic acid was established using finite, infinite and perfusion feeding. The amount of uptake and incorporation was quantified at 0, 1, 2, 3, 5 and 7 d after application. The experiment was

conducted using fruit sampled at 62 days after full bloom (DAFB). The number of replicates was 15.

The concentration response of uptake and incorporation of ^{14}C labelled oleic acid was quantified. Dosing solutions were prepared at concentrations of 0, 25, 50, 100 and 200 mg L^{-1}. The ES were sampled 3 d after application. The experiment was conducted using fruit sampled at 83 DAFB. The number of replicates was 15.

Uptake and incorporation of ^{14}C-labelled oleic acid and ^{14}C-labelled palmitic acid was compared in fruit sampled at 43 DAFB. Uptake and incorporation of ^{14}C-labelled oleic and palmitic acid were quantified 3 d after application The number of replicates was 15.

The developmental time course was established at 43, 62, 83, 112 and 140 DAFB. Uptake and incorporation of ^{14}C-labelled oleic acid was quantified 3 d after application. The number of replicates was 15.

4.4. In Vivo Assay Using ^{13}C

4.4.1. ^{13}C Fatty Acid Solution Preparation

Uniformly ^{13}C-labelled oleic acid (>95% purity) and uniformly labelled palmitic acid (>98% purity) were obtained from Larodan (Larodan AB, Solna, Sweden). Donor solutions were prepared at concentrations of 300 mg L^{-1} in 0.05% surfactant solution (Glucopon 215 UP/MB; BASF SE, Ludwigshafen, Germany). Immediately after preparation, the solutions were vortexed for a minimum of 3 min and again shortly before the application in the field.

4.4.2. Feeding ^{13}C Fatty Acid

The three feeding methods described above were also used in the field with minor modification. Fruit were selected for normal development and freedom from visual defects. Fruit hanging vertically from their spur were selected for ease of application. Cylindrical tubes were used for finite dose feeding or tubes with a tapered end and a hole in the tip for infinite dose feeding. Tubes were mounted as described above. 300 µL of donor solution containing ^{13}C fatty acids were pipetted into the tubes. In infinite dose feeding, the solution was applied through the hole in the tip of the tapered tube. The hole was then sealed using silicone rubber. This setup prevented evaporation of the donor solution in the infinite dose system. In contrast, in finite dose feeding a cylindrical tube with an open top was used for feeding. Here, the donor solution evaporated within about 3 h and a dried deposit formed on the fruit surface. Unless specified otherwise, all tubes were removed after 7 d of feeding. The original footprint of the tube was marked using a permanent marker. This portion of the fruit surface was then rinsed with deionized water. Fruit were sampled 14 d after termination of the 7 d feeding period. The total time for incorporation thus was 21 d for all three feeding methods.

For feeding by perfusion, 50 µL of ^{13}C fatty acid solution was directly injected into the flesh very close to the dermal tissue of the fruit using a micro-syringe. To control the depth of perfusion, the hypodermic needle was cut to 2.5 mm length. The portion of the fruit surface in contact with the injected solution was easily visible from the outside. This portion of the surface was immediately marked using a permanent marker. Subsequently, the hole in the fruit surface resulting from the perfusion was sealed with silicone rubber. Treated fruits were sampled 21 d after perfusion.

4.4.3. Sample Processing and ^{13}C Analysis

At the end of the feeding period, samples were fractionated in analogy to the procedure used for the ^{14}C labelled samples, with minor modifications. The footprint of the fruit surface exposed to the fatty acid solution was rinsed with 1% glucopon surfactant solution and then blotted. The ES were excised from the treated surface using a biopsy punch (12 mm diameter, Acuderm, Terrace, FL, USA) and incubated in the enzyme so-

lution as described earlier. The enzyme solution was regularly refreshed until the CMs separated from adhering tissue. CMs were cleaned using fine soft paintbrushes and rinsed with deionized water. Thereafter, CMs were dried overnight at 40 °C, weighed on a microbalance (CPA2P; Sartorius, Göttingen, Germany). The mass per unit area was calculated.

The quantity of C and the composition of regular carbon (^{12}C) and stable isotopic carbon (^{13}C) were measured on an elemental analyzer (Isotope Cube; Elementar, Hanau, Germany) coupled with an isotopic ratio mass spectrometer (Isoprime precisION; Isoprime-Elementar, Manchester, UK). Small pieces of dried CM (approx. 0.25 mg) were weighed into aluminum pans (6 × 6 × 12 mm^3; LabNeed GmbH, Nidderau, Germany) and the pans crimped. The samples were burnt in the oxidation reactor of the elemental analyser at 1080 °C under a pulse of oxygen. Combustion to CO_2 was catalyzed by the CeO_2 filling of the oxidation reactor. The C content was quantified using a heat conductivity detector. The detector was calibrated for each measurement using a commercial sediment standard.

Online calibration of C isotopes was done by perfusion of one pulse of reference gas. Isotopic composition of C isotopes was calculated in the delta notation referenced against Vienna Pee Dee Belemnite (VPDB) for C. Further calculations of isotope mass balancing (see equations below) were done based on the at% notation. Referencing was done using international isotope standards from the International Atomic Energy Agency (IAEA, Vienna, Austria).

Sucrose (IAEA-CH-6), cellulose (IAEA-CH-3) and caffeine (IAEA-600) were used as standards for C isotopic composition. An in-house standard made of spruce litter was used as an internal standard for quality control of C composition and the referenced isotopic composition.

The relative contribution of tracer derived carbon (R_{Tracer}) (new carbon) to total carbon pool (old plus new carbon) was calculated using a two-pool dilution model following Gearing et al. [49] and the following equation:

$$R_{Tracer} = \frac{at\%\,L - at\%\,C}{at\%\,T - at\%\,C} \times 100.$$

In this equation at% represents the at% value of tracer ($at\%T$) and labelled ($at\%L$) or unlabelled control ($at\%C$) CM material. Then, total mass of tracer in the whole CM sample (M_{Tracer}) was calculated using the following equation:

$$M_{Tracer} = \frac{R_{Tracer} \times M_{Sample} \times \%C}{m_{sample}}.$$

Here M_{Sample} represents the total mass of sample used for the labeling procedure, $\%C$ the carbon content of the respective sample, and m_{sample} represents the molar C-weight in the sample. All % values used in the above equations were divided by 100 prior to calculation.

Using this setup, the amounts of uptake and incorporation of ^{14}C and ^{13}C oleic acid into the cutin and wax fraction were quantified in developing 'Idared' apple fruit at 62 to 65 DAFB. Donor solutions were prepared at 200 mg L^{-1} (^{14}C oleic acid) or 300 mg L^{-1} (^{13}C oleic acid). The precursors were fed to the ES using the perfusion, the infinite or the finite dosing procedure described above. The ^{13}C oleic acid was fed to developing apple fruit that remained attached to the tree in the field. Due to safety regulations, the ^{14}C oleic acid was fed to detached fruit in the lab. The treated fruit were selected from the same trees as those used for the ^{14}C treatment in the lab.

Supplementary Materials: The following are available online at www.mdpi.com/2223-7747/10/3/497/s1, Figure S1**:** Sketch illustrating three different feeding methods for the ^{14}C labelled fatty acids used in the in vitro laboratory assay. In finite and infinite dose feeding, a polyethylene (PE) tube is mounted on the skin of the epidermal segment (ES). The donor solution containing the fatty acid is pipetted into the PE tube. In finite dose feeding the donor solution dries leaving behind a dried down deposit containing the fatty acid on the skin. In in-

finite dose feeding, the donor solution is prevented from drying. The fatty acid stays in solution throughout the feeding period. In contrast, in perfusion feeding, the donor solution is applied onto the cut surface of the ES.

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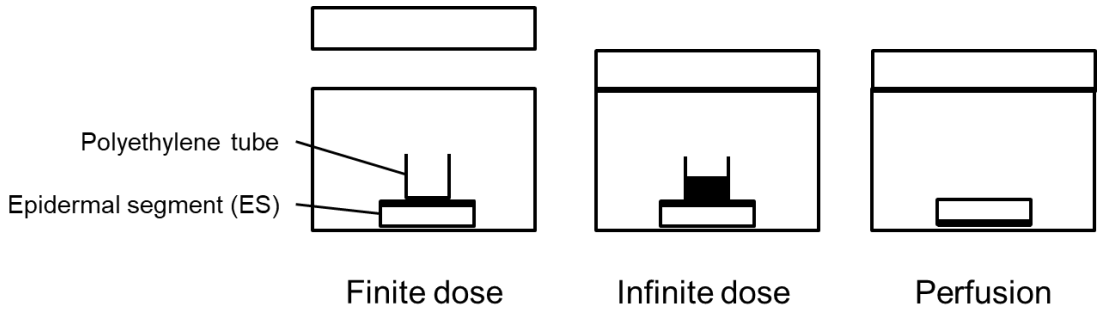

Figure S1. Sketch illustrating three different feeding methods for the ^{14}C labelled fatty acids used in the in vitro laboratory assay. In finite and infinite dose feeding, a polyethylene (PE) tube is mounted on the skin of the epidermal segment (ES). The donor solution containing the fatty acid is pipetted into the PE tube. In finite dose feeding the donor solution dries leaving behind a dried down deposit containing the fatty acid on the skin. In infinite dose feeding, the donor solution is prevented from drying. The fatty acid stays in solution throughout the feeding period. In contrast, in perfusion feeding, the donor solution is applied onto the cut surface of the ES.

2.2 Factors affecting cuticle synthesis in apple fruit identified under field conditions

Yiru Si, Bishnu P. Khanal and Moritz Knoche

Institute of Horticultural Production Systems, Fruit Science Section, Leibniz University Hannover, Herrenhäuser Straße 2, 30419 Hannover, Germany

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Factors affecting cuticle synthesis in apple fruit identified under field conditions

Yiru Si, Bishnu P. Khanal, Moritz Knoche *

Institute of Horticultural Production Systems, Leibniz University Hannover, Herrenhäuser Straße 2, 30419 Hannover, Germany

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ABSTRACT

Microscopic cracks are causal in a number of commercially-important fruit skin disorders of apple, including russeting. Microcracks result from ongoing skin strain during fruit growth. Continuous cuticle deposition in developing apple fruit reduces strain and thus, microcracking. The objective was to identify factors that affect cuticle deposition under field conditions in developing apples. We fed ^{13}C-labelled oleic acid as a precursor for cutin monomers to the surface of developing apple fruit.

Cutin accounted for most of the incorporation of the oleic acid. Incorporation was limited to the surface in direct contact with the feeding solution, there was very little lateral diffusion. Increasing the concentration of the feeding solution generally increased the incorporation of ^{13}C-labelled oleic acid. Incorporation was initially rapid, but then slowed down and approached an asymptote within three days. The duration of the incorporation period after feeding had no effect on the amount of precursor incorporated. All incorporation was essentially instantaneous. During fruit development, incorporation rate was maximal between 64 and 78 days after full bloom (DAFB), then decreased towards maturity. There was no effect of natural (foliar) shading on cutin deposition. Across five apple cultivars, incorporation of the precursor was lowest in 'Gala' and highest in 'Pinova'. Those cultivars whose cuticles had higher mass per unit area also had higher incorporations of the ^{13}C-labelled oleic acid in the cutin, but not in the wax.

The potential commercial benefits of applying cutin monomer precursors in the field are discussed.

1. Introduction

In most fresh-fruit species, the appearance of the skin is key in determining market value. Skin disorders lower the visual quality and so have serious economic consequences for the grower. Commercially relevant examples for apple include russeting (Faust and Shear, 1972; Khanal et al., 2013a) and skin spot (Grimm et al., 2012). Both disorders result from failure of the cuticle during fruit development. In other words, production of high quality fruit requires cuticular integrity be maintained throughout fruit development.

The cuticle is the thin, outermost layer of the fruit skin. It is a hydrophobic membrane formed on the outer wall of the epidermal cells and comprises a polymeric network of cutin, cell wall polysaccharides and non-polymeric waxes. The latter are deposited both within the cutin network and also on its surface. For reviews on the synthesis and composition of cutin and wax the reader is referred to Kolattukudy (2001), Heredia (2003), Kunst and Samuels (2003), Samuels et al. (2008) and Yeats and Rose (2013). The cuticle functions as a barrier to the passage of all substances – solid, liquid and gas phase – across all leaf and fruit surfaces (Kerstiens, 1996; Riederer and Schreiber, 2001; Schreiber and Schönherr, 2009; Yeats and Rose, 2013). It also serves as a barrier against invasion by pathogens (Huang, 2001; Köller, 1991; Serrano et al., 2014; Yeats and Rose, 2013).

During fruit growth the cuticle is subjected to an extended period of strain (Knoche and Lang, 2017). While the cellular components of the fruit skin accommodate increases in surface area by cell division, cell elongation and a change in aspect ratio from portrait to landscape (Maguire, 1998), the ongoing increases in surface area present a special challenge to the polymeric cuticle. As a non-living polymer, the cuticle is essentially dragged along by the extending cell wall surface just beneath. To cope with this continuing strain, some species, including apple, continuously deposit cuticle during fruit growth (Lai et al., 2016), thereby offsetting the thinning of the cuticle as it is stretched over an ever-increasing area of epidermis.

Cuticular deposition on the fruit surface also 'fixes' the cuticular strain (Khanal et al., 2014), while deposition of wax inside the polymeric

* Corresponding author.
E-mail address: moritz.knoche@obst.uni-hannover.de (M. Knoche).

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network 'fixes' the elastic strain (Khanal et al., 2013b). So, these two processes work together to help limit the extension of microcracks from the older, outer layer of the cuticular membrane (CM) into the increasingly younger, inner layers. The consequence is reduced microcracking (Maguire et al., 1999). Thus, from a fruit production point of view, these two developmental processes are of great interest. Hence, increasing our understanding of them and, ultimately, being able to manipulate them via managing the deposition of cuticular material is potentially of high importance.

The processes of cuticular synthesis have been investigated since the 1960s. Briefly, radiolabelled ^{14}C or ^{3}H labelled fatty acids were fed to leaves and fruit and the amounts of radioactivity incorporated were determined after known incubation periods (Heredia, 2003; Kolattukudy, 1970a, 1970b, 1980; Kolattukudy et al., 1973; Lendzian and Schönherr, 1983). Often, wax was extracted, the cutin depolymerised and the incorporation determined by liquid scintillation counting. However, with today's safety regulations, studies employing radioactive isotopes are prohibited in the field. Meanwhile, if conducted in a growth chamber using potted trees, the studies become very expensive because of the high cost of disposing of all the radioactive waste.

In response to this impasse, we have developed a novel system that allows quantification of cutin deposition in the field. It employs the feeding of fatty acids labelled with ^{13}C (Si et al., 2021). There are no restrictions around the field use of ^{13}C which is a stable C isotope making up about 1% of all natural C. Moreover, in our system, the fruit remains attached to the tree in the canopy under natural ambient conditions, so artifacts due to detachment, transport and *in vitro* incubation in a laboratory are eliminated. This now allows the quantification of cutin deposition under realistic conditions. Because it is technically not possible to distinguish between a precursor that has simply partitioned into the wax phase vs. one that is metabolised and deposited as a wax constituent, the procedure is limited to the quantification of cutin deposition.

The objective of the present study was to identify factors that affect cuticle deposition in developing apple fruit under field conditions. We fed ^{13}C labelled precursor to the fruit surface and investigated the time course of incorporation into the cuticle, the effect of precursor concentration in the donor solution, the developmental stage at the time of feeding and the effects of using a range of different apple cultivars.

2. Materials and methods

2.1. Plant material

'Idared' apple (*Malus* × *domestica* Borkh.) grafted on M9 rootstocks were grown in an experimental orchard of the horticultural research station of the Leibniz University Hannover at Ruthe, Germany (lat. 52°14′ N, long. 9°49′ E). Trees were cultivated according to current regulations for integrated fruit production (Anonymous, 2006). Incorporation was also compared across a small range of five different apple cultivars. For this we used 'Idared', 'Pinova', 'Gala', 'Jonagold' and 'Golden Delicious', all grafted on M9 rootstocks. We selected and tagged developing fruit that were free of visible defects and fed them with labelled precursor.

2.2. Methods

2.2.1. Feeding ^{13}C labelled precursors

Donor solutions were prepared using uniformly ^{13}C-labelled oleic acid (> 95% purity, Larodan AB, Solna, Sweden) or ^{13}C-labelled palmitic acid (> 98% purity, Larodan AB, Solna, Sweden). These fatty acids were dissolved in 0.05% aqueous surfactant solutions (Glucopon 215 UP/Mb; BASF SE, Ludwigshafen am Rhein, Germany) at final concentrations of 167 μM (equivalent to 50 mg L^{-1}). Solutions were vortexed for at least 3 min immediately after preparation and again a few minutes before feeding in the field (Si et al., 2021). The donor solutions were applied to the surfaces of developing fruits using an 'infinite-dose' feeding system. Earlier studies established that this system yielded consistently high incorporation of labelled precursor into the cutin fraction of developing apple (Si et al., 2021). In infinite-dose feeding, a dilute donor solution is fed to the skin. The donor solution remains liquid during the whole feeding period. Potentially, this allows continuing penetration and, hence, incorporation. For dosing, a vial cut from a Falcon tube (26 mm high, 14 mm inner diameter; Carl Roth, Karlsruhe, Germany) was mounted on the fruit surface using a non-phytotoxic silicone rubber (Dowsil™ SE 9186 Clear Sealant; Dow Toray, Tokyo, Japan) (Supplementary Fig. 1A). After curing, 400 μL of donor solution was injected into the vial through a hole in the tip. The hole was subsequently sealed with silicone rubber. Unless otherwise specified, the feeding solution remained on the fruit surface for 7 d. Thereafter, the remaining donor solution was removed from the tube and the tube detached from the surface. The fruit skin was carefully blotted dry using a soft tissue paper, the treated area was marked with a permanent marker and rinsed with deionised water. Following feeding, the fruit were left on the tree for an additional 14 d period to allow further incorporation before sampling.

2.2.2. Cuticle isolation

Epidermal segments (ES) were excised from the treated areas using a biopsy punch (12 mm diameter; Acuderm Inc., Fl, USA). Cuticular membranes were isolated by incubating the ES in 50 mM citric acid buffer containing pectinase (90 mL L^{-1}; Panzym Super E flüssig, Novozymes A/S, Krogshoejvej, Bagsvaerd, Denmark) and cellulase (5 mL L^{-1}; Cellubrix L; Novozymes A/S) at ambient temperature. The pH of the solution was adjusted to pH 4.0 using NaOH. To prevent the growth of microbes, sodium azide (NaN_3) was added at a final concentration of 30 mM. The enzyme solution was periodically refreshed until the CM separated from adhering tissue. Isolated CMs were carefully cleaned with a soft camel-hair brush and rinsed with deionised water. Clean CMs were dried to constant weight at 40 °C and weighed on a microbalance (CPA2P; Sartorius, Göttingen, Germany).

2.2.3. ^{13}C quantification by mass spectrometry

To quantify the incorporation of ^{13}C, a small piece of CM (approx. 1/8) was cut from the 12 mm CM disc, weighed in an aluminium pan (6 × 6 × 12 mm^3; LabNeed GmbH, Nidderau, Germany) and the pan crimped. For dewaxed CM (DCM), approx. 1/4 of the 12 mm disc was incubated in 2.0 mL chloroform/methanol (1:1, v/v) for 24 h at room temperature. The DCM was again rinsed with 0.5 mL chloroform/methanol solution and dried overnight at 40 °C. Thereafter, dried DCMs were weighed in aluminium pans and the pans crimped.

The amount of ^{13}C incorporated into the CM/DCM samples was quantified using an element analyser (Isotope Cube; Elementar, Hanau, Germany) coupled with an isotopic ratio mass spectrometer (IRMS; Isoprime precisION; Isoprime-Elementar, Manchester, UK). Briefly, samples were burnt at 1080 °C in the oxidation reactor of the element analyser under a pulse of O_2. Combustion to CO_2 was catalysed by CeO_2. The resultant CO_2 was introduced to the IRMS and the C content quantified using a heat conductivity detector. The detector was calibrated for each measurement using a certified commercial sediment standard (IVA33802150; Distributor IVA-Analysentechnik, Meerbusch, Germany) (Si et al., 2021).

The C isotope ratio was calibrated by perfusion of a pulse of reference gas. The isotopic composition was calculated in the delta notation, referenced against Vienna Pee Dee Belemnite (VPDB) for C. Referencing was done by using international isotope standards from the International Atomic Energy Agency (IAEA, Vienna - Austria). Sucrose (IAEA-CH-6), cellulose (IAEA-CH-3) and caffeine (IAEA-600) were used as standards for C isotopic composition. An in-house standard made from spruce litter was used to control the quality of the C composition and the referenced isotopic composition.

The relative contributions of tracer derived carbon (R_{Tracer}) (new carbon resulting from feeding) to total carbon pool (old plus new

carbon) was calculated using a two-pool dilution model (Gearing et al., 1991) and the following equation:

$$R_{Tracer} = \frac{at\%\ L\ -at\%\ C}{at\%\ T\ -at\%\ C} \times 100$$

In this equation, at% represents the atomic percentage value of the applied ^{13}C in tracer (T) and ^{13}C detected in the labelled sample (L) or ^{13}C from the natural C-pool, i.e., in the unlabelled control sample (C). The total amount of new carbon (M_{Tracer}) was calculated using the equation:

$$M_{Tracer} = \frac{R_{Tracer} \times M_{Sample} \times \%C}{m_{sample}}$$

In this equation M_{sample} represents the total mass of sample used for the labelling procedure, %C the C-content of the respective sample and m_{sample} the molar mass of C in the sample. All% values used in the above equations were divided by 100 prior to calculation.

2.3. Factors affecting incorporation of ^{13}C labelled precursors

Incorporation of ^{13}C oleic acid or ^{13}C palmitic acid was compared in 'Idared' apple at 83 days after full bloom (DAFB) following a 7-d feeding and a 14-d incorporation period.

The spatial distribution of ^{13}C on the surface of a fruit relative to the footprint of the feeding vial was quantified. Fruits were fed with ^{13}C labelled oleic acid for 7 days beginning at 80 DAFB. Following a 14-d incorporation period, fruit were harvested, the ES excised using a biopsy punch (12 mm diam.) and the CM isolated enzymatically. The ES were taken from three different positions: (1) the treated area of the footprint of the dosing vial ('central'; 12 mm inner diam.), (2) a concentric ring ('ring', 17 mm outer diam. (using a 17 mm cork borer), 12 mm inner diam.) around and immediately adjacent to the footprint of the dosing vial, (3) an adjacent area (12 mm diam.) on the same face of the fruit, next to the position of the feeding vial and (4) and an area of fruit surface directly opposite the feeding area (12 mm diam.). The area of the central position and that of the ring were 113 and 114 mm^2. For a schematic drawing see Supplementary Fig. 1B.

The effect of the precursor concentration in the feeding solution on the ^{13}C content of the cuticle was studied for concentrations of 0, 83, 167, 333 and 667 μM (equiv. 0, 25, 50, 100 and 200 mg L^{-1}) of oleic acid with 'Idared' apple. Fruits were fed with ^{13}C labelled oleic acid for 7 days beginning at 61 DAFB. Following a 14-d incorporation period, fruit were harvested, ES excised and the CM isolated.

The effects of the duration of the feeding period and of the duration of the incorporation period of the precursor were studied. The duration of the feeding period was varied from 1 to 2, 3, 5 or 7 d; thereafter, followed by a 14-d incorporation period. The experiment was carried out in 'Idared' apple at 62 DAFB. The effect of duration of the incorporation period was studied in 'Idared' at 69 DAFB following a 7-d feeding period. Fruits were sampled 0, 1, 3, 5, 7 or 14 d following termination of the feeding period and processed as described above.

The developmental time course of change in fruit mass, mass per unit area of the CM, cutin and wax were studied in 'Idared' apple. The change in incorporation of ^{13}C oleic acid was quantified following a 7-d feeding period and a 14-d incorporation period beginning at 50, 64, 78, 92, 113 or 134 DAFB. For earlier developmental stages (< 50 DAFB) the fruit were too small to allow mounting of the dosing vial.

The effect of light on incorporation of ^{13}C oleic acid was studied in 'Idared' at 83 DAFB. For the sun and the shade treatments, fruit were selected that were either well exposed to direct sunlight or that were well shaded by surrounding leaves. These fruit were fed for 7 d, followed by a 14-d incorporation period.

Potential differences in incorporation of ^{13}C oleic acid in the CM, the cutin and the wax factions of 'Idared', 'Pinova', 'Gala', 'Jonagold' and 'Golden Delicious' were established at 80 DAFB. The donor solutions containing ^{13}C oleic acid were fed for 7 d, followed by a 14-d period for incorporation. The number of replications was 10.

2.4. Data analysis and presentation

Data are presented as means and standard errors. The only exception is Fig. 7 where individual observations are shown. Incorporation data were subjected to analysis of variance and to regression analyses using SAS (SAS Institute, Cary, NC, USA). Means were compared using Tukey's Studentised range test ($P < 0.05$). Significance of coefficients of determination at the 1 and 5% level is indicated by ** and *.

3. Results

The incorporations of ^{13}C labelled oleic acid into CM and cutin were significantly higher than those of ^{13}C labelled palmitic acid in 'Idared' apple fruit at 83 DAFB (Fig. 1). Incorporation into the wax fraction was low and was independent of the chain length of these two fatty acids.

Incorporation was highest in the region immediately underlying the dosing vial, followed by the region of the concentric ring surrounding the footprint of the vial (Fig. 2). There was no incorporation in cuticles from regions adjacent to the feeding site, nor in those from the opposite side of the fruit.

Incorporation of ^{13}C oleic acid increased nearly linearly with increasing concentration in the donor solution (Fig 3). Again, cutin accounted for most of the incorporation into the CM, the amount incorporated in the wax was low.

Incorporation into the CM and cutin fraction was rapid and reached an asymptote within 3 d of feeding (Fig. 4A). The incorporation in the wax fraction was low. The duration of the incorporation period had no significant effect on incorporation (Fig. 4B).

Fruit mass increased sigmoidally with time until maturity (Fig. 5A). The mass per unit area of CM, cutin and wax increased and approached an asymptote (Fig. 5B). The rate of cuticle deposition was highest during early development and then decreased steadily (Fig. 5C). Incorporation of oleic acid depended on the stage of development; it increased towards a maximum between 64 and 78 DAFB and then decreased steadily towards maturity. Incorporation in the wax fraction was low throughout development.

There were no significant effects of natural (foliar) shading on the incorporation of ^{13}C labelled oleic acid into the CM, cutin or wax (Fig. 6).

Amongst the five different cultivars, the lowest incorporation of oleic acid into the CM and cutin fractions was observed in 'Gala' and the highest in 'Pinova' (Table 1). Incorporation in the wax fraction was low and did not differ significantly amongst cultivars. Regression analyses

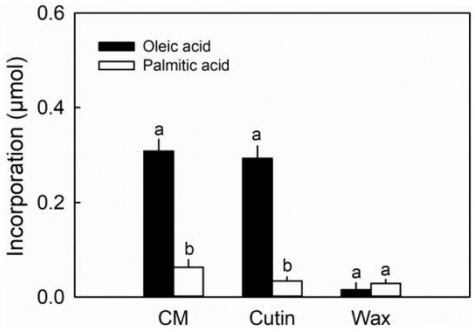

Fig. 1. Incorporation of ^{13}C labelled fatty acids in the cuticular membrane (CM), the cutin and the wax fractions of 'Idared' apple. Data represent means ± SE, $n = 9$ to 10. Means followed by the same letter are not significantly different, Tukey's HSD test at $P \leq 0.001$.

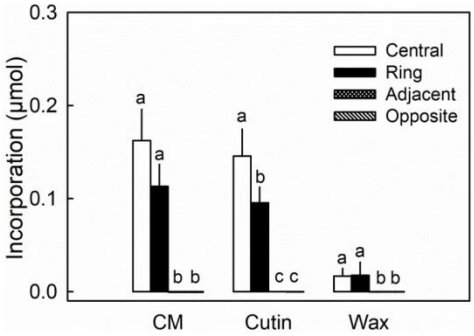

Fig. 2. Incorporation of ^{13}C labelled oleic acid into the cuticular membrane (CM), the cutin and wax fractions of 'Idared' apple. Fruits were fed with ^{13}C labelled oleic acid, the CM isolated, and the amount of ^{13}C quantified in the following positions: The central area (12 mm diam. disc) beneath the vial (14 mm inner diam.) containing the feeding solution ('central'). A concentric ring immediately adjacent to the 'central' region ('ring'; 17 mm outer diam., 12 mm inner diam.). This ring was located at the boundary between fruit skin with, and without, direct contact with the feeding solution. A region adjacent to the 'ring' that had no contact with the feeding solution ('adjacent'). A region on the opposite side of the fruit ('opposite'). Data represent means ± SE, $n = 10$. Means followed by the same letter are not significantly different, Tukey's HSD test at $P \leq 0.001$.

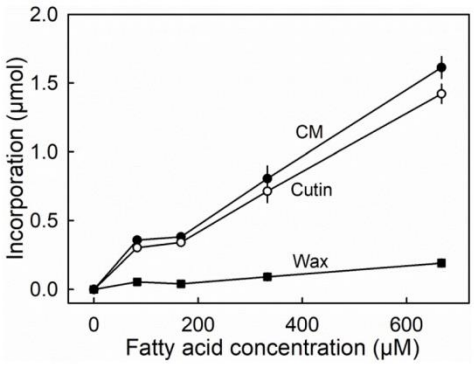

Fig. 3. Effect of the concentration of oleic acid on the incorporation into the cuticular membrane (CM), the cutin, and the wax factions of developing 'Idared' apple. The donor solution containing ^{13}C oleic acid was fed to the fruit surface at 61 days after full bloom (DAFB). Data represent means ± SE, $n = 10$.

between the incorporation of ^{13}C oleic acid into the CM and the cutin fraction and the mass of that fraction at that particular stage of development were significant. Those CMs that had a high cutin mass also had high incorporations of oleic acid, and vice versa (Fig. 7A and B). Only for the wax fraction was the amount of incorporation independent of the wax mass (Fig. 7C).

4. Discussion

The cutin of apple fruit was classified as a mix type comprising C16 and C18 monomers (Holloway, 1982). Oleic acid is a precursor of hydroxy C18 acids such as 18-hydroxyoctadecenoic acid, 10,18-dihydroxyoctadecanoic acid, and 9,10,18-trihydroxyoctadecanoic acid (Kolattukudy et al., 1971, 1973). These are major monomers of the apple fruit cutin (Holloway, 1973; Straube et al., 2021; Walton and Kolattukudy, 1972). Feeding the ^{13}C labelled oleic acid precursor to developing apples resulted in significant incorporation. This demonstrates that the method developed earlier in the laboratory using ^{14}C-labelled

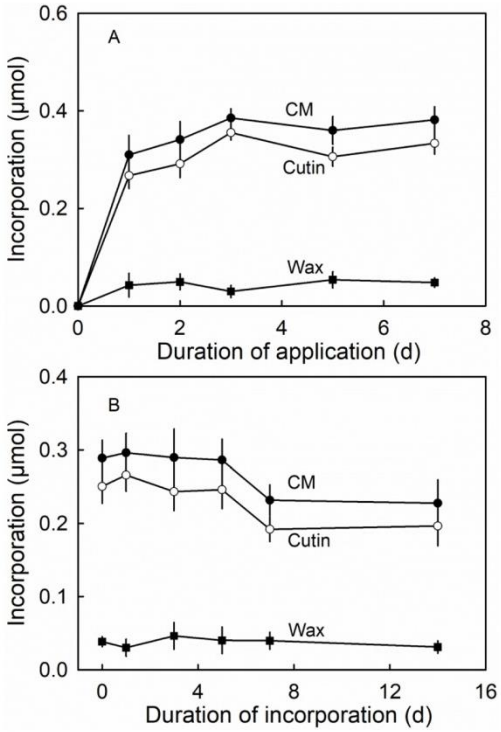

Fig. 4. Effect of the duration of the feeding period (A) and duration of the incorporation period following feeding (B) on the amount of ^{13}C labelled oleic acid incorporated into the cuticular membrane (CM), the cutin, and the wax fraction of developing 'Idared' apple. Data represent means ± SE, $n = 9$ - 10.

fatty acids is also applicable under field conditions using the same chemical compounds but labelled with ^{13}C (Si et al., 2021). As in this earlier study, incorporation of the ^{13}C-labelled precursors was higher for oleic than for palmitic acid. Also, more label was found in the cutin fraction as compared to the wax fraction. Both findings are consistent with earlier observations. As argued earlier, the label in the cutin fraction results from an incorporation in the polymer, whereas the label associated with the wax is likely to result from simple partitioning of the fatty acid into the wax fraction. The incorporation of oleic acid was strictly limited to the site on the fruit surface in immediate contact with the feeding solution. There was little lateral diffusion into the ring zone, but essentially nothing beyond it. The slight decrease in incorporation in the ring zone probably resulted from only partial exposure to the donor solution, while a significant portion was underlying the rim of the vial and the silicone glue. There was no transport beyond this ring, indicating a 'contact type' mode of action.

Amongst the factors investigated, the concentration of the precursor in the donor solution, the duration of its application, the stage of fruit development at application and the cultivar, all affected incorporation.

Increasing concentrations of oleic acid resulted in increasing incorporations. The incorporation of oleic acid requires that the precursor penetrates the cuticle and is taken up into the epidermal cells. In this process, penetration of the cuticle is the rate-limiting step (Buchholz, 2006; Schönherr and Baur, 1994). Cuticular penetration is a physical process where the concentration gradient of precursor across the cuticle represents the driving force (Schönherr and Riederer, 1989; Schreiber and Schönherr, 2009). Thus, higher concentrations will result in higher penetrations. That incorporation into the cutin fraction also increased, indicates that the synthetic pathways for cutin monomers, the export of

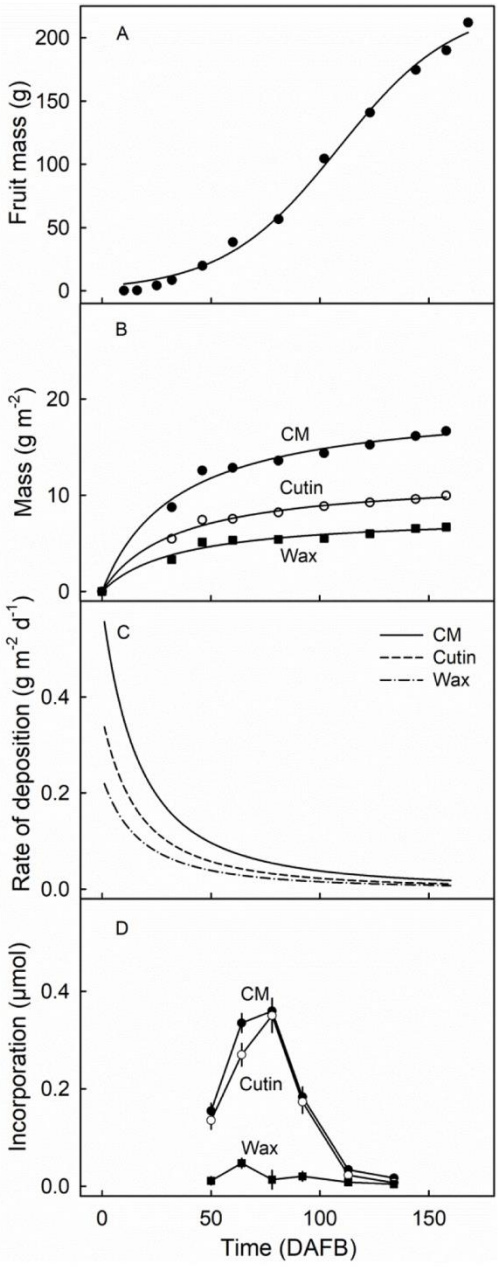

Fig. 5. Developmental time course of increase in fruit mass (A), the increase in mass per unit area of the cuticular membrane (CM), the cutin and wax fractions (B), and of the calculated rates of deposition of CM, cutin, and wax (C) in developing 'Idared' apple. Effect of fruit development on the incorporation of ^{13}C labelled oleic acid in CM, cutin and wax of 'Idared' apple (D). A donor solution was fed to the skin of developing 'Idared' apples and the ^{13}C incorporation in the CM, the cutin and the wax fraction determined. Data represent means ± SE, n = 10 - 15.

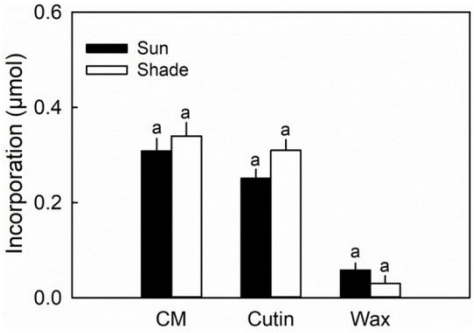

Fig. 6. Effect of shading of developing 'Idared' apple fruit on the incorporation of ^{13}C labelled oleic acid into the cuticular membrane (CM), cutin, and wax. The donor solution containing the precursor was fed to the fruit skin and the ^{13}C incorporation in the CM, the cutin and the wax fraction determined. The fruit surface was exposed to direct sun light or shaded by leaves. Values are means ± SE, n = 10 – 15. Means followed by the same letter are not significantly different, Tukey's HSD test at $P \leq 0.001$.

Table 1
Incorporation of ^{13}C labelled oleic acid (167 µM equiv. to 50 mg L^{-1}) in the cuticular membrane (CM), the cutin and the wax fraction of selected apple cultivars at 80 days after full bloom. The values are means ± SE, n = 10.

Cultivars	Incorporation (µmol)		
	CM	Cutin	Wax
Idared	0.29 ± 0.04ab[z]	0.24 ± 0.03bc	0.05 ± 0.01
Pinova	0.39 ± 0.02a	0.37 ± 0.02a	0.04 ± 0.01
Gala	0.22 ± 0.03b	0.18 ± 0.02c	0.04 ± 0.01
Jonagold	0.36 ± 0.04a	0.31 ± 0.04ab	0.06 ± 0.01
Golden Delicious	0.40 ± 0.03a	0.34 ± 0.03ab	0.07 ± 0.02
Mean	0.33 ± 0.02	0.29 ± 0.02	0.05 ± 0.01

[z] Means separation within columns by Tukey's HSD test at $P \leq 0.05$.

the monomers into the apoplast and their assembly in the extracellular cutin polymer are all unsaturated processes. Incorporation proceeds at a rate that is a simple function of concentration.

The time course of duration of application revealed a decrease in rates of incorporation with time until at about 3 d when no further penetration occurred. This decrease was unexpected. First, incorporation of monomers in the cuticle is a multi-step process comprising (a) uptake through the cuticle/cell wall/plasma membrane composite, (b) metabolic conversion of oleic acid into cutin monomers (most abundant C18 monomers are 18-hydroxyoctadecenoic acid, 10,18-dihydroxyoctadecanoic acid 18-hydroxy-9,10-epoxyoctadecanoic acid and the 9,10,18-trihydroxyoctadecanoic acid; Heredia, 2003; Holloway, 1973; Straube et al., 2021; Walton and Kolattukudy, 1972), (c) export through the plasma membrane/cell wall/cuticle composite and (d) polymerisation into the cutin. Based on the linearity of the concentration response, apparently none of these post-penetration processes was saturated. Second, we used an infinite-dose system where the donor solution is prevented from drying. Hence, a limitation of penetration due to the drying of the donor solution (as is often the case in finite-dose systems) can be excluded as a factor (Knoche and Petracek, 2014). The most likely explanation for the decreasing rate of penetration with time, is a steady decrease in the concentration of the donor solution so that the driving force for penetration gradually decreases. This finding was unexpected but is not judged unlikely in retrospect. In our earlier experiments where ^{14}C labelled oleic acid was fed to the ES, the amounts of oleic acid taken up from the donor solution ranged from 17.3 to 55.3% of the amounts initially present in the donor solution. These represent significant decreases in concentration and, hence, decreases in driving force. Oleic acid has a high octanol/water partition coefficient ($K_{oct/w}$) (Sangster,

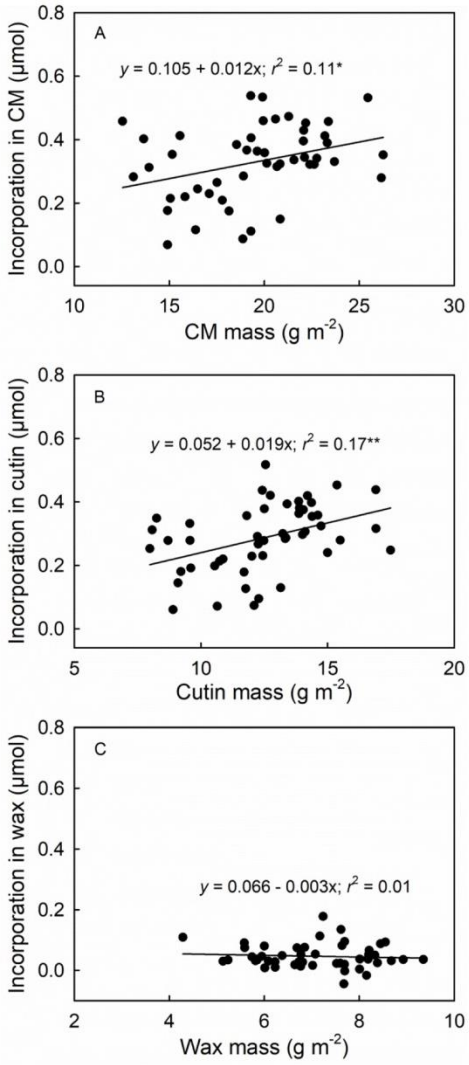

Fig. 7. Relationship between the amount of ^{13}C labelled oleic acid incorporated in the cuticular membrane (CM) (A), the cutin (B) and the wax fractions (C) of selected apple cultivars at 80 days after full bloom (DAFB) and the mass per unit surface area of the CM and the respective fraction. Data points represent individual replications.

1997) indicating high partitioning into the lipophilic cuticle (Kerler and Schönherr, 1988). Also, the molecular weight is 282 g mol^{-1} and, hence, not too high for significant penetration through the cuticle. These properties all favour cuticular penetration and the depletion of the donor solution.

The developmental stage of the fruit had a marked effect on uptake. Incorporation was at maximum between 64 and 78 DAFB but was markedly lower both before and after this period. Because, on a unit area basis, the rate of cuticle deposition decreased continuously throughout development, the most likely explanation for the observed biphasic incorporation is a change in the penetration rate. There are two arguments that make this likely. First, earlier studies have revealed a marked decrease in penetration of Ca in developing apple fruit after June drop (Schlegel and Schönherr, 2002). At this stage, trichomes are shed and the scars left behind on the surface are sealed. Also about this stage, stomata convert to lenticels. This stage is often associated with microcracking of the fruit surface (Ruess and Stösser, 1993). Second, the cuticles of developing apple fruit increase in thickness continuously (Lai et al., 2016). These two processes both serve to strengthen the barrier function with the result that penetration will decrease, and with it the incorporation of the precursor.

The reasons for the sharp increase in penetration and hence, incorporation from 50 DAFB to 64 DAFB is not known. Potential factors involved are environmental factors that affect penetration in the field, such as increases in temperature (Baur and Schönherr, 1995; Schönherr and Baur, 1996). Water-induced microcracking is unlikely a factor since the surface was continuously exposed to the water of the donor solution at all feeding times.

It is interesting that at the time of application, differences amongst the apple cultivars in cuticle thickness were significantly correlated with the rate of incorporation. The significant correlation for the cuticle resulted from a highly significant correlation with the cutin fraction. There was no such relationship for the wax. This is consistent with the arguments presented above.

5. Conclusions

From a commercial point of view our results raise some interesting possibilities: Could cutin deposition be increased by foliar applications of cutin monomers and/or wax constituents in the field? We predict a number of benefits from increased cutin deposition, including reduced elastic strain. This in turn should reduce microcracking and russeting and even fruit cracking (Khanal et al., 2014).

Our results suggest that - in principle at least – these management interventions are a possibility. They would require frequent applications and high doses to have detectable effects. As far as we are aware, this possibility has not been tested experimentally. However, there are several potential limitations. First, incorporation will be localised and limited to places where droplets of a spray solution are deposited and dry down on the surface. Second, incorporation would be higher in positions where droplets collect. For example, in the later stages of development of an apple fruit, a stem cavity forms where the spray solution will collect to form a puddle. Similarly, pendant droplets can collect on the calyx lobes. Third, high concentrations of the dosing solution could be phytotoxic. Lastly, there is no indication that the application of oleic acid increases wax deposition. This would have created an additional benefit in converting elastic into plastic strain in the cuticles of developing apple (Khanal et al., 2013b). Clearly, the potential benefits of this approach merit further study for its commercial feasibility.

CRediT authorship contribution statement

Yiru Si: Methodology, Investigation, Validation, Visualization, Formal analysis, Data curation, Writing – original draft, Writing – review & editing. **Bishnu P. Khanal:** Funding acquisition, Conceptualization, Methodology, Supervision, Project administration, Formal analysis, Data curation, Writing – original draft, Writing – review & editing. **Moritz Knoche:** Funding acquisition, Conceptualization, Methodology, Supervision, Project administration, Writing – original draft, Writing – review & editing.

Declaration of Competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare no conflict of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.scienta.2021.110512.

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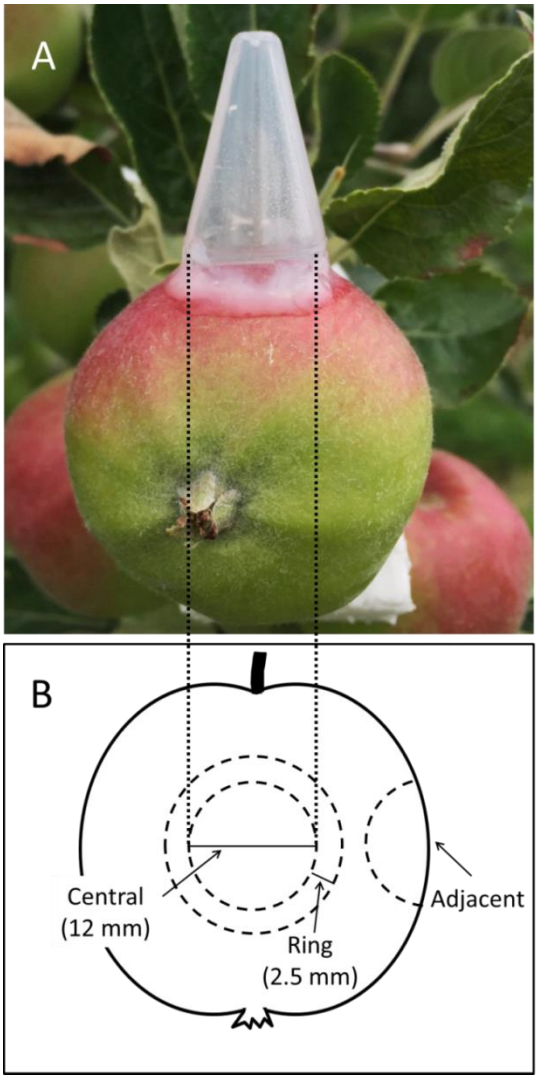

Supplementary Figure 1. (A) Apple fruit during feeding of ^{13}C labeled oleic acid at 62 days after full bloom. A polyethylene tube containing the feeding solution was mounted on the fruit surface using a non-phytotoxic, fast curing silicone rubber. (B) Sketch demonstrating the location of the central sampling area relative to the position of the dosing vial mounted on the surface of a developing apple fruit. The vial contained the ^{13}C labelled oleic acid that was fed for 7 days to the fruit surface.

2.3 Direct evidence for a radial gradient in age of the apple fruit cuticle

Yiru Si[1], Bishnu P. Khanal[1], Oliver K. Schlüter[2] and Moritz Knoche[1]

[1]Fruit Science Section, Institute of Horticultural Production Systems, Leibniz University Hannover, Hannover, Germany

[2]Department of Horticultural Engineering, Leibniz Institute for Agricultural Engineering and Bioeconomy (ATB), Potsdam, Germany

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Direct evidence for a radial gradient in age of the apple fruit cuticle

Yiru Si[1], Bishnu P. Khanal[1], Oliver K. Schlüter[2] and Moritz Knoche[1*]

[1]Institute of Horticultural Production Systems, Fruit Science Section, Leibniz University Hannover, Hannover, Germany

[2]Department of Horticultural Engineering, Leibniz Institute for Agricultural Engineering and Bioeconomy (ATB), Potsdam, Germany

*** Correspondence:**
Moritz Knoche
moritz.knoche@obst.uni-hannover.de

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Abstract

The pattern of cuticle deposition plays an important role in managing strain buildup in fruit cuticles. Cuticular strain is the primary trigger for numerous fruit-surface disorders in many fruitcrop species. Recent evidence indicates a strain gradient may exist within the apple fruit cuticle. The outer layers of the cuticle are more strained and thus more susceptible to microcracking than the inner layers. A radial gradient in cuticle age is the most likely explanation. Our study aims to establish whether (or not) deposition of new cutin in a developing apple fruit occurs on the inner surface of the cuticle i.e., immediately abutting the outward-facing epidermal cell wall.

Developing apples were fed with ^{13}C oleic acid through the skin. Following a 14-d period for incorporation, fruit were harvested and the cuticular membranes (CMs) isolated enzymatically. The CMs were then ablated to varying extents from the inner or from the outer surfaces, using cold atmospheric pressure plasma (CAPP). Afterwards, the ablated CMs were dewaxed and the ^{13}C contents determined by mass spectrometry.

Incorporation of ^{13}C in the cutin fraction was higher than in the wax fraction. The ^{13}C content was highest in non-ablated, dewaxed CM (DCM) and decreased as ablation depth from the inner surface increased. There was no change in ^{13}C content when ablation was carried out

from the outer surface. As fruit development proceeded, more ^{13}C label was found towards the middle of the DCM.

These results offer direct evidence for deposition of cutin being on the inner surface of the cuticle, resulting in a radial gradient in cuticular age - the most recent deposition (youngest) being on the inner cuticle surface (abutting the epidermal cell wall) and the earliest deposition (oldest) being on the outer surface (abutting the atmosphere).

1. Introduction

A cuticular membrane (CM) covers the outside of all primary-skin surfaces of all organs of terrestrial plants: all leaves, many stems and fruit. The CM is a non-living extracellular polymer, deposited on the outer surface of the cell walls of the epidermis. It comprises an insoluble polymer matrix 'cutin' and solvent-soluble lipids 'waxes' and cell wall polysaccharides (Schreiber and Schönherr, 1990; Dominguez et al., 2011; Yeats and Rose, 2013). The waxes are deposited within the CM as intracuticular waxes and also on the CM surface as epicuticular waxes. The primary function of the cuticle is to present a barrier against uncontrolled exchanges of respiratory gases (Jeffree, 2006) and water (Riederer and Schreiber, 2001; Kerstiens, 2006) and against invasion by pathogens (Huang, 2001; Heredia, 2003; Serrano et al., 2014). To continue its barrier functions, the cuticle must maintain its functional integrity throughout the life of a leaf or a fruit.

Maintenance of functional integrity presents a particular challenge to the cuticles of fruit. In contrast to leaves, a fruit skin is subject to an extended period of strain, as a fruit surface usually continues to extend from flowering through to fruit maturity – commonly a period of about five months. The epidermal and hypodermal cells beneath the cuticle cope with these growth strains by a combination of cell division, cell expansion and, in some fruit skins, by a change in epidermal cell aspect ratio (Maguire, 1998; Knoche and Lang, 2017). However, the polymeric CM cannot grow or divide but is simply stretched out by the ongoing area growth of the underlying epidermis (Knoche et al., 2004). This ongoing strain can result in the formation of cuticular microcracks that compromise its barrier functions. Microcracking is aggravated by surface wetness (Khanal et al., 2021). Moreover, microcracks are the first visible symptoms of a number of important fruit-surface disorders, including russet, skin spots, neck shrivel and macrocracks (Skene, 1980; Knoche and Lang, 2017). Throughout fruit development, the cuticle of an apple copes with the ongoing strain by the ongoing deposition

of new cutin and wax (Lai et al., 2016) – else the stretched CM would become thinner and thinner. The continuing addition of new cutin to the extending cuticle and its impregnation with intracuticular waxes 'fix' the strain, converting the elastic strain component into a plastic component (Khanal et al., 2013a).

Previous studies have shown that these processes result in the development of a radial gradient of strain across the CM, with the material on the inner side (abutting the cell walls) being less strained and that on the outer side (abutting the atmosphere) being more strained (Khanal et al., 2014). In this way, it is most common for a microcrack to appear first on the outer side of the CM and for this crack gradually to propagate deeper into the cuticle as straining continues, so as eventually to traverse the CM through to the inner (cell) side (Knoche et al., 2018). The most likely explanation for the observed radial gradient in strain is a corresponding gradient in the deposition and thus age of the cuticle. Additional factors that may contribute to a radial gradient in strain are the presence of polysaccharides on the cuticle's inner side (Dominguez et al., 2011), a compositional gradient of C16/C18 cutin monomers within the cuticle with C18 fatty acids having a higher impact on cuticle integrity (Kolattukudy and Walton, 1972; Walton and Kolattukudy, 1972; Kolattukudy et al., 1974; Kolattukudy, 1980; Straube et al., 2021) and/or a changing status of cutin polymerization (Espana et al., 2014; Martin and Rose, 2014).

It is hypothesized that with cutin being added preferentially to the inner side of the CM, this region will be younger, and so it will have suffered a shorter history of expansion, and so it will be less strained than the outer side. Taking the opposite view, the cutin on the outer side of the CM will have been deposited earlier on in the life of the fruit, and so be older, and so have suffered a longer history of expansion, and so be more strained. This hypothesis would explain why microcracking usually begins on the outer side of the CM. It would also explain why dewaxed CMs (DCM) usually 'curl' following extraction of wax. Unfortunately, direct evidence for the deposition and age gradients in the cuticle is lacking – i.e., that deposition occurs on the inner surface of the cuticle.

Therefore, the objective of this study was to provide direct evidence for a radial gradient in cuticle deposition and age. We first fed ^{13}C labeled oleic acid to the fruit surface. This was incorporated into the CM (Si et al., 2021a,b). Following feeding and incorporation, the cuticle was enzymatically isolated and then ablated from its inner surface, or from its outer surface, using a cold atmospheric pressure plasma (CAPP). Thereafter, the ^{13}C content of the ablated CM was determined. We focused on the cutin fraction, since an association of ^{13}C with the

wax fraction may have simply resulted from partitioning (Si et al., 2021a). We chose 'Idared' apple for our study, because 'Idared' is a russet non-susceptible cultivar where surface wetness during feeding does not trigger russet formation (Khanal et al., 2013b, 2021; Chen et al., 2020).

2. Material and Methods

2.1 Plant material

'Idared' apple (*Malus* × *domestica* Borkh.) trees grafted on M9 rootstocks were cultivated in the Horticultural Research Station of the Leibniz University Hannover at Ruthe, Germany (lat. 52°14'N, long. 9°49'E) according to current EU regulations for integrated fruit production. Representative fruit of normal growth and free from visible blemishes were selected for the experiments.

2.2 Methods

2.2.1 Fruit growth and cuticle deposition

Fruits were harvested at different stages of development and the mass of each was recorded. The surface area was calculated assuming sphericity and a mean density of 1 kg dm^{-3}. A sigmoid regression line was fitted through plots of fruit surface area vs time in days after full bloom (DAFB) and fruit mass vs DAFB. The number of replicates at each time was 60.

Cutin and wax deposition was quantified using enzymatically isolated CM using standard procedures. Briefly, epidermal skin segments (ES) were excised from the equatorial plane of a fruit using a biopsy punch (8 mm, Acuderm Inc., Fl, USA). The ES were incubated at ambient laboratory temperature in an isolation medium containing pectinase (90 mL L^{-1}, Panzym Super E flüssig; Novozymes A/S, Krogshoejvej, Bagsvaerd, Denmark) and cellulase (5 mL L^{-1}, Cellubrix L; Novozymes A/S). The enzyme solution was buffered in 50 mM citric acid and the pH adjusted to 4 using sodium hydroxide (NaOH). To avoid microbial growth, sodium azide (NaN_3) was added at a final concentration of 30 mM. The enzyme solution was periodically refreshed until the CMs separated from the underlying tissue. The CMs were cleaned using a soft camel-hair brush and thoroughly rinsed with deionized water.

To determine the mass per unit area, CMs were dried overnight at 40°C and then weighed on a microbalance (CPA2P; Sartorius, Göttingen, Germany). Subsequently, the CMs were

Soxhlet extracted using a chloroform : methanol mixture (1 : 1 v/v) for 2.5 h. Dewaxed CMs (DCM) were again dried overnight and weighed.

2.2.2 Dosing procedure and cuticle preparation

2.2.2.1 Feeding ^{13}C labeled oleic acid

The solutions were prepared by dissolving uniformly ^{13}C labeled oleic acid (> 95% purity, Larodan AB, Solna, Sweden) in 0.05% surfactant solution (Glucopon 215 UP/Mb; BASF, Ludwigshafen, Germany) at a final concentration of 167 µM (equiv. to 50 mg L^{-1}). Solutions were vortexed for at least 3 min immediately after preparation and again for 3 min immediately before application to the fruit surface. Donor solutions were always prepared fresh on the day of use.

The solution was applied as described earlier (Si et al., 2021a,b). Briefly, polyethylene tubes (25 mm height, 14 mm diameter) with a tapered tip and a minute hole in the tip were mounted in the equatorial region of the apple fruit using a non-phytotoxic silicon rubber (SE 9186 RTV; Dow Toray, Tokyo, Japan). A volume of 400 µL of donor solution was injected through the hole in the tip of the tube, and the hole sealed using silicone rubber to prevent drying of the donor solution (Figure 1A). Feeding was terminated after 7 d when the tubes were removed. The original footprint of the tube was then marked with a permanent marker and the marked area rinsed with deionized water. Fruits were sampled either 14 d after termination of feeding or at commercial maturity.

2.2.2.2 Cuticle isolation

After harvest, the marked area of the fruit surface was rinsed with 1% surfactant solution (Glucopon 215 UP/Mb; BASF) and blotted dry. A 12 mm diameter ES was excised from the central region of the marked area using a biopsy punch (Acuderm Inc., Fl, USA). The CMs were isolated from the ES as described above. Isolated and cleaned CMs were stored in deionized water at ambient temperature until use.

2.2.3 Cold atmospheric pressure plasma (CAPP) treatment

The CMs were dried overnight at 40°C and weighed on a microbalance. The CM discs were mounted between two discs of thick paper. The upper paper disc had an 8 mm diameter hole in the center. The paper/CM/paper ‘sandwich’ was then positioned on a custom made sample holder such that the CM surface was exposed to the plasma jet (Figure 1B,C). This setup prevented any movement of the CM during exposure to CAPP. The CAPP was generated

from a mixture of 99.9% argon and 0.1 % oxygen (Air Liquide, Düsseldorf, Germany) using an 8 W plasma jet (kINPen 09; Neoplas tools, Greifswald, Germany) at ambient temperature and pressure (Weltmann et al. 2009). When the mixture of gases passed through the electrode operated at a high frequency voltage (1.1 MHz; 2–6 kV peak-to-peak voltage), the CAPP was generated at the tip of the electrode (Figure 1B,C). The flow rate of the gas mixture was set at 5.4 L min^{-1} (Multi gas controller, 647C; MKS Instruments, Andover, MA, USA). The power supplied to the plasma jet was 65 V at a resonance balancing of 0.05 A.

The CM discs mounted on the sample holder were subjected to CAPP treatment of the inner or outer side for durations of 0-, 5-, 10-, 15-, 20- or 25-min. Earlier studies established that increasing exposure times to CAPP results in increasing ablation of synthetic polymers (Clouet and Shi, 1992) and of isolated cuticles irrespective of the cuticle's orientation (Khanal et al., 2014).

The distance between the CM and the tip of the plasma jet was set to 8 mm. This setup produced a CAPP treated area of about 8 mm diameter in the center of the CM disc. Using these settings, the temperature of the CM disc always remained below 40°C (Khanal et al., 2014).

2.2.4 Scanning electron microscopy (SEM)

The effect of CAPP treatment on the outer and inner surfaces of the CMs was established using SEM. Non-ablated control CMs and CAPP treated CMs following wax extraction were observed in a Quanta 200 SEM (FEI Europe Main Office, Eindhoven, The Netherlands). Cross-sections were viewed following freeze fracturing in liquid nitrogen. Specimens were mounted on aluminum stubs using conducting carbon tape and sputter coated with gold. Calibrated images of the inner and outer surfaces were prepared at 1000 x, those of cross-sections at 500 x. The acceleration potential was 15 kV.

2.2.5 Measurement of CM and DCM mass

The mass loss during ablation of the CM by the CAPP treatment was quantified on a core disc excised from the ablated CMs. The core disc was of 4 mm diameter and was excised using a biopsy punch. The CMs were dried overnight at 40°C and weighed on a microbalance. The mass per unit area was calculated. Thereafter, the CMs were extracted in 2.0 mL chloroform : methanol (1 : 1, v/v) per disc for 24 h at ambient temperature. The dewaxed CMs were removed from the chloroform : methanol extraction mixture, rinsed once

with fresh 0.5 mL chloroform : methanol, then dried overnight at 40°C. The DCM discs were then weighed and their mass per unit area calculated.

2.2.6 ^{13}C quantification using isotope ratio mass spectrometry (IRMS)

The amount of unlabeled (^{12}C) and labeled carbon (^{13}C) in the 4 mm diameter CMs and DCMs (after CAPP treatment of the CMs) were measured on an elemental analyzer (Isotope Cube; Elementar, Hanau, Germany) coupled with an isotope ratio mass spectrometer (Isoprime precisION; Isoprime-Elementar, Manchester, UK). We followed the procedure used by Si et al. (2021a,b). The labeled CM and DCM discs were crimped in aluminum boats (one disc per boat) (6 × 6 × 12 mm^3; LabNeed GmbH, Nidderau, Germany). The samples were combusted at 1080°C under a pulse of oxygen. Cerium dioxide was supplied to catalyze the combustion. The resulting CO_2 was passed to an isotope ratio mass spectrometer where the standard and isotopic C contents were quantified by a heat conductivity detector. For each measurement, the detector was calibrated using a commercial sediment standard.

The C isotope ratio was calibrated online by injecting one pulse of reference gas. The isotopic composition of C was calculated in the delta notation (at%) and referenced against Vienna Pee Dee Belemnite (VPDB). Further, C (at%) was referenced using international standards supplied by the International Atomic Energy Agency (IAEA, Vienna, Austria).

Sucrose (IAEA-CH-6), cellulose (IAEA-CH-3) and caffeine (IAEA-600) were used as standards for isotopic composition and an in-house standard made from spruce litter was used as an internal standard for quality control of C composition and the referenced isotopic composition.

The relative amount of tracer derived C (R_{Tracer}) (new carbon) to total carbon pool (old plus new carbon) was calculated using equation (1) (Gearing, 1991).

$$R_{Tracer} = \frac{at\%\ L - at\%\ C}{at\%\ C - at\%\ T} \times 100 \quad (1)$$

In this equation, at% represents the at% value of tracer (T) and labeled (L) or unlabeled control (C) CM or DCM sample. Total mass of tracer in the whole CM or DCM sample (M_{Tracer}) was calculated using equation (2).

$$M_{Tracer} = \frac{R_{Tracer} \times M_{Sample} \times \%C}{m_{sample}} \quad (2)$$

where M_{Sample} represents the total mass of the 4 mm diameter CM or DCM disc combusted in the elemental analyzer, %C represents the carbon content of the respective sample, and m_{sample} represents the molar mass of carbon in the sample. All % values used in the above equations were divided by 100 prior to calculation.

2.3 Data analyses

All experiments were conducted and analysed using completely randomised designs. Data were analysed by linear regression analysis using the statistical software package SAS (version 9.1.3; SAS Institute, Cary, NC). Data are presented as means ± standard errors. Where not shown, the error bars were smaller than data symbols.

3. Results

Fruit surface area and fruit mass increased sigmoidally with time (Figure 2A). The masses of CM, DCM and wax per unit fruit surface area, all increased during fruit development (Figure 2B). The rates of deposition of cutin and wax were highest in the early stages of fruit development, decreasing steadily until maturity (Figure 2B,C).

Un-treated CMs revealed a typical pattern of imprints of epidermal cell walls with slight depressions above the anticlinal cell walls when viewed from the outer surface (Figure 3A). On the inner surface, there were cuticular ridges above the anticlinal cell walls (Figure 3B). Exposure of outer or inner surfaces of CMs to CAPP resulted in significant ablations of the CM as indexed by significant decreases in CM thickness (Figure 3C-F). The cuticular ridges present on the inner surface of the CM above the anticlinal epidermal cell walls had almost disappeared after CAPP treatment for 20 min (Figure 3B,D,G).

Mass loss per unit area of the CM (Figure 4A), DCM (Figure 4B) and wax (Figure 4C) increased linearly as the duration of CAPP treatment of the CM increased. The mass loss of the CM and the DCM was lower when the morphological outer surface was ablated, as compared with the ablation of the morphological inner surface. The reverse applied for wax mass. Here, ablation of the outer surface induced a larger loss in wax mass as compared with the ablation of the inner surface. This is not surprising considering the presence of epicuticular wax on the outer surface of the CM. Similar results were obtained with CM ablated after feeding at 103 or 138 DAFB (Table 1). When treating the outer surface, CAPP treatment ablated the entire epicuticular wax layer plus some amount of the cutin and

cuticular wax, whereas CAPP treatment of the inner surface ablated cutin plus any cuticular wax only.

The amount of ^{13}C in the CM and the DCM of fruit fed at 69 DAFB remained constant following ablation of the outer surface but decreased continuously when the inner surface was ablated (Figure 5A). Regardless of the duration of CAPP treatment of the inner side, the decrease in the amount of ^{13}C in the DCM was always higher than that in the CM indicating that incorporation was in the cutin matrix and not or less in the wax.

Qualitatively and quantitatively similar results were obtained when analyzing CMs and DCMs of fruit fed at 103 DAFB (Figure 5B). At 138 DAFB, the amounts of incorporation of ^{13}C oleic acid in the CM and the DCM were low compared with those incorporated at 69 and 103 DAFB. There was no effect of CAPP treatment on ^{13}C content at 138 DAFB (Figure 5C).

Plotting the ^{13}C content of the CM or the DCM as a function of the amount of mass loss following CAPP treatment revealed that increasing mass loss resulted in decreasing ^{13}C content of CM and DCM when ablating their inner surfaces, but not when ablating their outer surfaces. With ablation of the outer surface of the CM, there was no relationship between the ^{13}C amounts in the CM or DCM and the mass losses of the CM or DCM following ablation. Similar results were obtained for CMs and DCMs of fruit fed at 69 and 103 DAFB (Figure 6A-D). There were no effects of ablation on the ^{13}C content of the CM or DCM for fruit fed at 138 DAFB (Figure 6E,F). At this stage of development, the deposition of CM has nearly ceased (Figure 2B,C).

Comparison of the ^{13}C contents of the DCM of fruit harvested 14 d after feeding at 69 DAFB with those from fruit harvested at maturity revealed significant differences (Figure 7). CAPP ablation of the CM of the fruit 14 d after feeding yielded an immediate decrease in ^{13}C content of the DCMs when carried out on the inner surface. However, when fruit was allowed to grow until maturity after feeding, ablation had no effects on the ^{13}C content up to a mass loss of about 4 g m^{-2}. Beyond this threshold, further ablation decreased the ^{13}C content as mass loss increased (Figure 7A). For DCMs of fruit fed at 103 DAFB and harvested at maturity, the ^{13}C contents began to decrease for a mass loss of about 2 g m^{-2} (Figure 7B). In DCMs of fruit harvested 14 d after termination of feeding, the ^{13}C content decreased continuously as mass loss increased. Fruit fed at 138 DAFB had very low ^{13}C contents in the DCM. Consequently, ablation by CAPP had little effect on the ^{13}C contents of the DCM

(Figure 7C). This is consistent with the cessation of CM deposition at 138 DAFB (Figure 2B,C).

4. Discussion

Our results evidence a radial gradient in the deposition and hence in age of the cuticle of developing apple fruit. Feeding apple fruit with ^{13}C oleic acid resulted in the incorporation and the deposition of labeled material on the inner surface of the CM. Consequently, the inner surface of the CM is younger, whereas the outer surface was deposited early on in fruit development, and so is older. The evidence for this conclusion is two-fold.

First, we obtained a gradient in ^{13}C content of the DCM of fruit that (1) incorporated ^{13}C oleic acid in the cuticle (69 and 103 DAFB) and that (2) was harvested 14 d after termination of the feeding period. The rate of this decrease was initially rapid but slowed as the duration of CAPP treatment increased and as the mass-loss increased.

Second, when the fruit was fed with ^{13}C oleic acid at 69 or 103 DAFB and then remained on the tree until maturity, the label was incorporated during the feeding period and immediately thereafter. However, un-labeled monomers were later incorporated in the cuticle on the inner surface. As cuticle deposition continued during development, the layer of label was progressively overlaid and so 'retreated' deeper into the cuticle as indicated in the sketch in Figure 8. Support for this view comes from the following observation. When the inner surface of the CM was ablated, short periods of ablation removed only the un-labeled portion of the cuticle, whereas longer ablations began to remove the labeled cuticle in ever deeper layers, and closer to the outer surface (Figure 8). The duration of the initial period without a decrease in ^{13}C and the magnitude of the mass loss before the removal of the ^{13}C labeled layer began, depended on the thickness of the un-labeled layer, deposited after termination of the feeding period. The duration of ablation before progressing into the labeled layer was longer for the feeding at 69 DAFB than for that at 103 DAFB. This interpretation is also consistent with the observation that treatment from the outer surface had no effect on the ^{13}C content of the polymer matrix. The above conclusions also account for the radial gradient in strain in apple fruit CM that has been reported previously (Khanal et al., 2014). Due to the earlier deposition, the outer CM has a longer history of strain and is therefore more strained, whereas the inner layer was deposited later and, hence, will have experienced less strain. This conclusion is also consistent with the structural characteristics of the apple cuticle (de Vries,

1968; Konarska, 2013). A cuticle proper (CP) that is rich in wax is distinguished from the underlying cuticular layer (CL) (Jeffree, 1996; Yeats and Rose, 2013). The CL is rich in cutin and contains embedded polysaccharides. The development of the CP precedes that of the CL (Jeffree, 2006).

The question arises as to what might be the chemical nature of the label incorporated in the cuticle. Since there was very little label associated with the wax, most of the incorporation was in the dewaxed CM. This indicates chemical binding, not simply partitioning into the CM (Si et al., 2021a). The two major constituents of the dewaxed CM are cutin and polysaccharides (Schreiber and Schönherr, 1990). Several arguments suggest this incorporation occurred in the cutin fraction.

First, the incorporation pattern was similar to that of CM deposition – i.e., it was higher during the early developmental stages, but lower in the late stage close to maturity. This is consistent with the deposition pattern of cutin during the development of the apple fruit (Lai et al., 2016). Second, feeding ^{14}C labelled oleic acids to apple skin discs resulted in incorporation of the label in hydroxy C18 acids such as 18-hydroxyoctadecenoic acid, 10,18-dihydroxyoctadecanoic acid, and 9,10,18-trihydroxyoctadecanoic acid (Kolattukudy et al., 1971, 1973). These are major monomers of the apple fruit cutin (Walton and Kolattukudy, 1972; Straube et al., 2021). Also, until now the composition of apple cutin has been found to be consistent among all cultivars investigated (Holloway, 1973; Legay et al., 2017; Straube et al., 2021). Third, the cutin of apple fruit has been classified as a "mixed-type cutin" comprising C16 and C18 monomers (Holloway, 1982). This is in line with earlier observations from our laboratory, that when developing apple fruit were fed with ^{14}C palmitic acid and ^{14}C oleic acid incorporation in the CM of ^{14}C palmitic acid was very much lower than of ^{14}C oleic acid (Si et al., 2021a). This incorporation occurs in all apple cultivars investigated and at a rate that is significantly correlated with the mass of the CM per unit fruit surface area (Si et al., 2021b).

It may be argued that oleic acid is also a precursor for suberin and that the exposure of the apple fruit surface to the feeding solution may have resulted in microcracking and then periderm formation (Chen et al., 2020; Khanal et al., 2021). However, we consider this possibility extremely unlikely. First, 'Idared' is a cultivar that is known not to be susceptible to russeting (Khanal et al., 2013b). Russeting involves the formation of a periderm where the phellem typically has heavily suberized cell walls. Second, the feeding treatments in our study were done long after the period of greatest russet susceptibility was over (Chen et al.,

2020; Khanal et al., 2021). Apple fruit is most susceptible to russet during the first 28 days after full bloom. Third, the apple fruit fed with oleic acid all had intact cuticles. There were no indications of either microcracking or russeting either to the naked eye or under SEM regardless of whether the fruit was harvested after a 7 day feeding plus the 14 day incorporation period or later at maturity. By this time, any russeting would have been visible on the fruit surface. Fourth, suberin is deposited inside the cell wall (Franke and Schreiber, 2007; Pollard et al., 2008). We obtained clean CMs after enzymatic isolation (see also Fig. 3). Furthermore, a 5-min CAPP treatment of the inner surface decreased CM mass by about 8% (Khanal et al., 2014). This would have been sufficient to remove any hypothetical suberized cell walls that might have been present. This would have left no label in the dewaxed CM. However, in fruit harvested at maturity the label from the early feeding was incorporated deep into the CM.

These arguments demonstrate that both, the label detected in the inner surface of the dewaxed CM from fruit harvested after the feeding and the incorporation period, and the label found deeper in the dewaxed CM of fruit harvested at maturity, most likely represent hydroxy C18 monomers polymerized in the cutin.

Practical implications

The deposition pattern of cutin on the inner side of the cuticle represents an important and critical mechanism that delays or prevents the formation of deep microcracks. A high rate of cutin deposition also maintains a minimum thickness of the CM during phases of rapid fruit expansion. Because the deposition occurs on the inner surface of the CM, the likelihood of formation of microcracks that traverse the cuticle decreases. Microcracks that traverse the cuticle dramatically impair the cuticle's barrier functions and trigger the formation of a periderm, which led to russeting.

The results obtained in apple are thought likely to apply also to other fruitcrop species that deposit cuticle throughout development. Further study should explore the possibility of stimulating the deposition of cutin during periods of rapid fruit growth, to help prevent deep propagation of microcracks.

5. Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as potential conflicts of interest.

6. Author Contributions

BK and MK obtained the funds to support the study. YS, BK and MK planned the experiments. YS and OS conducted the experiments. YS and BK analyzed the data. YS, BK and MK wrote, revised and edited the manuscript.

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9. Data Availability Statement

Datasets are available on request:

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

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Table

Table 1. Parameters of linear regression equations describing the relationships between mass loss (g m^{-2}) and the duration of ablation using a cold atmospheric pressure plasma (CAPP) on the morphological inner and outer sides of isolated cuticular membranes (CM) of 'Idared' apple. Fruit was fed using ^{13}C oleic acid for 7 d beginning at 69, 103, and 138 days after full bloom (DAFB). After 7 d, feeding was terminated. Fruit were harvested 14 d after terminating of feeding and the CMs were isolated. Wax was extracted after CAPP treatment. Dewaxed CMs are referred to as DCMs. Since the intercept term was not significantly different from zero, all regression lines were forced through the origin.

Stage (DAFB)	Fraction	Morphological side	Slope ± SE	Coefficient of determination
69	CM	Inner	0.33 ± 0.01	0.999***
		Outer	0.23 ± 0.02	0.956***
	DCM	Inner	0.36 ± 0.02	0.992***
		Outer	0.10 ± 0.01	0.932**
	Wax	Inner	0.08 ± 0.00	0.990***
		Outer	0.13 ± 0.02	0.916**
103	CM	Inner	0.41 ± 0.01	0.999***
		Outer	0.32 ± 0.01	0.994***
	DCM	Inner	0.32 ± 0.02	0.979***
		Outer	0.12 ± 0.01	0.983***
	Wax	Inner	0.04 ± 0.00	0.941**
		Outer	0.22 ± 0.02	0.975***
138	CM	Inner	0.42 ± 0.01	0.995***
		Outer	0.21 ± 0.01	0.986***
	DCM	Inner	0.33 ± 0.02	0.985***
		Outer	0.08 ± 0.01	0.949**
	Wax	Inner	0.10 ± 0.00	0.995***
		Outer	0.20 ± 0.01	0.983***

SE standard error of the estimate

Significance of the coefficients of determination at the 1 and 0.1% levels are indicated by ** and ***, respectively.

Figures

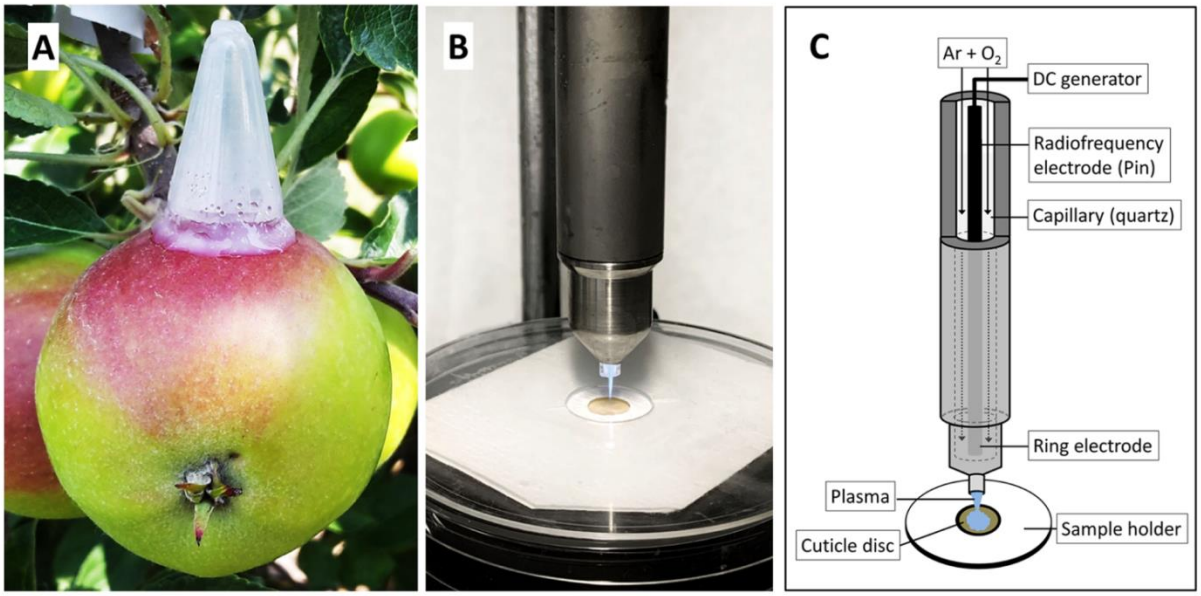

Figure 1. Apple fruit with mounted polyethylene tube for feeding ^{13}C labeled oleic acid under field conditions (A). Cold atmospheric pressure plasma (CAPP) generated by a plasma jet during ablation of the cuticular membrane (CM) (B). Sketch of plasma jet and CM sample holder illustrating the various parts of the plasma jet and the experimental setup (C). The sketch was adapted from Bußler (2017).

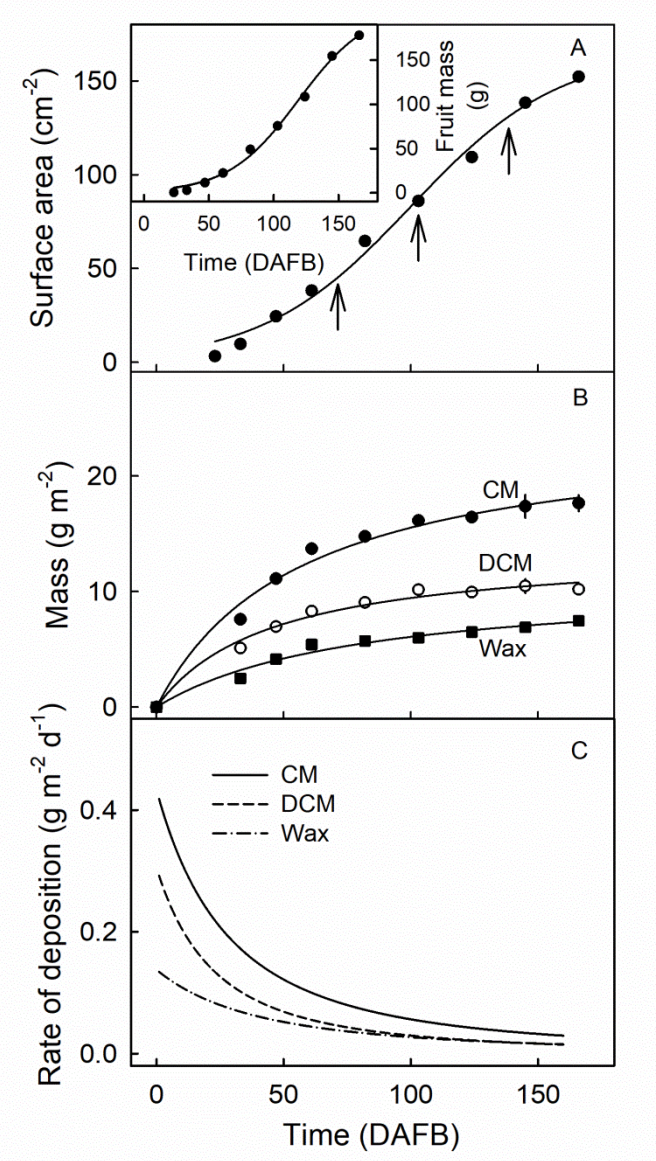

Figure 2. Fruit surface area (A), fruit mass (A, inset) and mass per unit area of the cuticular membrane (CM), the dewaxed CM, and the wax (B) during the development of apple fruit. (C) Rates of deposition of CM, DCM and wax. The x-axis scale is in days after full bloom. The vertical arrows in A indicate the beginning of the feeding periods of ^{13}C oleic acid to the outer surface of developing apple fruit. Data points represent means ± SE, n = 15 – 60.

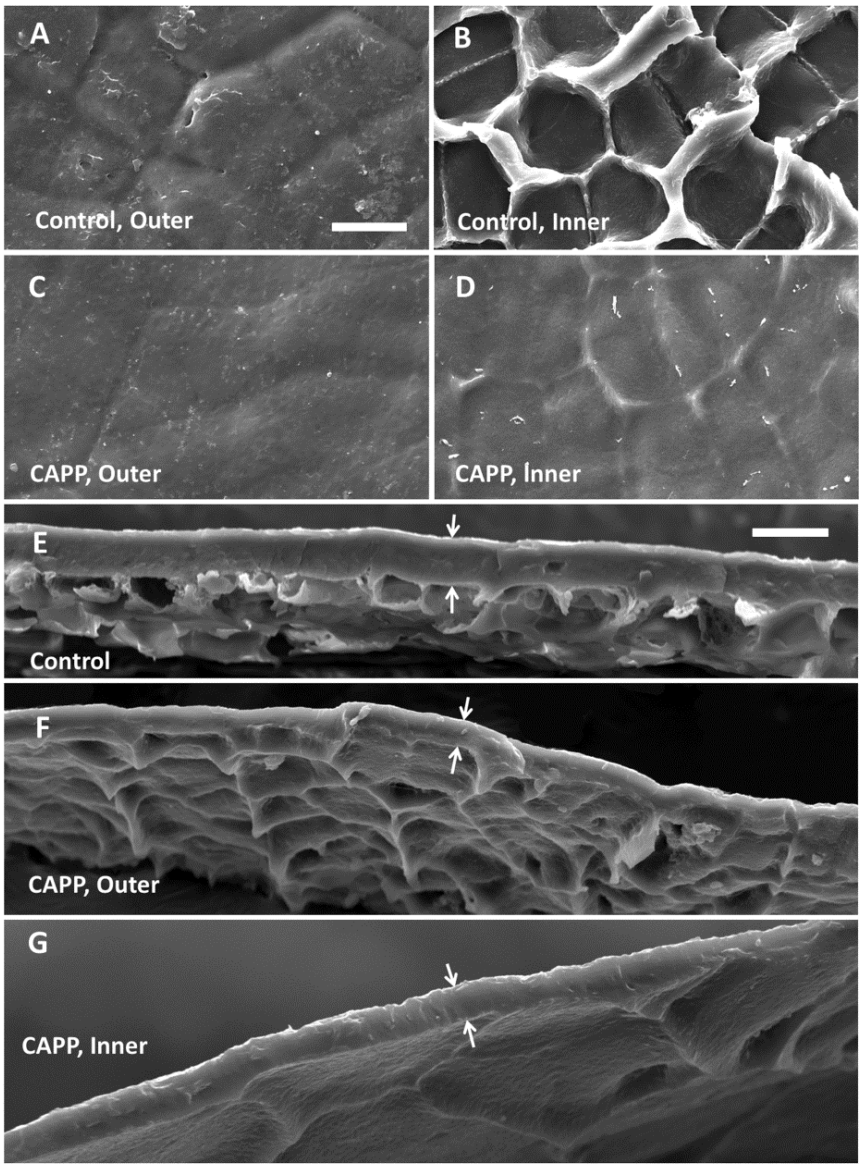

Figure 3. Scanning electron micrographs of outer surfaces (A,C), inner surfaces (B,D), and cross-sections (E-G) of cuticular membranes (CMs) of apple fruit (fed with ^{13}C oleic acid at 69 days after full bloom (DAFB) and harvested at 178 DAFB) with ablation (C,D,F,G) and without ablation using a cold atmospheric pressure plasma (CAPP) (A,B,E) following wax extraction. The white arrows indicate outer and inner surfaces of the CMs. Thickness of the CM differed significantly and was 11.0 ± 0.3 µm for the control, 7.4 ± 0.7 µm for the CAPP treatment of the outer side and 5.5 ± 0.4 µm for the CAPP treatment of the inner side. Data on thickness represent means ± SE, n = 3 – 5.

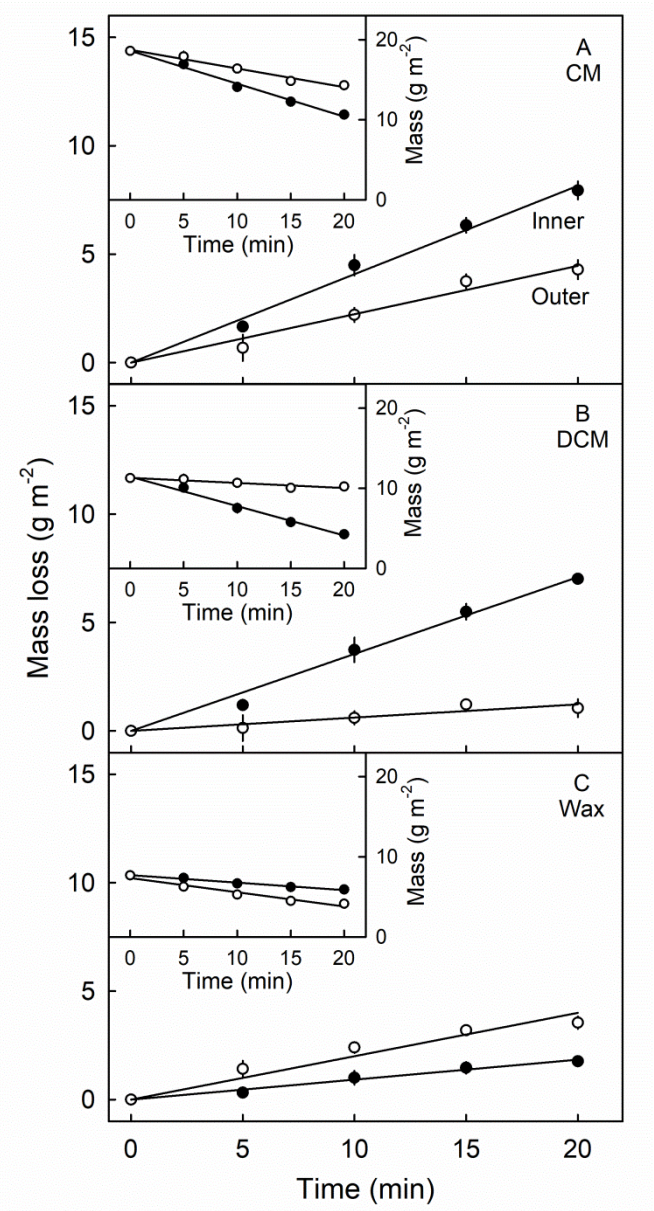

Figure 4. Effect of duration of ablation of cuticular membrane (CM) of apple fruit using cold atmospheric pressure plasma (CAPP) on the mass loss (main figures) and absolute mass (inset figures) of the CM (A), the dewaxed CM (DCM) (B) and the wax (C). The inner or the outer surface of the CM was ablated using CAPP and the mass loss of the CM, the cutin and wax fraction determined. Fruit were fed 138 days after full bloom and harvested 14 days after termination of the feeding. Data points represent means ± SE, n = 6 – 16.

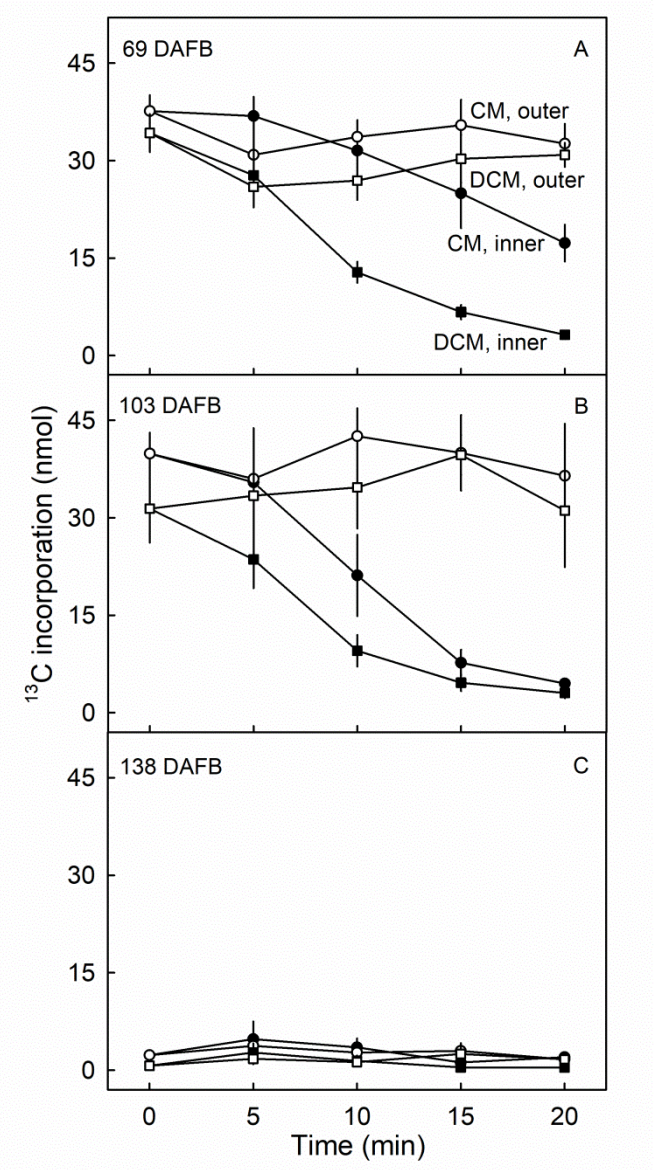

Figure 5. Effect of duration of ablation of cuticular membranes (CM) of developing apple fruit using cold atmospheric pressure plasma (CAPP) on the amount of ^{13}C in the CM and the dewaxed CM (DCM). The inner or the outer surface of the CM was ablated using CAPP. Developing apple fruit were fed for 7 d using ^{13}C labeled oleic acid at 69 (A), 103 (B), 138 (C) days after full bloom (DAFB). Fruit were sampled 14 d after termination of the feeding and the CMs were isolated. Data points represent means ± SE, n = 6 – 8.

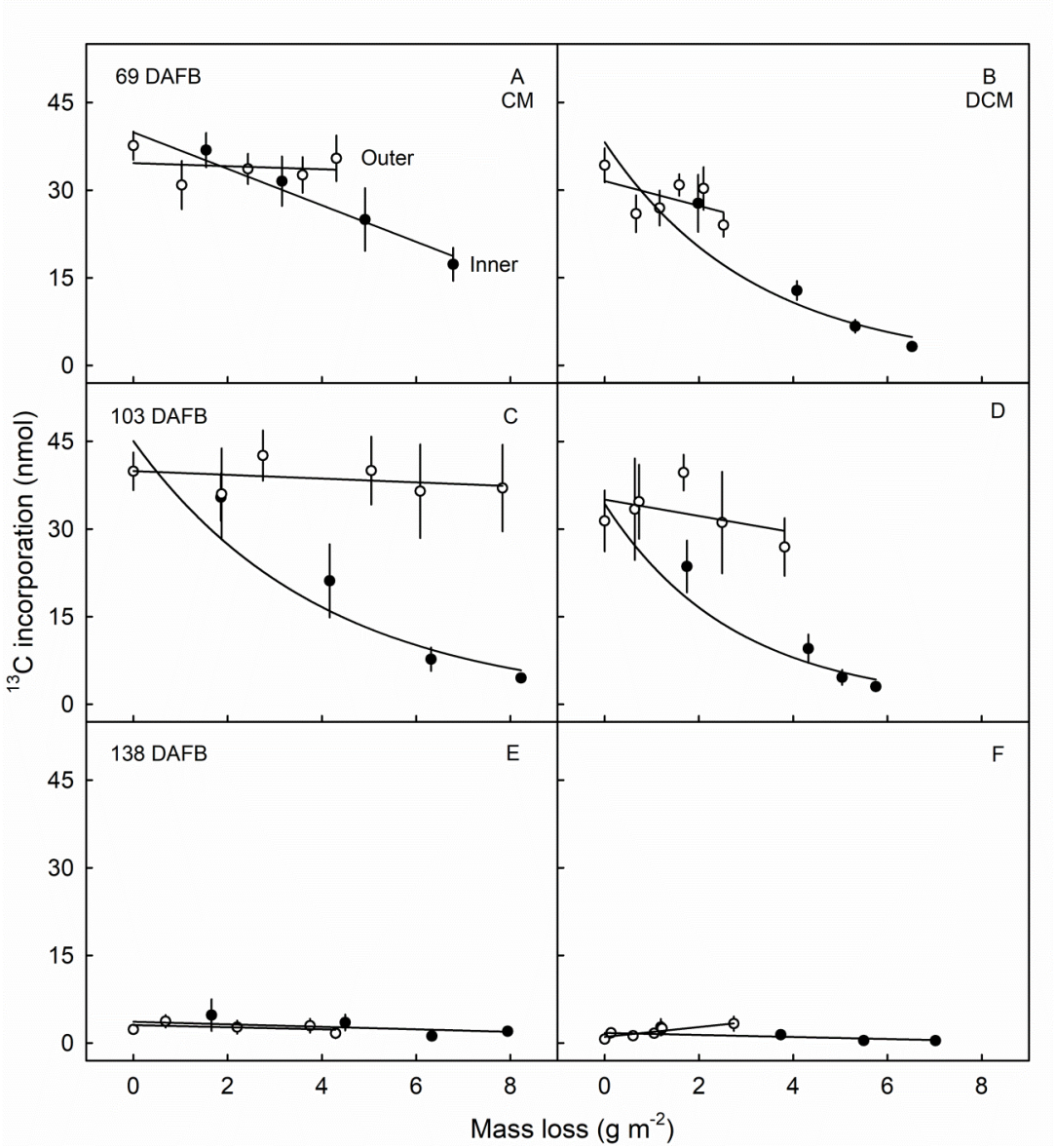

Figure 6. Relationship between the amount of ^{13}C in the cuticular membrane (CM) (A,C,E) or in the dewaxed CM (DCM) (B,D,F) and the mass loss of the CM or DCM that resulted from ablation of the inner or outer surface of the CM using a cold atmospheric pressure plasma (CAPP). Developing apple fruit were fed for 7 d using ^{13}C labeled oleic acid at 69 (A,B), 103 (C,D), and 138 (E,F) days after full bloom (DAFB). Fruit were sampled 14 d after termination of feeding and the CMs were isolated. Data points represent means ± SE, n = 6 – 8. For data on the relationships between mass loss and duration of ablation of the CM by CAPP see Table 1.

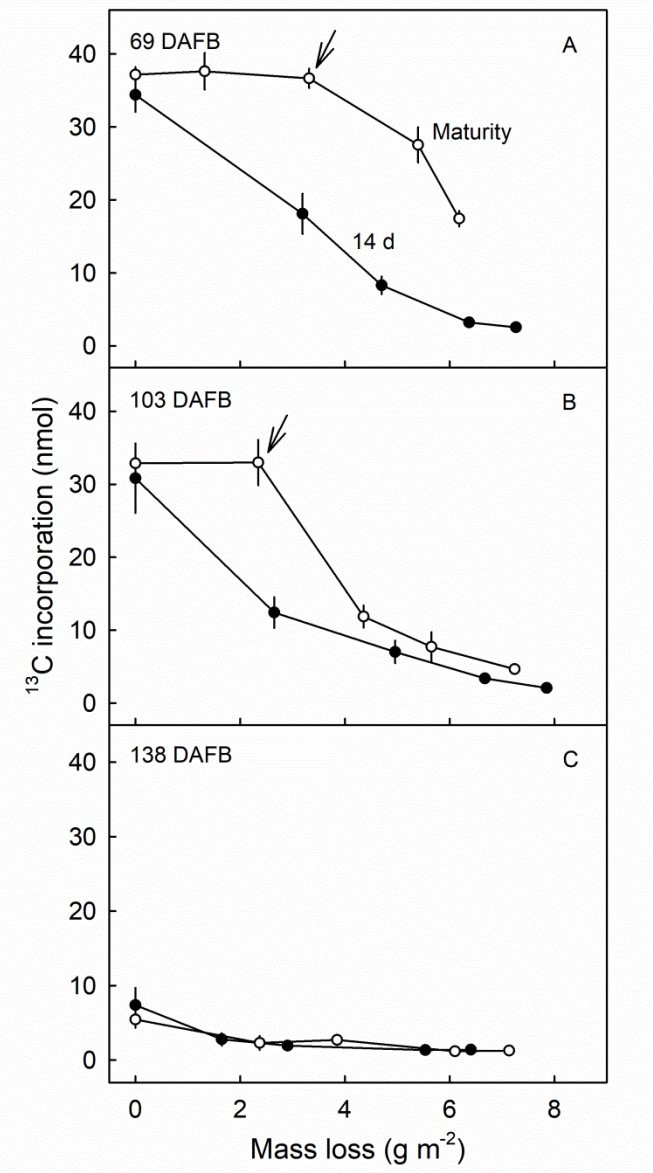

Figure 7. Relationship between the amount of ^{13}C in the dewaxed cuticular membrane (DCM) and the mass loss of the DCM that resulted from ablation of the inner surface of the CM using a cold atmospheric pressure plasma (CAPP). Developing apple fruit were fed for 7 d using ^{13}C labeled oleic acid at 69 (A), 103 (B), and 138 (C) days after full bloom (DAFB). Fruit were sampled either 14 d after termination of the feeding or at maturity and the CMs isolated. Data points represent means ± SE, n = 8 – 10. For data on the relationships between mass loss and duration of ablation of the CM by CAPP see Supplementary Table 1.

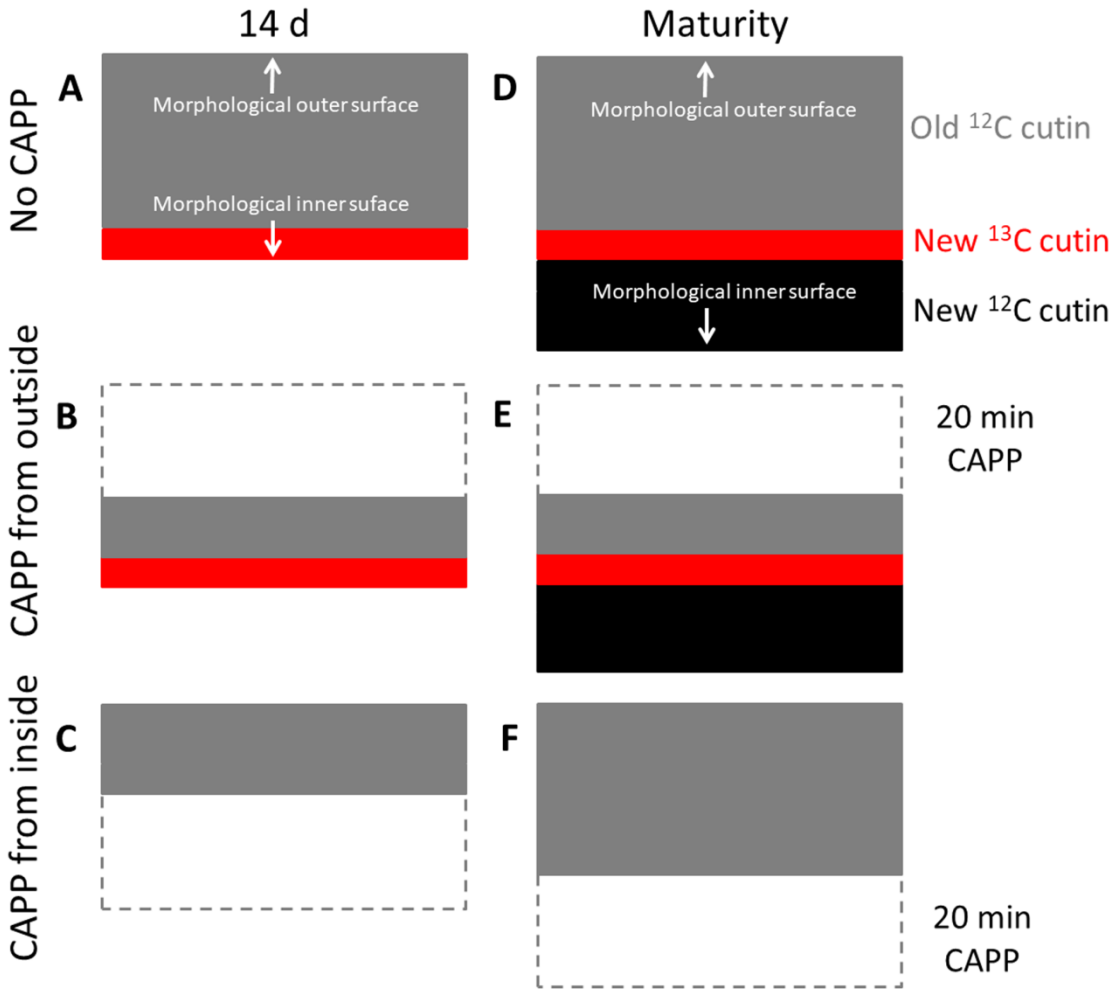

Figure 8. Sketch illustrating the location of the ^{13}C labelled layer within the cuticular membrane (CM) that resulted from feeding the developing apple fruit using ^{13}C labelled oleic acid for 7 d. The ^{13}C labelled precursor is incorporated in the inner side of the CM (A). Isolation of the CM at this stage and ablation from its outer side has no effect on the ^{13}C content until the ^{13}C labelled layer of the CM is ablated (B), ablation from the inner side decreases the ^{13}C content of the CM as mass loss increases (C). In contrast, when fruit remains on the tree until maturity, CM deposition continues on the inner side. The layer resulting from feeding ^{13}C oleic acid now "moves" into the CM (D). Ablation from the outer side still has no effect on the ^{13}C content until the ^{13}C labelled layer of the CM is ablated (E). When ablation from the inner side, ablation will not affect the ^{13}C content of the CM until the labelled layer is reached (F). Further ablation then will decrease the ^{13}C content.

Supplementary Table 1. Parameters of linear regression equations describing the relationships between the mass loss (g m^{-2}) and duration of ablating the inner side of cuticular membrane (CM) of developing 'Idared' apple fruit using a cold atmospheric pressure plasma (CAPP). Fruit were fed for 7 d using a ^{13}C oleic acid solution at 69, 103 and 138 days after full bloom (DAFB) and harvested 14 d after termination of the feeding period or at maturity. The CMs were isolated. Wax was extracted after CAPP treatment. Dewaxed CMs are referred to as DCMs.
Since the intercept term was not significantly different from zero, all regression lines were forced through the origin.

Stage (DAFB)	Fraction	Harvest time	Slope ± SE	Coefficient of determination
69	CM	14 d	0.52 ± 0.03	0.982***
		Maturity	0.44 ± 0.01	0.996***
	DCM	14 d	0.41 ± 0.03	0.980***
		Maturity	0.35 ± 0.01	0.995***
	Wax	14 d	0.11 ± 0.01	0.953***
		Maturity	0.08 ± 0.01	0.975***
103	CM	14 d	0.54 ± 0.02	0.992***
		Maturity	0.52 ± 0.03	0.989***
	DCM	14 d	0.43 ± 0.02	0.991***
		Maturity	0.38 ± 0.01	0.994***
	Wax	14 d	0.12 ± 0.01	0.991***
		Maturity	0.16 ± 0.02	0.956***
138	CM	14 d	0.45 ± 0.01	0.996***
		Maturity	0.53 ± 0.02	0.994***
	DCM	14 d	0.33 ± 0.01	0.993***
		Maturity	0.38 ± 0.01	0.995***
	Wax	14 d	0.11 ± 0.01	0.956***
		Maturity	0.16 ± 0.01	0.988***

SE standard error of the estimate

Significance of the coefficients of determination at the 0.1% level is indicated by ***.

3. General Discussion

The results of this work present several new and important findings:

(1) The feeding procedure using ^{13}C oleic acid allowed the quantification of cutin deposition in developing apple fruit both *in vitro* in the laboratory and *in vivo* in the field. The ^{13}C incorporation was highest using the 'infinite dosing' procedure (rather than the 'finite dosing' method) and using oleic acid (rather than palmitic acid). The oleic acid must have been incorporated and most likely polymerized within the cutin matrix. For the wax fraction, incorporation cannot be distinguished from simple partitioning (Chapter 2.1).

(2) The incorporation from a donor solution and cutin deposition were affected by: • the concentration of solutes, • the duration of the dosing procedure, • the developmental stage of the apple fruit and • by the apple cultivar (Chapter 2.2).

(3) Direct evidence was provided for a radial gradient of cutin deposition across the CM and a consequent gradient in cutin age. The new cutin is deposited on the inner surface of the CM, thus the cutin in the inner CM surface is younger, and that in the outer CM surface is older (Chapter 2.3).

For detailed discussions with respect to the above the reader is referred to the respective chapters of the thesis. The general discussion below will concentrate on the implications of this study both for future research and for horticultural practice.

3.1 Potential implications for future research

An established feeding system with ^{13}C labelled fatty acid precursors safely allows quantification of cutin deposition under field conditions. This opens up interesting possibilities for new horticultural research which requires a fruit to continue growing and thus remain *in situ* and attached to the tree.

3.1.1 Investigation of the external factors

Cuticle deposition is likely to be affected by a range of external factors such as by environmental and orchard conditions as well as by cultural practices. The system established in our study permits comprehensive investigation of factors without risk of radioactive contamination. The limitation of earlier laboratory assays of cuticle deposition (Heredia, 2003; Kolattukudy, 2001; Lendzian and Schönherr, 1983), apart from using radioactive precursors, was that the experimental setup factors such as the concentration of the donor solution and the duration of incubation were selected empirically. These earlier studies

allowed an observation of certain amount of applied labelled precursor but without knowing whether the maximum incorporation was achieved or not. The setup we establish in this study was selected experimentally and allows delivery of a clear comparison of effects indicated by a high level of incorporation into the cutin fraction. In general, the most efficient setup that achieved the considerable incorporation was to apply ^{13}C labelled oleic acid solution at 25 to 50 mg L^{-1} using an 'infinite dosing' method and to retain the dosing solution on the fruit surface for about three days.

3.1.2 Investigation of russet susceptible cultivars

'Idared' was chosen in this study owing to its non-susceptibility to russeting. It seems to offer the following advantages: 1) the fruit have a homogenous primary fruit skin with an intact and continuous cuticle and there is hardly ever any russet; 2) recent studies of russet susceptible and non-susceptible cultivars find marked differences between these cultivars in the cell morphology of the epidermis and hypodermis: compared with the russet susceptible cultivars, in the russet non-susceptible cultivars, the epidermal and hypodermal cells are less variable in size, shape and arrangement, the cuticle is accordingly of relatively uniform thickness (Khanal et al., 2020; Knoche and Lang, 2017). These factors allow clear observations to be made of cuticle deposition during the entire period of fruit development. The cuticles of the russet-susceptible apple cultivars are confronted with a greater incidence of skin disorders. Thus, the study of cuticle deposition in russet susceptible cultivars is of great importance. This can be done using the system established herein.

However, the method we developed does have some limitations.

First, exposing russet susceptible cultivars to surface moisture induces russeting (Chen et al., 2020; Khanal et al., 2021). Hence, infinite dose feeding may also induce russeting in susceptible cultivars. Initially, microcracks form and these increase penetration of the donor solution. Subsequently, the incorporation into the cutin may be diminished by the formation of suberin. Second, the highly variable cuticle thickness in russet-susceptible cultivars interferes with the CAPP treatment. Uniform ablation of the CM is not possible. However, the method is applicable to non-susceptible cultivars without modification. For susceptible cultivars, finite dose feeding should be tested. In finite dose feeding the solution is subjected to a drying process. As a result, exposure to surface moisture is limited to the short period before drying (perhaps about few hours, depending on field conditions). This makes russet induction by dosing much less likely.

3.2 Potential implications for horticultural practice

The results in this thesis demonstrate that an exogenously applied precursor results in significant incorporation in the cutin fraction. It may therefore be hypothesized that cuticle deposition can be stimulated by spray applications of the cutin precursor, in the field, under the practical conditions of commercial horticulture.

Stimulation of cutin deposition would have several advantages:

The apple fruit cuticle is subjected to a gradient in strain, with the outer CM surface being more strained than the inner CM surface. Thus, microcracking begins at the outer CM surface and the crack then propagates radially inwards towards the inner CM surface. Since cutin deposition occurs on the inner surface (see Chapter 2.3), the traverse of a cuticle by a microcrack is delayed or prevented if cutin deposition keeps up with crack propagation.

Microcrack formation and extension may result from either of the following factors: (i) rapid fruit expansion growth causing rapid increases in strain and (ii) surface wetness that alters the cuticle's mechanical properties resulting in increased microcracking due perhaps to decreased stiffness and decreased fracture forces (Khanal and Knoche, 2017). Thus, spray applications of cutin precursors before or during phases of rapid fruit growth or during or after exposures to surface moisture could help reduce/prevent microcracking and hence, russeting. In apple, the first 40 days after full bloom are considered to be the phase of maximum russet susceptibility (Faust and Shear, 1972; Knoche et al., 2011; Skene, 1982).

It is unlikely that registration of a potential commercial spray product will pose a problem, since rape seed oil contains significant amounts of oleic acid (Matthaus et al., 2016; Sagan et al., 2019) and rape seed oil is already registered for agricultural purposes (Agricultural marketing resource center, 2018; Woźniak et al., 2019).

However, several difficulties will have to be overcome if increasing cutin deposition by spray application of precursors.

(1) The results from chapter 2 of this thesis indicate the application of the precursors results in a 'contact type' mode of action. There was essentially no lateral diffusion away of the dosing site. When spraying, coverage is not 100% uniform. Droplets collect and form residues, adjacent areas may be without deposit. If the distribution pattern on the fruit is not uniform, the 'contact action' implies that incorporation of the precursor will be restricted to the places where the spray droplets adhere and collect. For example, in high volume spraying applications, droplets tend to pool in the stem cavity and as pendant droplets on calyx lobes. Using a surfactant will alleviate these problems, but only silicone surfactants will present a

uniform film due to their very low surface tension (Knoche, 1994). It is important to note that surfactants will not eliminate pooling in the stem cavity or pendant drops on the calyx lobes. Whatever surfactant is selected, it must not cause phytotoxicity.

(2) The efficiency of spray application of precursors is likely to be lower than that achieved in the wetted area in the present study using infinite dose feeding. This study shows that feeding 50 mg L^{-1} oleic acid solution with infinite dosing results in significant increases in the masses of the CM and the cutin (feeding vs. non-feeding: 16.86 ± 0.26 g m^{-2} vs. 14.76 ± 0.22 g m^{-2} for the mass of CM, 10.59 ± 0.26 g m^{-2} vs. 9.05 ± 0.16 g m^{-2} for the mass of cutin). However, compared to spray application in the field, infinite dose feeding only mimics the liquid phase of the droplet following spray application. After droplet drying, a deposit has formed in which several changes relevant to penetration take place: (i) the concentration will increase which tends to increase the driving force for uptake; (ii) the droplet/fruit contact area decreases which tends to decrease uptake (Bukovac, 2005) and (iii) in some cases the active ingredient (ai) may even crystallize causing the driving force to decrease to zero and penetration to stop (Knoche and Bukovac, 2000). The net effect of these three components determines the effect of droplet drying on penetration. This net effect is unknown for spray application of oleic acid. A preliminary spray application experiment conducted in the field in the current season revealed a slight but significant increase (approx. 4%) in cuticle mass per unit area following weekly spray applications of oleic acid at 10 g L^{-1} (Winkler, unpublished data). This observation indicates that cuticle deposition may indeed be stimulated by the application of suitable precursors.

Based on the markedly lower efficiency of the spray application process as compared to the controlled infinite dose feeding, multiple application at higher dose rates may be needed to reduce microcracking and hence, russeting.

To further optimize the spray application, ai of small molecular size could be used instead of fatty acids (Schönherr and Baur, 1994). The penetration of ai, therefore, could be maintained using appropriate formulations, e.g., by employing acetate. Acetate is activated into acetyl-CoA and participates in the synthesis of fatty acid precursors. It is much smaller in terms of chain length and molecular weight than the fatty acids. So, using acetate instead of fatty acids may increase the efficiency of a spray application.

(3) Schlegel and Schönherr (2002) demonstrated that the permeability of the apple fruit surface depends on the stage of fruit development when applying an aqueous $CaCl_2$ solution. Permeability is high during early fruit development, before trichomes are shed and when

microcracks are present. The permeability, however, decreases as fruit development proceeds. Thus precursor applications during early fruit development will probably be more effective than later on.

This topic merits further study.

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List of Publications

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Si, Y., Khanal, B.P., Sauheitl, L., Knoche, M. (2021) Cutin synthesis in developing, field-grown apple fruit examined by external feeding of labelled precursors. *Plants* 10, 1–14. doi: 10.3390/plants10030497

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Curriculum Vitae

Name:	Yiru Si
Gender:	Female
Date of birth:	21.07.1991
Place of birth:	Changzhou (P.R. China)
Nationality:	Chinese

Education

03/2017 – present	**Doctoral candidate** Institute of Horticultural Production Systems: Fruit Science Section, Leibniz University Hannover, Germany Thesis: Cuticle deposition in apple fruit: A radial gradient in cuticle age
10/2013 – 09/2015	**M.Sc International Horticulture** Institute of Horticultural Production Systems: Tree Nursery Science Section, Leibniz University Hannover, Germany Thesis: Influence of nutrient loading on *Corylus avellana* and consequences for early frost reactions
09/2009 – 06/2013	**B.Sc Horticulture** Institute of Horticulture Jinling Institute of Technology, China Thesis: Effects of phosphate-solubilizing fungi on rooting and growth of bamboo willow cutting seedlings
2006 – 2009	Changzhou Beijiao High School, China
2003 – 2006	Changzhou Beijiao Middle School, China
1997 – 2003	Changzhou Xixinqiao Primary School, China