On the origin of internal browning in pears

(*Pyrus communis* L. cv Conference)

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Abbreviations and symbols

AA	L-ascorbic acid
ACC	1-Aminocyclopropane-1-carboxilic acid
ACP	Anaerobic extinction point
ADH	Alcohol dehydrogenase
ADP	Adenosine diphosphate
AEC	Adenylate energy charge
AMP	Adenosine monophosphate
AO	Ascorbate oxidase
APX	Ascorbate peroxidase
ATP	Adenosine triphosphate
CA	Controlled atmosphere
CO_2	Carbon dioxide
DCS	Dynamic Control System
DHA	Dehydroascorbic acid
DW	Dry weight
L-DOPA	Precursor of dopamine
EC	Enzyme Commission (enzyme taxonomy)
EP	Extinction point (fermentation)
Eq(s)	Equation(s)
FW	Fresh weight
GSH	Glutathione (GSH, L-Y-glutamyl-L-cysteinyl-glycine)
GSSH	Glutathione (oxidised form)
HPLC	High performance liquid chromatography
IA	Image analysis
Km	Michaelis-Menten constant
MAP	Modified atmosphere packaging
MCP	Methyl cyclopropane
ME	Maintenance Energy
MDHA	Monodehydroascorbic acid
NADH	Nicotinamide adenine dinucleotide (reduced form)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
O_2	Oxygen
PA	Photoacoustic
PDC	Pyruvate decarboxylase
PEP	Phosphoenol Pyruvate
PPO	Polyphenol oxidase
PS-1	Photosystem I
ROS	Reactive oxygen species
Rpm	rounds per minute
SDS	Sodium dodecyl sulphate
SOD	Superoxide dismutase
TBC	4-*Tert*-butyl catechol
TCA	Trichloroacetic acid
ULO	Ultra low oxygen

Stellingen

I. De membraanschade die decompartimentalisatie veroorzaakt is waarschijnlijk te wijten aan een combinatie van vrije zuurstofradicalen, een tekort aan antioxidanten om deze radicalen te inactiveren en een energietekort als gevolg van een sterk verlaagde zuurstof concentratie.

II. De gevoeligheid van Conference peren voor de ontwikkeling van interne bruinverkleuring heeft geen aantoonbare relatie met de weerstand van de schil voor zuurstof en kooldioxide.

III. Na decompartimentalisatie kan de pH-verandering in de cel die hierop volgt verantwoordelijk zijn voor de activering van cytoplasmatisch polyfenol oxidase.

IV. De vondst van hominide resten in Afrika, zoals van Louis Leaky en de vondst van Lucy (*Australopithecus afarensis*) in 1974, spreekt bij veel mensen tot de verbeelding. Echter, deze ontdekkingen lijken maar niet te leiden tot een duidelijke naamgeving en taxonomie. De vraag is hoe een antropoloog te werk zou gaan als hij of zij naast elkaar een schedel van een Zweed van 2,10 meter en een pygmee in dezelfde aardlaag zou aantreffen.

V. De bepérking van onderwerpen en middelen in zijn creatieve werk scherpt de geest van de kunstenaar, en leidt tot resultaten. Absolute vrijheid leidt tot niets.

VI. Hoe meer individualisme in de maatschappij, des te meer oranjegevoel tijdens EK en WK.

VII. Door de verregaande communicatie lijkt onze wereld bijna zo groot als de aarde. Toch kennen steeds minder mensen hun buren.

Stellingen behorend bij het proefschrift: 'On the origin of internal browning in pears (*Pyrus communis* L. cv Conference'.

Rob. H. Veltman, 14 mei 2002

1. General Introduction

Packed full of vitamins, minerals, fibres and antioxidants fruits and vegetables are nutrient-dense commodities. Research continues to strengthen the positive association between consumption of fruits and vegetables and decreased risk of heart disease, some cancers, and other health issues. Apples and pears are grown in Europe and more specifically in The Netherlands in large quantities. The total pear production in Europe is estimated 2.4 million tons (2000). In The Netherlands this production is about 180,000 tons. In The Netherlands two cultivars of the world-wide *Pyrus communis* species are grown almost exclusively: Conference (about two third of the total Dutch production) and Doyenné du Comice (about one sixth of the total Dutch production). On a European scale 20% of all grown pears is a Conference pear [1].

Pyrus communis is –like apple (*Malus*) and hawthorn (*Crataegus*)- a member of the *Rosaceae* family. The Greek cultivated pears as early as 800 BC; their cultivation is already documented in the Odyssey. The history of pear cultivation in The Netherlands goes back to the Roman Empire. Likely, the Romans brought the pear here, and in old Rome different pear cultivars were already known. Certain is the introduction of pear-growing by Charles the Great. He ordered the cultivation of pears on his manors around the year 800. Some of these manors were located in The Netherlands, like in Gennep near Nijmegen. In 1052 pear cultivation was mentioned near Maastricht. Also in the province Utrecht and Zeeland fruit growing started. Furthermore, pears were traditionally grown near monasteries and castles. From the

15th century on fruit growing developed very rapidly in Holland. Wertheim [2] speaks of a so-called Golden Age of the pear, which lasted from about 1750 to 1875. Improvement of pear cultivars was fashionable at that time, and the known fruit-expert Knoop mentioned about 150 cultivars. In 1874 even 1100 cultivars were described in a Belgian book. In the same time pear growing has mainly moved to just over the Dutch borders, to present-day Belgium and Northern France [2].

1.1 The history of fruit CA storage [1]

Table 1.1. Benefits of controlled atmosphere storage (CA).

- A higher quality after storage
- Possibility of product-accumulation before marketing
- A better price
- Avoidance of specific disorders like scald
- Extension of the availability of a given commodity

The idea to preserve fruits and vegetables is not new, and the favourable effect of certain gases during storage on crops was discovered long ago. Early storage of crops in pits with restricted ventilation in the United Kingdom, for instance, sometimes improved storage life. Early scientific studies (Bernard, France, 1819) showed that fruits absorb O_2 and release CO_2. The same study revealed that fruits did not ripen under anoxia, and that ripening was resumed when fruits were re-subjected to normoxia. This kind of experiments showed that anoxic storage resulted in a prolonged storage-life of 4 weeks for peaches, apricots and prunes, and a life of 3 months to apples and pears. In 1856 B. Nice built one of the first commercial storage facilities. In this facility, with airtight doors, the temperature was kept below 1°C with ice. He claimed that apples could be preserved for 11 months, and that the

[1] This paragraph is among others based on 'Postharvest technology of fruits and vegetables' by A.K. Thompson (chapter 5). Blackwell Science Ltd, 1996. ISBN 0-632-04037-8

O_2 concentration during storage was so low that a flame would not burn. In 1907 Fulton observed that fruits could be damaged when large amounts of CO_2 were introduced in the store. R.W. Thatcher experimented with apples in sealed boxes, and concluded that CO_2 inhibits ripening, and Kidd [3] described the effect of different levels of this gas on fruit respiration. In 1927 Kidd and West carried out the first comprehensive and well-documented studies on the effect of CO_2 and O_2 on fruits and vegetables during storage, and called the new storage technique 'gas storage'. In the 1940s this term was changed in Controlled Atmosphere (CA) storage.

Studies on CA application have shown possibilities for a wide range of fruits and vegetables, however, CA is still mainly applied for apples and pears. Basically, the original concept of CA storage from the beginning of the 20th century hasn't changed much. CA storage started with controlling CO_2 levels. The storage facility was refrigerated mechanically, and CO_2 was fixed at a level that was cultivar dependent. By means of ventilation the excess of CO_2 was removed from the facility, creating an equilibrium between the condition in the storage facility and the outside atmosphere [4]. In such a system the amount of nitrogen was kept constant at 79 kPa, the same as in normal air. When CO_2, for instance, was fixed at 3 kPa, O_2 was 18 kPa constantly. In the present storage facilities both O_2 and CO_2 levels are controlled. Moreover, CO_2-scrubbers are introduced, first lime-scrubbers, and nowadays charcoal scrubbers. Cell air is directed through these scrubbers, and next the cleaned air is pumped back into the facility. During 'passive scrubbing' the CO_2-absorbing material is placed in the storage facility (e.g. limestone), a technique that is still used in container transports of fruits and vegetables. When respiration of the product is used to reach the optimal CA O_2 concentration, one speaks of 'product generated CA'. This technique is also used in modern packages nowadays, and is then called Modified Atmosphere Packaging (MAP). In an MA package optimal gas conditions are achieved by choosing such a foil that O_2 and CO_2 asymptotically stabilise at the desired gas concentrations after a predictable time and at a certain temperature.

1.2 Fruit ripening

Ripening is a complex, natural process, which improves the organoleptic characteristics of fruits. The main goal of lowering the storage temperature and applying CA is to slow down ripening and senescence, and to extend storage duration while maintaining all quality attributes of a certain commodity, also after ripening on the shelf. Kidd and West [5] discovered that a burst of respiratory activity accompanies the onset of ripening in apples – a phenomenon they named the climacteric. In apples, as in many other fruits, this respiration climax is accompanied by a spate of autocatalytic ethylene production [6]. Ethylene is a volatile plant hormone. It was the Russian physiologist Dimitry N. Neljubow who first established that ethylene affects the growth of plants [7]. In climacteric fruits low levels of ethylene (sometimes as low as <0.1 ppm) can already induce ripening. In response to ethylene, fruits change the pre-climacteric stage for the climacteric, respiration rises, and many processes connected to fruit ripening, including softening, colour changes and flavour development are started. Non-climacteric fruits, however, produce little ethylene and ripening in these fruits is not induced by ethylene. A characteristic of climacteric fruits is the autocatalytic ethylene-production. This autocatalysis is triggered by an ethylene peak (system I), which is followed by a massive increase in the ethylene production (system II) [8]. Non-climacteric fruits lack system II, and thus the induction of autocatalytic ethylene production [9]. Some examples of climacteric and non-climacteric fruits are given in Table 1.2.

Ethylene is synthesised from the amino acid methionine with 1-amino-cyclopropane-1-carboxylic acid (ACC) as an intermediate [9]. Its synthesis requires O_2, and it is not formed under anoxia. Addition of CO_2 to the storage facility not only inhibits respiration, but also ethylene production and thus ripening. CO_2 is also known to decrease the sensitivity of fruits towards ethylene. Burg and Burg [10] postulated that CO_2 competes with ethylene for ethylene binding sites. In pear, ethylene production was inhibited by 5 to 20 kPa CO_2 [11]. However, De Wild *et al.* [12] showed that in 1-MCP-treated pears (MCP is an effective blocker of ethylene effects, probably by binding to the ethylene receptor) CO_2 had an additive inhibitory effect on

the ethylene production. This suggests that inhibition of the ethylene production by CO_2 does not (solely) take place at the receptor level.

Table 1.2. Examples of climacteric- and non-climacteric fruits.

Climacteric	*Non-climacteric*
Apple	Orange
Pear	Strawberry
Banana	Lemon
Avocado	Cherry
Peach	Pineapple
Kiwifruit	Grape
Tomato	Cucumber
Mango	Berries

Low O_2 and high CO_2 concentrations both have an inhibitory effect on ripening and ethylene production. In cultivars with a high ethylene production rate the main advantage of CA may be the inhibitory effect on the action of ethylene. In cultivars with a low ethylene production CA may act as an inhibitor of both ethylene-production and ethylene-action [13].

1.3 Current storage conditions and physical disorders

Nowadays about 80% of the apples and pears in The Netherlands is stored under CA conditions. In the first place the respiration rate of fruits is decreased by a lowered storage temperature. Ke and Kader [14] showed that fruits like pears, stone fruits, blue berry and strawberry stored at temperatures ranging from 0 to 5°C up to 10 days, tolerate O_2 levels between 0.25 and 1 kPa (Ultra Low Oxygen, ULO), while apples tolerate these levels for much longer periods. Under CA, pears are normally subjected to circa 2-3 kPa O_2. Apples are usually stored at 1-2 kPa O_2 often combined with CO_2 levels up to 4 kPa. Respiration of fruits is further decreased when crops are stored at lower O_2 concentrations with the addition of CO_2 [15].

A side effect of low O_2 is the accumulation of ethanol and acetaldehyde to concentrations that can result in off flavours [16-19]. Ethanol is an end-product of an

alternative, anaerobic way of sugar degradation (Fig. 1.1), and acetaldehyde is an intermediate of this pathway. This metabolic route is called the fermentative pathway. Fermentation takes place in the cytosol, and does not make use of the mitochondrial respiratory chain. The onset of fermentation can be used as a functional marker to indicate the minimal amount of O_2 below which anoxic sugar breakdown starts. Practical trials have shown that apples can be stored at extremely low O_2 concentrations. In the so-called Dynamic Control System (DCS), a kind of interactive ULO storage, low O_2 levels in the storage facility are regulated on the basis of ethanol levels (*product response* control) [20, 21].

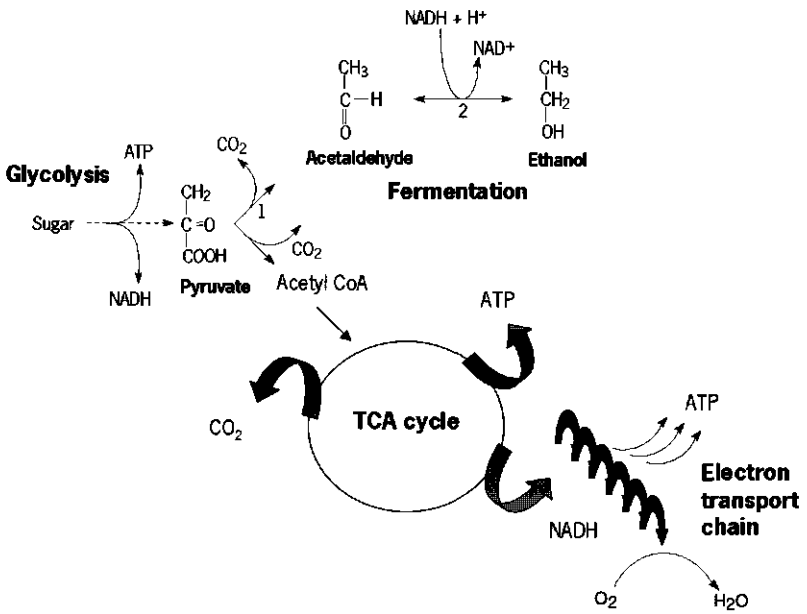


Figure 1.1 Simplified representation of the fermentative pathway (ending in ethanol) and aerobic respiration, consisting of the TCA cycle and an electron transport chain located in the mitochondrion. Enzymes involved in fermentation: 1. Pyruvate decarboxylase (PDC), 2. Alcohol dehydrogenase (ADH)

Regular CA practice may be described as a means to suppress respiration to the lowest possible level, without inducing excessive fermentation. This concept is called the Extinction Point approach (EP). This approach is defined by the lowest O_2 concentration where no fermentation takes place. However, it seems that fermentation can even take place in some fruits under normoxia [22]. A decreasing O_2

gradient can exists between the storage atmosphere and the cytosol or mitochondria of the cells in the fruit flesh, the peel being one of the diffusion resistances [23]. Gas penetration through cells and the cuticle of the fruit affects this gradient. In establishing correct storage conditions the biological variation of fruits with respect to O_2 tolerance plays an important role as well.

Figure 1.2. Typical example of brown core in Conference pears. Brown core is a result of enhanced CO_2 levels during storage. Sensitivity can increase when O_2 levels are lowered (below 3 kPa), or when pears are harvested late.

Because ethanol synthesis can probably never be excluded completely, the Anaerobic Compensation Point, ACP, defined as the O_2 concentration where CO_2 production is lowest, is probably a better concept.

Besides lowering O_2 concentrations the storage life of pome fruit varieties can be extended further by addition of CO_2 to the storage room. However, CO_2 concentrations are often restricted because they can induce physical or physiological disorders like brown core (also called brown heart) in apples and pears [24]. Brown core can be described as a CA disorder. It is an abnormal condition of the fruit resulting from the death and subsequent browning of parts of the internal fleshy tissue, while the peripheral flesh generally remains sound. Brown patches, which are mostly sharply delimited from the living tissue, often appear to originate in vascular

bundle tissue, especially that of the ten main vascular bundles. If a pear is removed from the conditions that gave rise to brown core, the brown areas do not increase in size, but the affected tissue gradually dries out, and in this way cavities are formed lined with leathery tissue [25]. For Conference pears it has been suggested that the incidence of internal browning tends to increase with increased water losses [24].

High CO_2 concentrations are known to suppress respiration. It is suggested that CO_2 acts as a controller of the TCA-cycle by suppressing the oxidation of succinate and citrate, which explains the accumulation of these acids [26]. The succinoxidase system of apple mitochondria is extremely sensitive to CO_2. The widespread effects of CO_2 on the mitochondrial metabolism could also be attributed to pH changes within the mitochondria. Shifts in internal pH due to the acidification of the cytosol and other cellular compartments by bi-carbonate affect enzyme activities and oxidation rates. Prolonged storage under high CO_2 concentrations reduces the rate of oxidative phosphorylation, although CO_2 does not act as a strong 'uncoupler'; the reduced rate may be the consequence of e.g. a declined NADH-production within the cell.

1.4 Polyphenol oxidase

Fruits and vegetables sometimes suffer from internal and/or external tissue discolorations (browning) during storage and processing, like for instance brown core disorder in Conference pears as defined above. Most of these discolorations are owed to enzymatic browning (instead of non-enzymatic browning like for instance the Maillard reaction). Enzymatic browning can be described as the oxidation of polyphenols to corresponding coloured quinones. It is mainly associated with the enzyme class of the PPO's. The classification of PPO's is complex. Catecholase (EC 1.10.3.1) catalyses the oxidation of monophenols in *o*-diphenols, which is also known as the creolase activity of the enzyme (Fig. 1.3, reaction 1). The catecholase activity of the same enzyme (reaction 2) is responsible for the conversion of the latter into *o*-quinones [27]. A second class of PPO's, the laccases (EC 1.10.3.2), oxidise *o*-diphenols and *p*-diphenols to their corresponding quinones (Fig. 1.3, reactions 2 and 3). But, laccase does not possess a creolase activity. Besides EC 1.10.3.1 and EC

1.10.3.2 a third category PPO's exists, the tyrosinases (EC 1.14.18.1, also called monophenol monooxygenase), which correspond to the same enzymes as the catecholases.

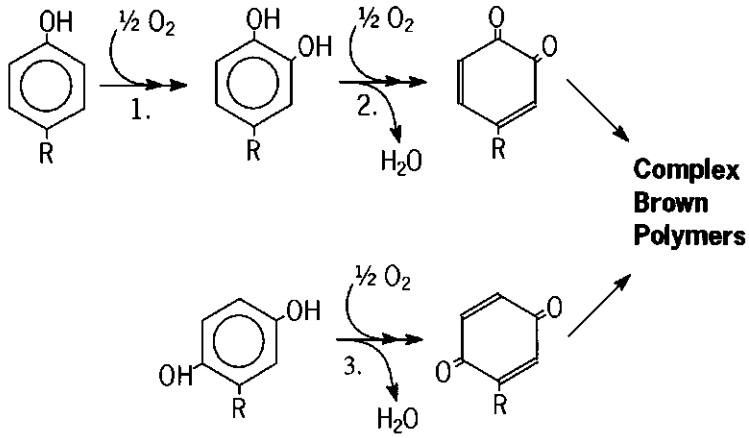
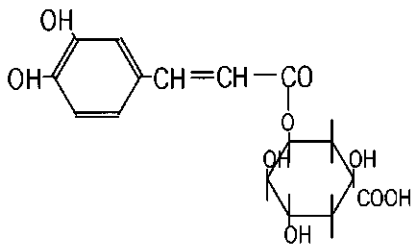
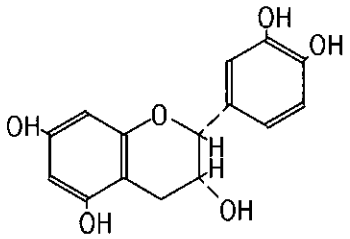


Figure 1.3. Reactions catalysed by PPO. Monophenols are hydroxylated to o-diphenols by PPO creolase activity (1). Dehydrogenation converts o-diphenols into o-quinones by the catecholase activity (2). Laccase possesses catecholase activity (2 and 3), but no creolase activity.

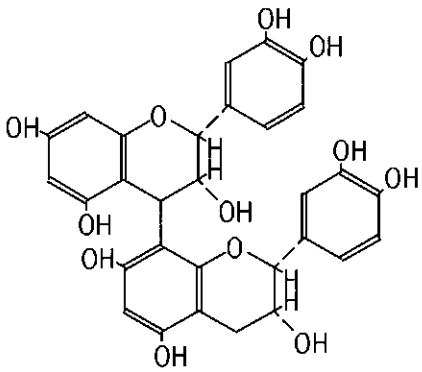
In the cortex tissue of apples [27, 28] and pears [29] three phenolics account for more than 90% of the total phenolic content: caffeoyl quinic acid (or chlorogenic acid, most abundant), (-)-epicatechin and procyanidine B2.



Chlorogenic acid



(-)-Epicatechin



Procyanidine B2

Figure 1.4. The three most abundant phenolics in apple tissue: chlorogenic acid [30]*, (-)-epicatechin* [30]*, and procyanidine B2* [31]

Although considerable efforts were done to determine the physiological function of PPO in plant cells, its role remains obscure. PPO has been found in chloroplasts or in the other plastids in both photosynthetic and non-photosynthetic tissues. In many extracts of plant tissues PPO's are found in a latent form, and proteolysis, detergents or other factors may be required for their activation. Laccase, however, is not known to exist in a latent state. PPO's from higher plants are able to oxidise a broad range of mono-, *o*-di- and *p*-diphenols. Km values for O_2 vary between 0.1 and 0.5 m*M*. The Km and Vmax values for different phenolics, are highly variable [27].

Tyrosinase normally possesses two conserved regions, CuA and CuB [32]. These active sites of the enzyme contain three histidine amino acids, which bind copper. A pair of copper ions in tyrosinase interacts with O_2 and the polyphenol substrates [30]. A

comparable mechanism is supposed to be active in laccases. At the expense of AA, and the formation of DHA, oxidation products of PPO can be regenerated. This prevents the formation of brownish pigments and discolorations. PPO is also known to oxidise AA directly, comparable to the enzyme ascorbate peroxidase (APX, EC 1.10.3.3) [33].

Although the *o*-quinones formed from phenolic substrates are themselves coloured from red to reddish-brown, the reaction rarely stops there [27], and secondary reactions lead to more intensively coloured products. These reactions include polymerisation of polyphenols after the initial quinone formation and complexing with amino acids and proteins.

1.5 Ascorbic acid metabolism

Ponting and Joslyn [34] already stated in 1948 that AA oxidation is intimately related to tissue browning, and that browning does not occur until all AA was oxidised. L-ascorbic acid (vitamin C, at that time called hexuronic acid) was discovered 20 years earlier by Albert Szent-Györgyi in 1928 [35]. It is an important vitamin in the human diet and is abundant in plant tissues. Primates and guinea pigs have lost the ability to synthesise AA, and they must acquire it by consuming plant material. In the absence of AA, collagen is insufficiently hydroxylated and hence cannot properly form collagen fibres, which can eventually lead to scurvy in humans. Because of its nutritional importance, the distribution of AA has been studied extensively in plants, but, relatively little consideration has been given to its function in the plant. AA has been shown to have an essential role in several physiological processes in plants, including in growth, differentiation and metabolism [36]. It functions as a reductant for several Reactive oxygen Species (ROS), thereby minimising the damage caused by oxidative stress; however, AA has also more specific functions. In ripening fruits it is probably involved in the conversion of ACC to ethylene [37], and in photosynthesis, as it removes hydrogen peroxide formed during photoreduction in photosystem I (PS-I) in the chloroplast [38]. Apparently, synthesis of AA occurs in the cytosol because a specific AA translocator has been identified in the chloroplast envelope. AA is synthesised from hexose sugars in higher plants. D-mannose and L-galactose are

efficient precursors for AA synthesis, and they are interconverted by an enzyme called GDP-D-mannose-3,5-epimerase. Wheeler *et al.* [39] identified an enzyme in pea and *Arabidopsis thaliana*, L-Galactose dehydrogenase, that catalyses the oxidation of L-galactose to L-Galactono-1,4-lactone, the direct precursor of AA (Fig. 1.5).

AA can directly scavenge oxygen free radicals with and without enzyme catalysts and can indirectly scavenge them by recycling α-tocopherol, bringing it back to the reduced form. By reacting with activated O_2 more readily than any other water-soluble component, AA protects vital macromolecules from oxidative damage. The reaction with the hydroxyl radical, for instance, is limited only by diffusion. The reaction with superoxide (O_2^-, Eq 1.1.1) may serve a physiologically similar role as superoxide dismutase (SOD, Eq 1.1.2). The superoxide radical was discovered in 1931 by Linus Pauling. He suggested that this radical is produced in minute quantities as an unwanted by-product of oxidative phosphorylation, and that it could have an enormous destructive capacity if not removed.

$$2\,O_2^- + 2H^+ + AA \longrightarrow 2\,H_2O_2 + DHA \qquad 1.1.1$$

$$2\,O_2^- + 2\,H^+ \xrightarrow{SOD} 2\,H_2O_2 + O_2 \qquad 1.1.2$$

The reaction with hydrogen peroxide is catalysed by APX [40]:

$$H_2O_2 + 2\,AA \longrightarrow 2\,H_2O + O_2 \qquad 1.2$$

AA regenerates the membrane-bound antioxidant α-tocopherol, which scavenges peroxyl radicals and singlet O_2:

$$LOO^{\bullet} + \alpha\text{- Tocopherol} \longrightarrow LOOH + \text{Tocopheryl radical} \qquad 1.3.1$$

$$\text{Tocopheryl radical} + AA \longrightarrow \alpha\text{- Tocopherol} + MDAA \qquad 1.3.2$$

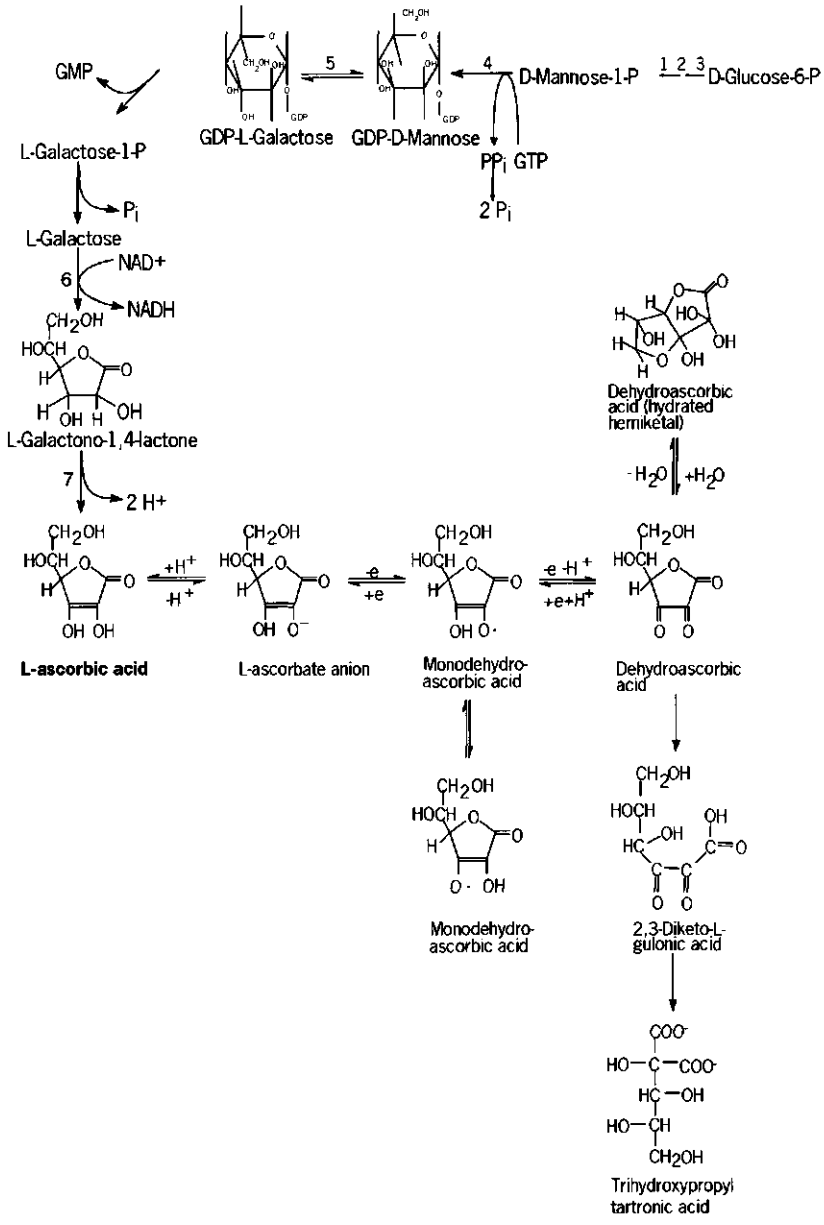


Figure 1.5. AA anabolism as proposed by Wheeler et al. [39] *AA catabolism according to Washko et al.* [41] *Numbers are referring to the following enzymes: 1. Hexose phosphate isomerase, 2. Phosphomannose isomerase, 3. Phosphomannose mutase, 4. GDP-D-Mannose pyrophosphorylase, 5. GDP-D-Mannose-3,5-epimerase, 6. L-Galactose dehydrogenase, 7. L-galactono-1,4-lactone dehydrogenase.*

The above reactions indicate that there are two different products of AA oxidation, MDHA (or semi-dehydro-ascorbate) and DHA that represent one and two electron transfers, respectively (Fig. 1.5). MDHA can either spontaneously dismutate (Eq 1.4) or is reduced to AA by NAD(P)H monodehydro-ascorbate reductase (Eq 1.5, Fig. 1.6):

$$2\ MDHA \longrightarrow AA + DHA \qquad 1.4$$

$$MDHA + NAD(P)H \longrightarrow AA + NAD(P) \qquad 1.5$$

The DHA is unstable at pH-values higher than 6, decomposing into tartrate and oxalate. The reduction of DHA to AA by dehydro-ascorbate reductase using reducing equivalents from glutathione (GSH) prevents this:

$$2\ GSH + DHA \longrightarrow GSSH + AA \qquad 1.6$$

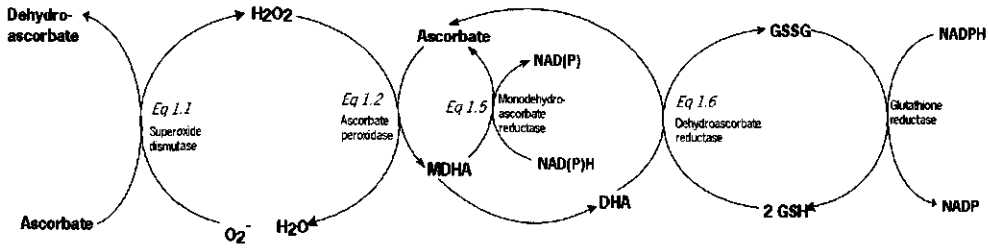


Figure 1.6. Enzymes and antioxidants involved in ROS breakdown. MDHA: mono-dehydro-AA (semi-DHA); GSH: glutathione; GSSH : glutathione, oxidised form.

AA is localised in the chloroplast, cytosol, vacuole and extra-cellular compartments of the cell. About 20-40% of the AA in the mesophyll leaf cell is located in the chloroplast. This organelle contains all the enzymes necessary to regenerate reduced AA from its oxidised products. MDHA has two regeneration routes, one via

MDHA reductase, and the other via DHA reductase consuming GSH (Fig. 1.6). The terminal electron donor of both routes is NAD(P)H. Both regeneration routes are involved in hydrogen peroxide detoxification that might otherwise participate in Fenton reactions (see Chapter 7, Eq 7.8). Although AA metabolism has been studied in most detail in the chloroplast, it is likely that all enzymes for its regeneration also exist in the cytosol of both photosynthetic and non-photosynthetic cells.

1.6 Outline of the thesis

Internal browning in pears is a disorder that appeared with the introduction of CA storage. Kidd and West [21] already described it in 1923 for apples and pears. However, still –after about 80 years- the biochemical and physiological background of internal browning has not been cleared up. The goal of this study was to understand what happens before and during the onset of brown core development in Conference pears, and to unravel the physical, physiological and biochemical mechanism behind it. In connection to this, the metabolism of AA during storage, the availability of energy, and the diffusion characteristics of O_2 and CO_2 in the fruit were investigated.

On an empirical level most of the factors that have an influence on the brown core disease in pears and apples were exhaustively investigated in the period in which CA storage was introduced world-wide. It is now known that the ripening stage (harvest date), the growing location, and the (both yearly and geographical) climate are important factors. For example, when pears are harvested too late, disorders are mostly inevitable, and losses can be massive. Furthermore, disorders are less severe in the South-European countries.

In this thesis the processes that lead to brown core in Conference pears are studied. Chapter 2 focuses on PPO activity and its role in the formation of brown pigments. In Chapter 3 the relation between AA concentrations in pears and the susceptibility towards brown core is depicted. In Chapter 4 PPO was investigated more profoundly. This chapter describes the partial purification of the enzyme, the determination of Vmax and Km values, and the reaction stoichiometry. The relation between AA and brown core development was further elaborated on a semi-practical

scale in Chapter 5. In this chapter AA levels were also investigated in the Rocha cultivar. In Chapter 6 energy metabolism in pears under various conditions is analysed, and an integral 'brown core theory' is formulated, which tries to describe the process of brown core initiation. In Chapter 7 [2] it was attempted to determine ethane emissions from pears. The development of brown core in pears was hypothesised to be partly caused by membrane peroxidation, and ethane is a putative by-product of this reaction. In Chapter 8 a model is presented that describes the relation between external applied O_2 and CO_2 concentrations, internal gas concentrations, diffusion characteristics, and respiration. In Chapter 9 (general discussion) the previous chapters are discussed and conclusions are drawn.

[2] This research was performed at the department of Molecular- and Laser Physics of the University of Nijmegen (KUN) under the supervision of Dr. Frans J.M. Harren and in co-operation with Dr. Stefan T. Persijn.

2. Polyphenol oxidase activity and polyphenol content [3]

Summary

Brown core is a disorder in pears that is frequently observed in the Conference cultivar. The brown colour is the result of an enzymatic oxidation of polyphenol substances to *o*-quinones, a reaction catalysed by PPO. In a later stage pears can form cavities in and nearby the core.

The goal of this research was to understand and predict the development of core browning. Pears from orchards in Spain and The Netherlands were compared to observe climate influences on the development of brown core.

PPO activity and total polyphenol content are not affected by harvest date, storage atmosphere and orchard, and both do not seem to be limiting during development of

[3] This chapter is based on: Veltman RH, Larrigaudière C, Wichers HJ, Van Schaik ACR, Van der Plas LHW, Oosterhaven J. 1999. Polyphenol oxidase activity and polyphenol content are not limiting factors during brown core development in pears (*Pyrus communis* L. cv Conference). The Journal of Plant Physiology 154: 697-702.

brown core. An observed decrease of tyrosinase activity might be the consequence of the decreased viability of brown tissue. It is hypothesised that core browning is induced by decompartmentation of intracellular membrane structures.

2.1 Introduction

The storage life of a number of pome fruit commodities may be prolonged considerably in atmospheres containing enhanced CO_2 and lowered O_2 concentrations (ULO) [14]. However, these controlled atmospheres are, in some cases, known to induce physiological disorders, such as brown core, an enzyme-induced aberration in *Pyrus communis* [24]. Browning is initiated in the core of the fruit and is assumed to expand concentrically through the cortex tissue. However, except for extreme cases, the outer appearance of affected fruits is normal. In these severe cases the affected tissue loses water to the adjacent tissue, and eventually cavities are formed. The susceptibility of pears for brown core depends on geographical factors [42], orchard, storage conditions, cultivar [29], growth year [43] and harvest date.

Browning in the core and the cortex tissue of pears is attributed to the action of polyphenoloxidase (PPO), which catalyses the oxidation of phenolic compounds to highly reactive *o*-quinones [44, 45]. These *o*-quinones undergo further oxidation and polymerisation and cause a brown or black pigmentation during processing and storage. These pigments are referred to as melanins [30, 46]. Tyrosinase (EC 1.14.18.1) is a PPO that exhibits a monooxygenase (or cresolase) activity, which oxidises monophenols to *o*-diphenols and an oxido-reductase (or catecholase) activity, which oxidises *o*-diphenols to quinones [32]. Laccases (EC 1.10.3.1) represent a second group of PPO's, which only exhibit *o*- and *p*-catecholase activity. Tyrosinase, in contrast to laccase, exhibits a latent and an active form. Enzymatic browning as a consequence of PPO action is described for various fruits and vegetables, like pear [47-49], apple [27, 50], spinach [51], cabbage [52], and mushroom [53].

There is a general agreement that, in higher plants, PPO is a plastidal enzyme [54], however, in apples PPO was found both in chloroplasts and mitochondria [27, 55]. Data on phenolics and PPO in pears are scanty. d'Anjou pears contain three PPO's which seem to exist in a latent form [48]. In apples phenolics are localised in the vacuole [56].

Attempts to correlate browning directly to enzyme (PPO) activity and phenolic content failed; studies on different apple cultivars turned out to be contradictory [27]. The susceptibility for browning may depend on PPO-activity, phenolic content or both [57, 58].

This chapter describes an investigation on the putative relations between susceptibility for browning in pears, PPO activity and total phenolic content.

2.2 Materials and methods

2.2.1 Plant materials

In The Netherlands, pears (*Pyrus communis* L. cv. Conference) were harvested at two locations: Strijen Sas (orchard 1) and Zuid Beyerland (orchard 2), both in the western part of the country (province South Holland). Pears from Strijen Sas are known to be relatively more sensitive to the development of brown core. Pears from both locations were picked at three dates. The optimal (second) harvest date for CA storage was 15 September, 1995. The other pears were harvested 6 September, (harvest date 1) and 26 September (harvest date 3).

Pears in Spain were obtained from an orchard in Lleida during summer 1996 (orchard 3). Pears were harvested one week after the commercial harvest date, 22 August, 1996, to ensure that they will show disorders.

2.2.2 Storage and treatment

In The Netherlands pears were stored in crates, placed in 650 litre containers (static system) with a water sealing, at -1°C air temperature and 2 kPa ± 0.1 O_2. Half of the batch was stored at <0.7 kPa ± 0.1 CO_2 (standard), the other half at 3 kPa ± 0.1 CO_2 (stress condition to induce browning). Relative humidity was kept at 97-99%. After 4 months storage, samples were taken for PPO and total phenolic measurements. For measurements on tyrosinase, pears (The Netherlands) were stored in a flow-through system [59]. After 25 days of storage at 2 kPa O_2 in combination with 0.2 kPa or 10 kPa CO_2, samples were taken. Pears in Spain were

placed in experimental chambers during 4 months at -1°C, 2 kPa ± 0.1 O_2 and 3 kPa ± 0.1 CO_2. Relative humidity was kept at 95%.

2.2.3 Image analysis system

Thin slices (about 0.4 cm) were cut from the pear longitudinally with a double-knife cutter. From this profile the total area and the percentile brown area was measured immediately after cutting using Image Analysis (IA). The method of Schouten *et al.* [60] was used with some software adaptations.

During visual judgement brown pears were classified in 4 classes: no browning (0), slight browning (I, <30% of the area), moderate browning (II, about 30-70% of the area) and severe browning (III, >70% of the area, with only the cortex fraction just underneath the peel not showing browning).

2.2.4 Polyphenol oxidase assay using L-DOPA as a substrate

Freeze-dried samples were ground in a mortar under liquid nitrogen and stored at -20°C. Pulverised samples were mixed with 100 m*M* NaPi buffer, pH 6.5, centrifuged (10 min, 10000 rpm), and mixed with O_2 saturated buffer with L-DOPA (final concentration 16.7 m*M*). For determination the of the tyrosinase activity the method of Wichers *et al.* [61] was used. To activate latent tyrosinase, and calculate the total tyrosinase activity in a sample, 0.11% v/v SDS was added to the substrate buffer [53]. For measurement of laccase 0.5 m*M* tropolone, a specific inhibitor for tyrosinase, was added [62].

Analysis of variance (Genstat) was used to determine correlations between parameters.

2.2.5 Polyphenol oxidase assay using 4-methyl catechol as a substrate

PPO was extracted from pear tissue by the acetone extraction method of Sciancalepore and Longone [63]. The acetone powder was stored in a desiccator until extraction. The extract was prepared by suspending 1 gram of acetone powder in 30 ml phosphate buffer (0.1 *M*, pH 7.0) with 0.5 m*M* cysteine. The extract was

homogenised, filtered through two layers of miracloth and centrifuged at 40,000 g for 20 minutes (4°C). The supernatant was loaded onto a Sephadex G-25 column (PD 10, Pharmacia), which was previously equilibrated with 20 ml phosphate buffer (pH 6.5). PPO was eluted with 3.5 ml of the same buffer. Activity was determined spectrophotometrically according to Sciancalepore and Longone [63]. The reaction mixture consisted of 1.9 ml citrate buffer (0.1 *M*, pH 5.3), 1.0 ml 4-methylcatechol (final concentration 0.02 *M*) in citrate buffer, and 0.1 ml of enzyme extract. The reaction was monitored for 3 min at 420 nm (30°C). The initial velocity was calculated from the slope of the linear part of the curve obtained.

2.2.6 Total phenolic content

The method is based on the reaction between the Folin-Ciocalteus reagent and substituted phenyl residues [64]. Freeze-dried tissue was homogenised in water. Cell remainders were removed by centrifugation (15-30 min, 11,000g). The homogenate (0.5 ml) was mixed with 0.5 ml 10% TCA to remove proteins. After vortexing and centrifugation (15-30 min, 11,000g) 0.75 ml of the sample was transferred to a 3 ml cuvette and mixed with 1.5 ml 1.4 *M* Na_2CO_3 and 0.45 ml Folin-Ciocalteus reagent (Merck 9001). After 30 min the samples were centrifuged again and the extinction was measured in triplicate at 650 nm. Calibration was done with 0.0-1.0 m*M* tyrosine.

2.3 Results.

2.3.1 Occurrence of brown core

Pears from orchard 1, stored in the static system at both <0.7 and 3.0 kPa CO_2, were more susceptible to brown core than pears from orchard 2. Pears from later harvest dates were more susceptible than pears from early harvest dates, and 3 kPa CO_2 clearly induced brown core during storage. These trends, shown in Fig. 2.1 and

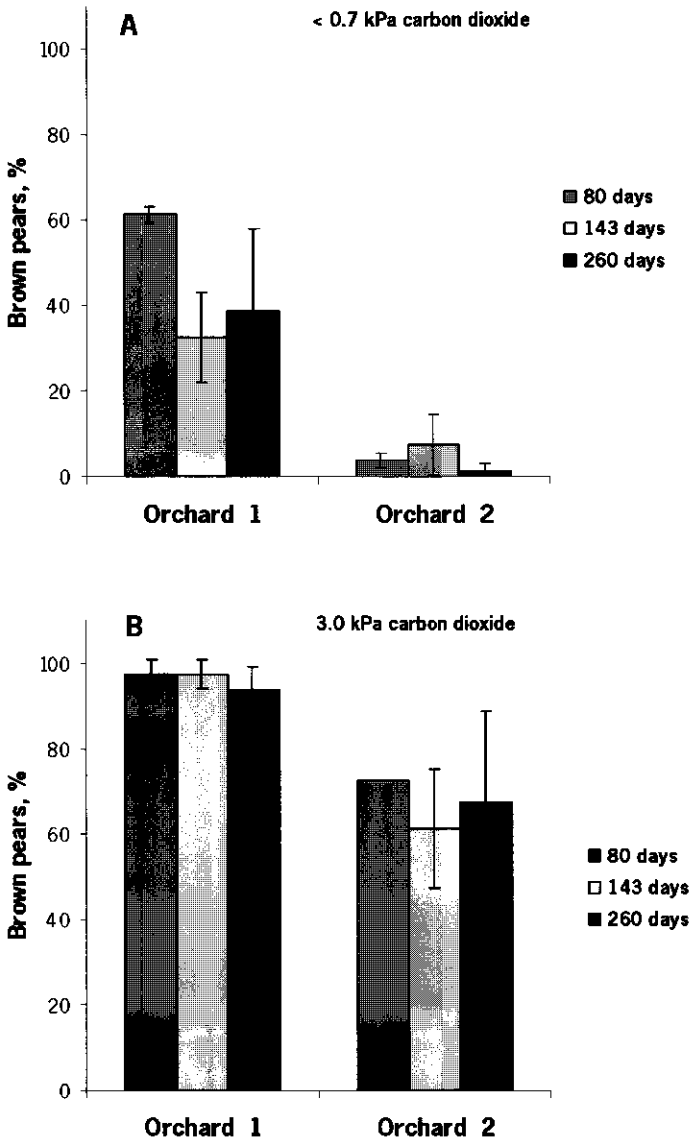


Figure 2.1. Influence of orchard and CO_2 on browning in Conference pears. Fruits were monitored and judged visually after 80, 143 and 260 days of storage respectively. Pears were grown in two orchards in The Netherlands and stored at 0.5 kPa (A) and 3.0 kPa (B) CO_2. All fruits were from the third harvest date (pick 3). Bars are the mean of four judgements of 20 pears (n=4) ± SE.

Fig. 2.2, were observed for several years. The initiation of brown core was also monitored in pears stored at enhanced CO_2 atmospheres (3 kPa) and a higher temperature (5°C) in the flow-through system. Brown core was already detected after five weeks in pears stored at these conditions (data not shown).

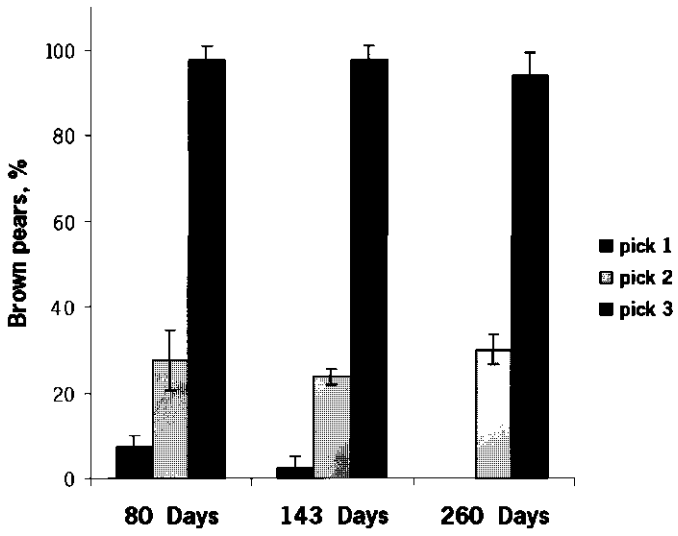


Figure 2.2. Influence of harvest date (pick 1, 2 and 3) on the development of brown core in Conference pears. Fruits were monitored and judged visually after 80, 143 and 260 days respectively. All pears were grown at orchard 1 in The Netherlands, and stored at 3 kPa CO_2. Bars are the mean of four judgements of 20 pears (n=4) ± SE.

2.3.2 PPO, total phenolic content and development of brown core

Brown core develops by the action of PPO [24]. PPO catalyses the formation of quinones, which polymerise into melanin products. In The Netherlands PPO activities were determined in pears from two growing locations (orchard 1 and 2) and three harvest dates (pick 1, 2 and 3) stored under <0.7 and 3 kPa CO_2. After 4 months storage, tyrosinase (latent and active form) and laccase activities were determined. The same samples were used to determine the total phenolic content. PPO activity was not, as expected, depending on gas conditions during storage, but a correlation with browning of the cortex tissue of pears *was* found. Generally, taken together the

results of all measurements (different conditions, harvest dates and growing locations), the percentage of active tyrosinase was 4.2% on average, with a high SD of 4.2 (n=85). Tyrosinase activity appeared to be inversely related to the appearance of brown core, which is shown in Fig. 2.3 (Panel A and B). Pears from the third harvest date of orchard 1 showed the severest incidences of browning (Fig. 2.3, panel A), while these pears exhibited the lowest tyrosinase activity (Fig. 2.3, panel B). A correlation between laccase activity and browning was not clear. Laccase activities were very low in pear tissue. No relation was found between total polyphenol content, growing location, harvest date and extent of browning, although shifts in concentrations of individual phenolics could not be excluded on basis of these results (results not shown). According to the results, total phenolic content was also not directly related to susceptibility for browning or the degree of browning. PPO activity or total phenolic content were also not related to the size of pears (results not shown). Although the active form of tyrosinase did not seem to be affected by gas conditions, total tyrosinase (after activation with SDS) did slightly, but significantly (Fig. 2.4). Pears stored at 10 kPa CO_2 in a flow-through system exhibited a lower total tyrosinase activity (pears were not brown at the moment activities were determined), and later during storage pears showed brown core.

In parallel, PPO activity was determined in Spanish pears (orchard 3), with 4-methyl-catechol as a substrate. Just as in Dutch pears (PPO measurements with L-DOPA as substrate, Fig. 2.3, Panel B) PPO activity in Spanish pears was also lower in fruits with brown core (Fig. 2.5).

The regions around cavities showed a red colour in *in situ* treatments of pear slices with L-DOPA, which can be attributed to the reaction product dopachrome (Fig. 2.6). Higher activities of laccase and tyrosinase in this region were not found (data not shown)

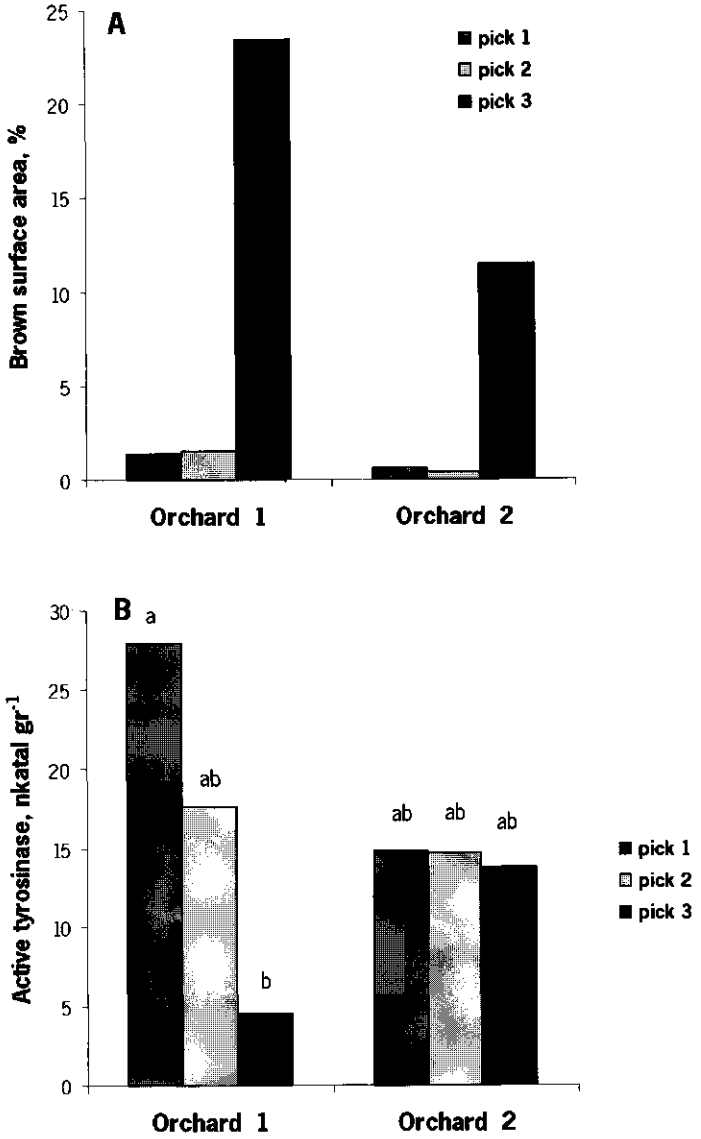


Figure 2.3. Pears were stored at 2 kPa O_2 and <0.7 or 3 kPa CO_2. Samples of cortex tissue were taken after 4 months. Panel A shows the presence of brown core, measured using the IA system, which calculates the percentile brown tissue area of the length-wise cut pear, in pears from two orchards and three harvest dates. Panel B describes the relation between active tyrosinase (nkatal g^{-1} DW), orchards and harvest date (pick 1, 2 and 3). Different letters describe significant differing values.

2.3.3 Polyphenol oxidase and peroxidase control

Peroxidase can disturb PPO assay by abstracting hydrogen atoms from phenolic compounds. The highest peroxidase activities were found in the core of the fruit (0.95 *p*kat, n=5). This value was about a factor 1000 lower than the laccase activity in the same sample (0.89 *n*kat). Because activities are relatively low, peroxidase is not likely to interfere with the PPO assay. No inhibition reactions were observed when higher extract quantities were used in the L-DOPA assay. The relation between the enzyme activity and extract concentration was linear.

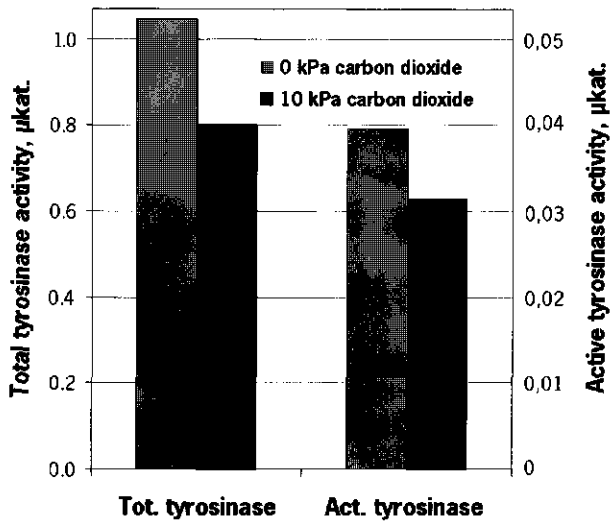


Figure 2.4. Tyrosinase activity after SDS treatment ('Tot. tyrosinase') and active tyrosinase (no SDS treatment, 'Act. Tyrosinase') in Conference pears, stored in a flow-through system. Pears did not show tissue browning. Activities are expressed in µkatal g^{-1} DW. Total tyrosinase in pears stored under the two conditions differed significantly, active tyrosinase did not ($p \leq 0.05$).

2.4 Discussion

Browning in pears is a result of PPO action. One of the main initiators of brown core, independent of important preharvest factors like picking date and orchard, is CO_2. CO_2 concentrations within the fruit are higher than in the storage environment [65],

because the porosity of pear tissue is relatively low, and diffusion resistance of the fruit hinders gas exchange [65] (Chapter 8). Enhanced CO_2 concentrations are known to potentially influence enzyme activities, but results in this article indicate that active tyrosinase was not affected. Total tyrosinase, on the contrary, was affected slightly, but significantly. The formation of brown pigments (melanins) in the core tissue of pears negatively affected tyrosinase activity in pears from The Netherlands and Spain (Fig. 2.3, Panel B; Fig. 2.5). Decreased activities can be a result of decreased cell viability, enzyme degradation, and senescence of the fruit. PPO may also be auto-inhibited by formed quinone products. It is generally accepted that PPO acts as a suicide enzyme, by inactivating or modifying of the enzyme protein through covalent, hydrogen, hydrophobic and ionic bounds [66]. No relation between harvest date, growing location, size of the fruit, storage condition and PPO activity could be observed when pears were not brown.

Apparently, PPO activities and the total phenolic content are crucial but not limiting factors in the process of brown core development. A low PPO activity is sufficient to account for browning. Polyphenols were present abundantly, but their availability might limit the browning reaction [56]. Possibly, shifts in concentrations of individual polyphenols are important [29].

There is a general agreement that, in higher plants, PPO is a plastidal enzyme. In non-senescent tissues it is mainly located in the thylakoid membrane of chloroplasts and in vesicles or other bodies in non-green plastid types [55, 67]. In pears PPO was 100% non-membrane-bound (Chapter 4), but it was not clear if PPO was located in the matrix of plastids or in the cytosolic fraction of the cell (see also Nicolas *et al.* [27]). According to Yamaki [56] 97% of the total phenolic content is localised in apple vacuoles, only three percent is located in the free space, and none in the cytoplasm. Apparently, PPO and phenolic substances are separated within the cell, even if PPO is found in the cytosolic fraction, and therefore browning can not be initiated.

Frenkel and Patterson (1974) showed that pears kept under CO_2 show ultra-structural alterations in various organelles, including mitochondria, vacuoles, plastids, and other membranous systems such as in the tonoplast and the cytoplasm [68]. They observed that the shape of the mitochondrion changes from elliptical to spherical. In

the plastids there was a replacement of the concentric lamellar system with spherical membrane inclusions. The tonoplast showed proliferation in daughter compartments, and the cytoplasmic matrix became extensively vacuolated.

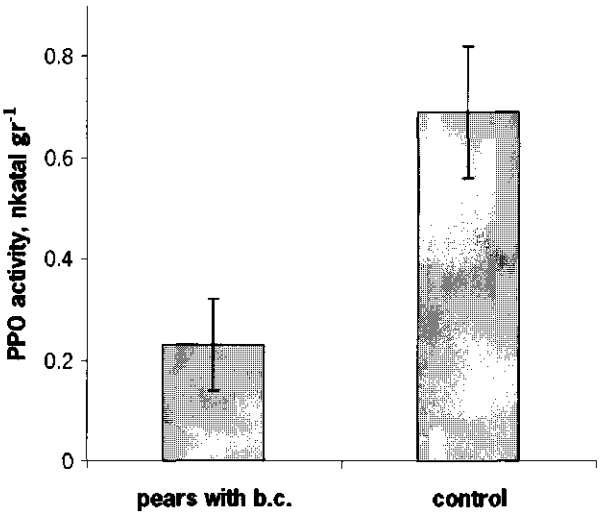


Figure 2.5. Relationship between brown core and PPO activity (n=6). 4-Methyl catechol was used as substrate. Pears were grown in Spain (orchard 3). 'b.c': fruits with brown core (presence of cavities); 'control': non-damaged fruits. Enzyme activities are expressed as nkatal g^{-1} *DW.*

Based on these and own observations we hypothesise that brown core is a consequence of decompartmentation of cellular compartments, caused by membrane disintegration. During senescence internal membrane damage results in leakage of phenolic compounds from the vacuole.

Borders between brown and healthy tissue are rather distinct, but no enhanced tyrosinase activity was observed at or in the neighbourhood of this border (data not shown), which indicates that initiation of browning is not caused by a local tyrosinase activation. Near cavities, a red-coloured band was shown in *in situ* experiments (Fig. 2.6).

Figure 2.6. In situ *staining of a longitudinally cut slice of pear tissue with L-DOPA solution (16.7 mM). A red band of dopachrome, the reaction product of L-DOPA, surrounds cavities.*

This band was attributed to the red oxidation product of L-DOPA, dopachrome. Again, no enhanced tyrosinase or laccase activity could be determined in tissue surrounding cavities (data not shown). This phenomenon is in agreement with the idea that there is a difference in PPO (or substrate) availability near tissue affected by brown core and cavities. Disintegration of internal membranes initiates the PPO reaction. This internal membrane damage might be induced by peroxidation of membrane lipids due to radicals. Another factor that could be involved in the disintegration of membranes is the level of maintenance energy in the cell [69]. Trippi *et al.* [70] showed a close relation between ATP concentrations and membrane permeability, which suggests a relation between 'energy status' and the start of cell disfunctioning [69]. Energy is also needed to regenerate antioxidants necessary to inactivate radicals, but is depleted by the action of CO_2 on the mitochondria [71].

Initiation of brown core can not be explained by PPO activity or phenolic concentrations in pear tissue. Probably, the availability of PPO, PPO substrates and the disappearance of the enzyme-substrate separation within the cell are the cause of brown core initiation.

Acknowledgements

The authors like to thank Jeroen Van Leeuwen for technical assistance and Huug de Vries, Herman Peppelenbos and Rob Schouten for stimulating discussions.

3. Decreased ascorbic acid levels and brown core development in pears [4]

Summary

Changes in AA content are measured in the cortex tissue of Conference pears stored at various compositions of CO_2 and O_2. Enhanced CO_2 levels cause AA concentrations to decline. Soon after AA declines below a certain value browning of the core tissue can be observed. Reducing CO_2 levels before this value is reached, causes AA levels to increase again, and prevents browning to a great extent. In preliminary experiments with a photoacoustic laser-based detection system it was shown that brown pears produce ethane, which is most likely a result of membrane peroxidation. Storage conditions, AA levels and browning in pears are discussed in relation to diffusion characteristics, energy metabolism, and energy maintenance levels of the fruit.

[4] This chapter is based on: Veltman RH, Sanders MG, Persijn ST, Peppelenbos HW, Oosterhaven J. 1999. Decreased ascorbic acid levels and brown core development in pears (*Pyrus communis* L. cv. 'Conference'). Physiologia Plantarum 107: 39-45.

3.1 Introduction

A fundamental step in browning of fruits and vegetables is the enzymatic oxidation of polyphenol compounds to *o*-quinones by PPO (EC 1.14.18.1) [46, 55]. Quinones are highly reactive molecules, which cross-link with a variety of cellular substituents, resulting in the formation of brown or black melanin-type polymers [30].

Although CO_2 is a cause of brown core in pear, no clear relation could be established between CO_2 in the storage atmosphere and PPO activity. In Chapter 2 it is hypothesised that neither the PPO activity nor the concentration of PPO substrates is limiting, but that brown core initiation is a consequence of cellular decompartmentation, caused by membrane disintegration.

During senescence, internal membrane damage results in leakage of phenolic compounds from the vacuole, which facilitates the PPO reaction. This internal membrane damage might be induced by peroxidation of membrane lipids due to the presence of radicals. Lipid peroxidation is the process in which molecular O_2 is incorporated into unsaturated lipids, to form lipid hydroperoxides. During peroxidation ethane and pentane, gaseous end-products of a process called β-scission, can be produced followed by hydrogen abstraction from ω-3 and ω-6 unsaturated fatty acids, respectively [72].

Increased lipid peroxidation and progressive loss of membrane integrity are characteristics of ageing potato tubers [73]. Longer stored tubers have a substantially higher respiration rate during the initial stage of sprouting, compared to younger tubers [74], which is required to equal the higher ATP consumption of older tubers. Since increased free-radical-mediated peroxidation of fatty acids occurs during tuber ageing, GSH-mediated free radical scavenging is supposed to be an ATP sink [71]. The AA-GSH cycle, which is important in the defence against hydrogen peroxide and free radicals, is present in plant mitochondria and peroxisomes. AA-GSH cycle enzymes were found in pea leaf mitochondria and peroxisomes [75]. AA is a crucial substance in the defence mechanism against free radicals [76]. It has been demonstrated to be either directly or indirectly involved in the removal of radicals [77]. Subsequently formed MDHA or ascorbate radicals are enzymatically or non-

enzymatically reduced (regenerated), or broken down. Furthermore, AA is able to convert enzymatically formed *o*-quinones back to their precursor phenols during the PPO reaction [27].

In this chapter the relation between free radical scavenger content (i.e. AA) and the development of brown core is discussed. We found a clear relation between enhanced CO_2 concentrations and decreased AA levels in pears during storage. Furthermore, a relation between the induction of browning during storage and the AA content of pears is demonstrated. Data on AA levels are used to predict the onset of browning. Next, with the use of an ultra-sensitive photoacoustic laser-based detection system, it is shown that pears with brown core emit ethane, which confirms membrane peroxidation.

3.2 Materials and methods

3.2.1 Plant material and storage

For the AA determinations at different gas conditions (Fig. 3.1) the same pears were used as in Chapter 2: orchard 1, harvest 2. Before experiments started fruits were stored in the facility (static system) described in the same chapter under 2 ± 0.1 kPa O_2 and <0.7 kPa CO_2 (standard CA). For the experiments, pears were transferred to 70-l containers connected to a flow-through system, and kept at 5°C and 0.3 ± 0.1 kPa O_2 or 2.0 ± 0.1 kPa O_2 in combination with either 0.2 ± 0.2 kPa or 10.0 ± 0.2 kPa CO_2. An enhanced temperature and an increased CO_2 concentration were used to accelerate the induction of browning. Relative humidity during storage was kept at 97-99%. Gas mixtures of nitrogen, O_2 and CO_2 were composed with Brooks 5850 TR series mass flow controllers. The total flow-rate was established between 490 and 510 ml min^{-1} [59]. Samples for AA measurements were taken from cortex, core or peel tissue in four-fold (n=4). Every sample was a mixture of 5 pears.

For experiments in which AA concentrations were related to ripening (Fig. 3.2, panel A and B), pears were harvested in Ingen (orchard 2), The Netherlands (province Gelderland), on 16 September 1997, and stored at standard CA conditions

for 21 days before experiments started. During ripening experiments the same flow-through system was used, but the temperature was raised to 10°C to accelerate ripening. Fruits were stored for 15 days at 21 kPa O_2 and 0.2 ± 0.2 kPa CO_2. During storage different ripening parameters were determined, like instrumental firmness (n=20), respiration (n=12), ethylene production (n=12) and AA content (n=20) (see: *3.2.2 AA measurements*). For AA determinations only the cortex tissue of the pear was used, without the peel and core fraction. Firmness and AA content were determined of individual fruits. The other parameters were determined in cuvettes with two fruits (see: *3.2.3 Respiration, ethylene and firmness*).

For the experiment in which the change in AA was followed (Fig. 3.3, Fig. 3.4 and Table 3.1), and conditions were adapted to avoid browning, pears were obtained from a local auction in February 1998 (orchard 3). Fruits were stored at the auction under standard CA conditions for 6 months before the experiment started. During the experiment fruits were stored in the flow-through system at 2.0 ± 0.1 kPa O_2 combined with 0.2 ± 0.2 kPa CO_2 (four-fold) or 10.0 ± 0.2 kPa (eight-fold) CO_2. After 40 days half of the containers with 10 kPa CO_2 (four-fold) were switched to 0.2 ± 0.2 kPa CO_2. AA was determined in two mixed samples of 10 pears in every container. Only the cortex tissue of the fruit was used (see: *3.2.2 AA measurements*). The experiment lasted 83 days in total. Every week containers were opened and pears were judged on browning.

3.2.2 Ascorbic acid measurements

High-pressure liquid chromatography (HPLC) was performed using a Waters (Milford, USA) chromatograph model 510 with a Waters 486 UV-VIS detector (251 nm). A Symmetry C-18 column (3.9 x 150 mm, particle size 5 µm, Waters), with a Sentry Guard column C-18 (Waters), was employed. Measurements were performed at 25°C.

The mobile phase consisted of 2.5 g tetrabutylammoniumhydrogensulfate (z.s. 818858, Merck, Dorset, UK) and 55 ml methanol (p.a. Merck 6009) dissolved in 942.5 grams Milli-Q water (Millipore (Waters), Milford, USA) [78]. Before use the eluent was filtrated and degassed with a 0.45 µm Millipore filter (HVLP 04700). The flow

rate during measurements was kept at 1 ml min^{-1}. Analyses were completed within 5.5 min including a post-column elution time of about 1 min. As a standard 177 μM AA (Sigma) prepared with Milli-Q water was used (stock I). One ml of stock I was diluted in 39 ml Milli-Q water with 5 ml 9.5% w/v oxalic acid (Merck 100495) and 5 ml methanol v/v (stock II). A dilution series from stock II was stored on ice and kept in the dark until injection. Stocks were prepared freshly every day.

Fruits were divided in a peel, cortex and core sample, and immediately frozen in liquid nitrogen for the experiment presented in Fig. 3.1. In other experiments only the cortex tissue was examined. The samples were crushed in a kitchen mixer (Braun, Germany). All the following steps were carried out in the refrigerator or on ice in diffuse light. Ten grams of sample were diluted with 5 ml 9.5% (w/v) oxalic acid (Merck 100495), 5 ml methanol (p.a.) and 30 ml Milli-Q water. The mixture was homogenised with an ultra turrax mixer and filtered through fluted paper (Schleicher & Schüll 595½, Dassel, Germany). The filtrate was passed through a unit consisting of a 0.45 μm sterile filter and a Sep-Pak C18 cartridge (Waters), and was directly injected in a manual-injector system with a 20 μl sample-loop. HPLC measurements were done directly after the extraction procedure. Results were analysed by means of the Millenium HPLC manager (Waters).

3.2.3 Respiration, ethylene and firmness

After 21 days of storage in the static system ripening of pears was monitored for 15 days in the flow-through system. Gas exchange characteristics and ethylene production were measured at 10°C after 2, 6, 9 and 13 days. Firmness and AA were determined after 0, 4, 6, 8, 11 and 15 days.

Fresh weight (FW) was determined before measurements were done. Two pears were placed in 1500 ml cuvettes, and connected to the containers of the flow-through system. Gas was sampled directly from the cuvettes, after they have been temporarily disconnected from the flow-through system. The exact time of measurement was logged. For every measurement two samples were taken, and only the second sample was used. The time period between the first and second measurement was 4.5 hours so that the difference in partial pressure between the

two measurements never exceeded 0.3 kPa O_2 or CO_2 at high O_2 and 0.1 kPa at low O_2 levels.

To convert gas levels from percentages to partial pressures, total pressure in the cuvettes was measured directly after the first measurement and before the second measurement (with a Druck PDI 265 manometer). After the second measurement, cuvettes were reconnected to the flow-through system. The difference in gas partial pressures between the first and the second measurement was converted to moles according to the Ideal Gas Law. Gas exchange rates were calculated by expressing the mole differences between the two measurements per unit time (s) and per unit weight (kg FW at the start of the experiment).

The ethylene production was determined by taking a 2.5 ml sample from the headspace directly after disconnecting the cuvettes, before respiration measurements, and injecting it into a gas chromatograph. After 15 min a second sample was taken. Before calculating the ethylene production the data were corrected for the pressures inside the cuvettes.

Firmness was calculated from stress-strain plots produced by compressing cubes of tissue taken from an equatorial slice of the pear. Cubes with a thickness of 8 mm were taken from the slice with a 17 mm corkbore. For measurements samples were taken from 20 individual pears. Instrumental firmness was measured with an Instron (model 4301, High Wycombe, UK) apparatus, fitted with a 100 N static load cell. The maximal compression was set on 0.5 mm and the compression rate was $1.67 \cdot 10^{-5}$ m s^{-1}. Firmness values are expressed in N.

3.2.4 Brown index

Internal browning in pears was classified as in Chapter 2. A brown index was calculated in which I, II, and III refer to the number of pears in the various browning classes (Eq 3.1). A browning index value 0% means no browning; 100% means maximal browning. The following brown-index was used:

$$\text{Brown Index} = \frac{\text{I} + 2\text{II} + 3\text{III}}{3(\text{O} + \text{I} + \text{II} + \text{III})} \qquad 3.1$$

3.2.5 Ethane measurements

Pears were stored in a static system at conditions (0.5 ± 0.1 kPa O_2 and 3.0 ± 0.2 kPa CO_2) that induce browning before ethane measurements started. For the ethane measurements, 3 pears were enclosed in a 2 l cuvette for 8 h, and hereafter the cuvette was connected to the flow-through system of the laser-based detector at 2.5 ± 0.1 kPa O_2 and 8.0 ± 0.2 kPa CO_2 (flow rate ca 5 l h^{-1}). The set-up of the laser equipment has been described by Bijnen *et al.* [79] and in Chapter 7. Ethane has been monitored for 4 h at room temperature. The detection limit of ethane was been about 1 part per billion (ppb). Additional equipment, for example a cold trap to pre-clean the incoming trace gas flow and tubing has been used to lower the detection limit for ethane to 1 ppb [80].

3.2.6 Statistical analysis

AA values were analysed for significant differences by analysis of variance (ANOVA) with the statistical package Genstat (release 5). When significant differences were found, comparisons between pairs of data were made using the least significant differences between means (LSD) at a significance level of 95% or 99%.

3.3 Results

3.3.1 Ascorbic acid as an indicator for brown core

Storage conditions clearly affected AA levels in pears (Fig. 3.1). Normally fruits are stored at standard CA conditions: 2.0 kPa O_2 and <0.7 kPa CO_2. However, here pears were stored at different conditions whereby AA concentrations were

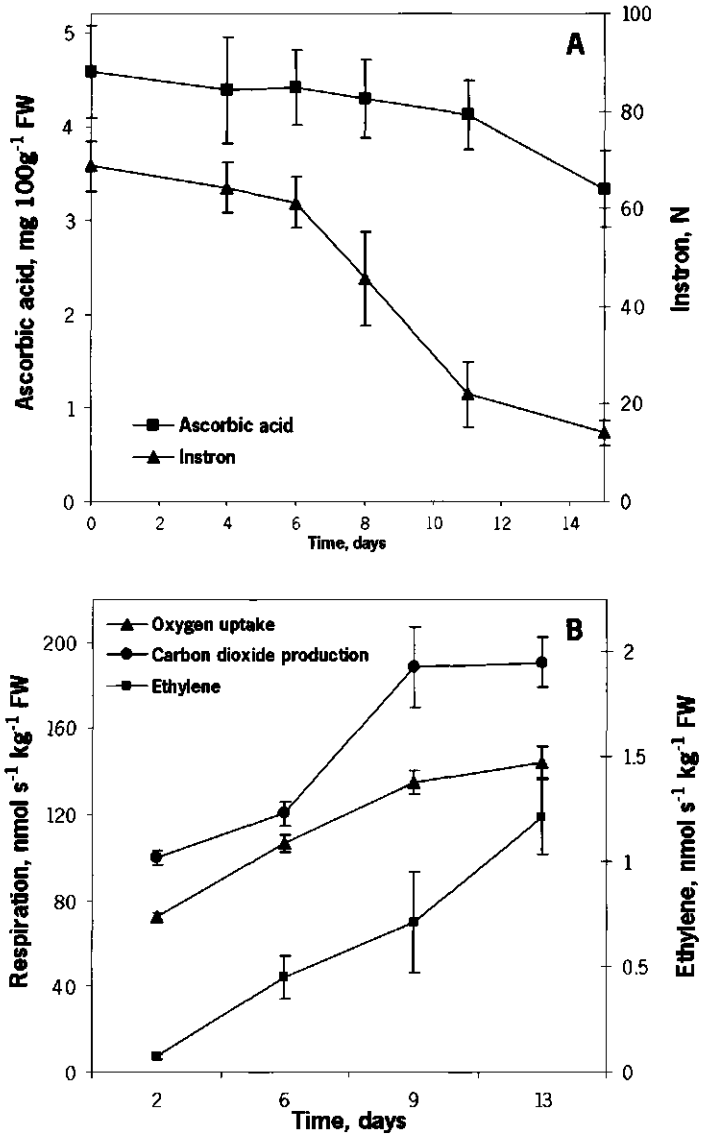


Figure 3.2. Ripening of Conference pears. Fruits were stored at 10°C and 21 kPa O_2 in the flow-through system (without CO_2). During ripening different ripening parameters, respiration rate and AA content were monitored. Panel A: (■) AA content expressed as mg 100g^{-1} FW (n=20) and (▲) firmness (Instron) values expressed in N (n=20). Panel B: (■) ethylene production expressed as nmol *s^{-1} kg^{-1} FW (n=12), (●) CO_2 production expressed as* nmol *s^{-1} kg^{-1} FW (n=12), (▲) O_2 uptake expressed as* nmol *s^{-1} kg^{-1} FW (n=12). All measurements were done on individual pears.*

increased, as expected, during the progression of ripening (Fig. 3.2, panel B). In contradiction to the results of Trautner and Somogyi [81] no clear decrease in AA levels could be observed during the first 10 days; only at day 15, values differed significantly from the values at day 0 ($p=0.01$), while these fruits were in a very ripe, consumable state. Generally pears did not reach this state of ripening during the storage experiments presented in this paper. Based on these results we conclude that differences in AA concentrations in Fig. 3.1 could not be attributed to differences in the stage of ripening.

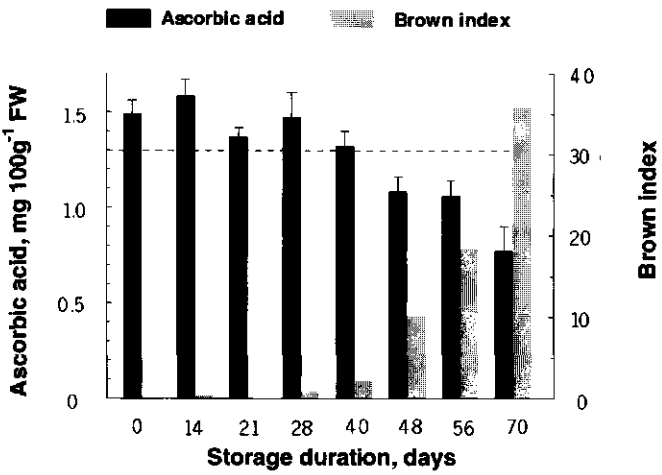


Figure 3.3. AA and browning in pears during storage at 2 kPa O_2 and 10 kPa CO_2 in the flow-through system (5°C). The AA content and the brown index of pears were monitored for 71 days. For the AA determinations two mixed samples of 10 pears each were taken from 4 containers (n=4). Before AA was determined, pears were judged on browning. When AA levels dropped below ca 1.3 mg 100g^{-1} FW (horizontal line in graph) browning seemed to be initiated.

When pears were stored at 2 kPa O_2, 10 kPa CO_2 and 5°C in a flow-through system, AA levels were slowly decreased (Fig. 3.3). After 40 days of storage, levels decreased from 1.5 mg 100g^{-1} FW at t=0 to 1.3 mg 100g^{-1} FW. When pears were stored more than 40 days at these conditions AA levels continued to drop, and after 48 days the first clear incidences of browning appeared. After 71 days AA levels dropped to 0.75 mg 100g^{-1} FW, while the brown index of the pears exceeded 35%.

In brown tissue AA levels were zero, but after 71 days storage, browning was not maximal (browning index 100%). However, healthy tissue close to brown tissue can contain 'normal' AA concentrations. In experiments presented here, AA in the whole cortex of the fruit was determined, explaining why were never zero. In this experiment an AA level of 1.3 mg $100g^{-1}$ FW on average was practically regarded as a limit. Beneath this limit browning of the pears was expected to be unavoidable.

3.3.2 Switching storage conditions

In different experiments we examined the possibility to use decreased AA levels of the fruit as a marker for the initiation of brown core. Under enhanced CO_2 concentrations AA levels drop; beneath a certain threshold value pears seem to become brown. The question might be asked if, just before AA values drop below this threshold value, and the formation of aberrations is initiated, browning can be avoided by changing gas conditions. In Fig. 3.4 conditions were switched from with enhanced CO_2 to without CO_2 when levels had dropped to 1.3 mg $100g^{-1}$ FW. AA levels in pears stored at standard CA conditions increased slowly to ca 2.9 mg $100g^{-1}$ FW after 30 days. Under enhanced CO_2 AA levels dropped slowly, and after switching, AA concentrations in the pears increased again, paralleling concentrations of control fruits after about 2 weeks (Fig. 3.4). Concentrations in pears stored under enhanced CO_2 showed a continued decrease. Instron values, measured at day 56, were 33, 64 and 55 N (n=20) for pears stored under standard conditions, enhanced CO_2 and switched conditions respectively. These measurements show that pears of the control are riper, but that AA levels are probably not affected by ripening when compared to Fig. 3.2. Ethylene production in containers where conditions were switched increased to the level of control pears at day 56: ca 0.15 *n*mol kg^{-1} s^{-1}. In the enhanced CO_2 containers the ethylene production was about 5 times lower: 0.03 *n*mol $kg^{-1}s^{-1}$. These values can be compared to the ethylene production between day 3 and 6 in the ripening experiment presented in Fig. 3.2. As far as a comparison can be made between Fig. 3.2 and Fig. 3.4 AA levels seem not to be affected by ripening at day 56.

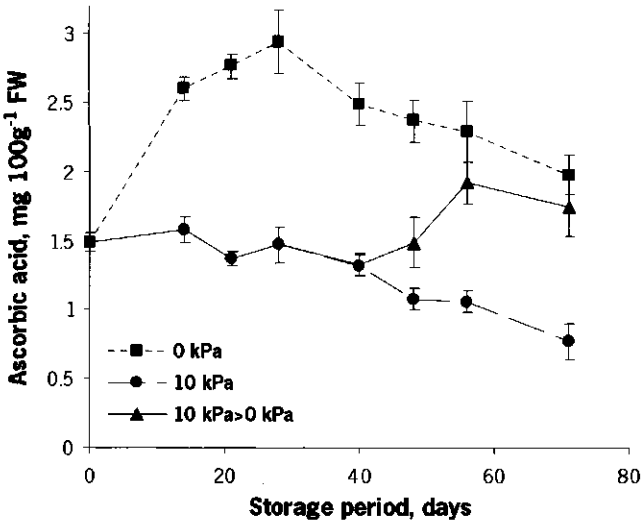


Figure 3.4. AA in pears as an indicator for browning before and after switching gas conditions. Fruits were stored at 2 kPa O_2 without CO_2 (control, n=4) or under 2 kPa O_2 and 10 kPa CO_2 (n=8). After 40 days of storage four of the enhanced CO_2 containers were switched to the control condition (10 kPa>0 kPa). During storage AA was determined in two mixed samples of 10 pears from each of the 12 containers. Values are expressed as mg $100g^{-1}$ FW ± SE.

After 40 days the first incidences of browning could be seen in pears stored at enhanced CO_2. After 56 days browning was also detected in the control pears. At switched conditions there were some incidences of browning. Table 3.1 gives an overview of brown induction after 83 days of storage. The effect of switching conditions from enhanced CO_2 to control conditions was clear; the percentage of healthy fruits was about 75% compared to about 25% when the pears were stored under CO_2 continuously. About 50% of the pears stored at enhanced CO_2 showed severe browning, compared to only 8% of the pears stored in containers in which gas conditions were switched.

3.3.3 Ethane

Due to lipid peroxidation in cell membranes ethane can be formed, which diffuses out of the fruit. In a preliminary test ethane emission has been monitored during storage at unfavourable, brown-inducing conditions (0.5 kPa O_2 and 3.0 kPa CO_2).

Three fruits were placed in 2-L cuvettes and connected to the flow-through system of a photoacoustic detector. The ethane emission was monitored for 4 h (Fig. 3.5). In one cuvette the average ethane concentration in the flow was 30 ppb, while one pear showed small cavities, the second showed moderate-sized cavities, and the third pear did not show aberrations. In a second cuvette the average ethane emission was 60 ppb, while two pears showed moderate browning and cavities, and the third pear did not show browning or cavities. Pears without browning or cavities did not show a detectable ethane emission.

Table 3.1. Brown core in pears after 83 days of storage. Pears were stored at 2 kPa O_2, with or without 10 kPa CO_2 (2-0 and 2-10, n=4). One series of containers was switched from high CO_2 (10 kPa) to no CO_2 (2-10>0) after 40 days (n=4). Pears were classified as healthy (class 0), and slightly (class I), moderately (class II) or severely (class III) brown. For every condition (2-0, 2-10 and 2-10>0) circa 120 fruits were examined. Values are expressed as percentages. From the percentages the brown index is calculated.

Percentage of pears with brown core			
Brown stage	2-0	2-10	2-10>0
Healthy (O)	41.3	23.9	74.7
Slightly (I)	36.7	14.7	6.1
Moderate (II)	16.6	13.0	11.6
Severe (III)	5.4	48.5	7.7
Brown index	*28.7*	*62.1*	*17.5*

3.4 Discussion

There were no clear differences in AA levels of pears from either different orchards or picking dates. Also no relation could be established between AA levels in these pears and browning later during storage ($p \leq 0.05$, results not shown). However, AA levels were relatively low in the core of the fruit, which fits well with the start of

brown core development in this part of the fruit. Generally, peel tissue and cortex tissue just beneath the peel of the fruit is not affected by browning. Except for extreme cases of the disorder, the outer appearance of affected fruits is normal. AA concentrations in the peel of the fruit were significantly higher than values in the fruit flesh. Ponting and Joslyn [34] stated already in 1948 that there is an intimate relation between browning of apple tissue and AA levels. Browning does not occur until all AA in the tissue is oxidised.

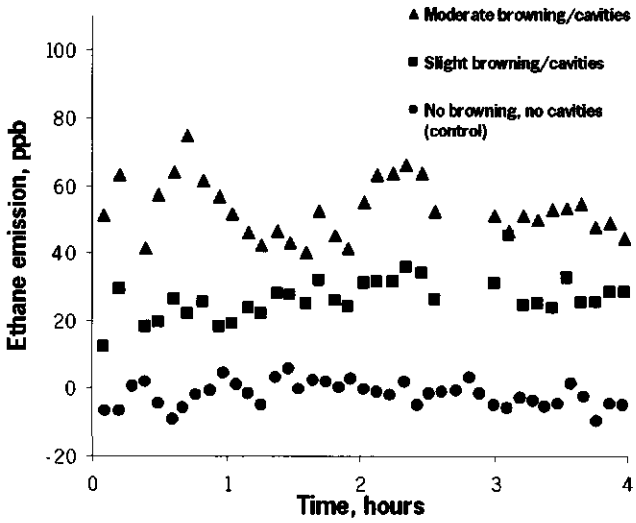


Figure 3.5. Photoacoustic laser measurements on the ethane emission of brown pears. Before the experiment fruits were stored at 0.5 kPa O_2 and 3.0 kPa CO_2 for 5 months, to initiate aberrations. Three cuvettes were connected to the flow-through system of a photoacoustic CO laser, all with three pears, at 2.5 kPa O_2 and 8 kPa CO_2. The total flow through the cuvette was 5 l h^{-1}. Ethane emission is expressed as ppb. Pears were judged afterwards. (▲): Moderate browning/cavities; (■): slight browning/cavities; (●) no browning or cavities.

Specific atmospheric conditions lead to continuously decreasing levels of AA, e.g. storage at lowered O_2 and increased CO_2 concentrations. Comparable results were found by Agar *et al.* [82] in several berry fruits. A rapid decrease of AA was monitored in strawberries, when stored at 15 kPa CO_2. Both, AA concentrations in pears and the susceptibility for brown core depend strongly on external gas conditions. Browning of the fruit-flesh occurred when AA levels dropped below ca 1.3 mg $100g^{-1}$

FW, while browning could be avoided to a great extent, and AA levels were enhanced, when CO_2 concentrations were lowered at the moment this level was reached. AA levels showed a continued drop when conditions were not changed, and severe browning became inevitable. At this moment it is not clear whether the drop in AA is the main step yielding brown core. The function of other antioxidants, like α-tocopherol and GSH, for instance, was not studied. However, AA may well serve as a marker for browning. As the results in this paper show, brown core can be avoided by monitoring AA levels.

In contradiction to the results of Trautner and Somogyi [81], the AA level was not clearly affected by the stage of ripening, as determined by respiration rate, ethylene production and firmness. Even when pears were in a soft, consumable state (Instron values lower than 20 N) AA levels did not significantly decrease. During dynamic measurements on AA Instron values dropped but levels were not affected by ripening. Only after 15 days ripening a significant decrease ($p \leq 0.01$) in AA could be recorded. Although the ripening control in this paper (Fig. 3.2) was performed at different storage conditions it seems very unlikely that AA levels in the experiments were affected. Instron values of pears stored at standard CA conditions for 56 days (Fig. 3.4) are comparable with values at day 10 of the ripening experiment (Fig. 3.2). At day 10 of the ripening experiment the ethylene emission is around 1 *n*mol kg^{-1} s^{-1}. In the experiment presented in Fig. 3.4 the ethylene emission is even much lower (circa 0.15 *n*mol kg^{-1} s^{-1}).

It is not clear how AA concentrations were affected during storage. Gas concentrations in the fruit are not comparable to concentrations in the storage environment. Williams and Patterson [23] found that CO_2 levels in the tissue of pears are higher than concentrations in the storage environment, because internal gas conditions are influenced by respiration rates and diffusion characteristics of the fruit [83] (Chapter 8). Hypoxia (0.25 kPa O_2) in Bartlett pears caused a decrease of 0.4 pH units and a drop in nucleotide triphosphate (NTP, mainly ATP) levels resulting in an increase in NDP [84], which points out the effect of gas conditions on the energy charge (AEC) of pears. A rapid decrease in ATP content and ATP/ADP ratio was caused in excised pea roots at short-term anoxia [85]. Mitochondrial functionality was partially lost by a 6 hours anoxic treatment. Elevated concentrations of CO_2 caused a

depression in respiration in plants. Bartlett pears stored at enhanced CO_2 concentrations showed a decreased succinic dehydrogenase activity, which was compatible with a decreased respiratory activity [68]. Pears kept at enhanced CO_2 showed ultra-structural alterations in various organelles including mitochondria, which display fragmentation and reduction in size. In Chapter 2 it was hypothesised that brown core in Conference pears is a consequence of cellular decompartmentation, caused by membrane disintegration. During senescence internal membrane damage results in leakage of phenolic compounds from the vacuole. A higher susceptibility for browning is neither caused by an enhanced activity of PPO, neither by a higher concentration of phenolics. Destructive rearrangements of the mitochondria and other organelles give grounds to suggest that these phenomena were caused by an energy deficiency. Probably maintenance energy (ME) requirements are not constant during CA storage of fruits [86]. Such changes were found in potato tissue, where increased lipid peroxidation, progressive loss of membrane integrity and substantially higher rates of Cyt-mediated respiration were characteristics of older potato tubers during sprouting [73]. As tubers age, membrane permeability to electrolytes and age induced loss of membrane integrity increased. Potential ATP sinks during ageing are ATPases in the plasmalemma that become leaky with advanced age. Older tubers respire with a faster rate to achieve the same AEC [71, 74]. In the case of pears stored in CA, respiration cannot be accelerated due to the limiting O_2 concentration in the storage room. A second parameter affecting respiration is the diffusion characteristics of the fruit. When ME requirements increase during ageing, this parameter could become of in increasing importance. An increase in diffusion resistance, for example, could be an explanation for browning in ripe pears at CA conditions, which is seen in Table 3.1. Browning can be seen at control conditions (2/0) after 83 days storage. Although AA levels slowly decreased in the control after 30 days storage, levels were not determined after 70 days, so that no statements can be made about the course of this decrease after 70 days.

While initiation of browning can probably be explained by energy deficiency, it is not clear why the appearance of browning takes so long. When pears are stored at 2 kPa O_2 and 10 kPa CO_2 (5°C) the first incidences of browning become clear after

more than 5 weeks.

Another ATP sink during storage is the *de novo* synthesis and regeneration of antioxidants, like GSH in potatoes [71]. The AA-GSH cycle, which is important in the defence against activated oxygen species, is present in plant mitochondria and peroxisomes. AA-GSH cycle enzymes were found in pea leaf mitochondria and peroxisomes [75]. Enhanced CO_2 and lowered O_2 concentrations inhibit respiration, lower AA levels, and can induce browning in Conference pears.

Fig. 3.5 indicates that brown pears and pears with cavities produce ethane. Konze and Elstner [87] reported that ethane is formed from linolenic acid in potato mitochondria. In their paper they stated that oxygen radicals mainly drive ethane (and ethylene) production by potato mitochondria. Ethane and pentane, end products of a process called β-scission, can be produced followed by hydrogen abstraction from ω-3 and ω-6 unsaturated fatty acids respectively [72].

Optimisation of gas conditions is established to reduce maturation of the fruit during storage. However, during storage energy requirements could increase, by which an energy shortage is created and vital processes, like the regeneration of antioxidants are abolished. Depending on parameters like diffusion characteristics of the fruit, browning of the tissue is initiated. Probably developing an interactive storage system, in which energy needs play a crucial role, can prevent disorders in pears and other fruits.

Acknowledgements

I'd like to thank Alex Van Schaik, Sandra Robat and Tjerk Lammers for technical assistance, and Huug de Vries, Frans Harren, Rob Schouten and Linus Van der Plas for stimulating discussions.

4. The oxidation of L-ascorbic acid catalysed by pear tyrosinase[5]

Summary

In this chapter the ability of partially purified pear tyrosinase to catalyse the oxidation of AA is investigated. The ascorbate oxidase (AO) activity of pear tyrosinase was studied by oxymetric assays. The activity was linearly related to the enzyme concentration with a Michaelis constant (Km) for AA of 0.55 ± 0.03 m*M* at pH 7. The stoichiometry was found to be 1 : 2 (O_2 : AA). The action of the tyrosinase inhibitors tropolone and sodium chloride was studied to exclude a possible interference of endogenous pear AO in the oxidation of AA. A possible role of the 'AA/PPO'-system in the browning of pears is proposed.

4.1 Introduction

The copper-containing enzyme tyrosinase (EC 1.14.18.1; PPO, PPO) can be found throughout the phylogenetic tree. This enzyme is of central importance in processes such as vertebrate pigmentation and the often undesirable enzymatic

[5] This chapter is based on: Espín JC, Veltman RH, Wichers HJ. 2000. The oxidation of L-ascorbic acid catalysed by pear tyrosinase. Physiologia Plantarum 109: 1-6.

browning of fruits and vegetables [88]. PPO activity can lead to a significantly diminished quality of foodstuffs by both decreasing the content of the phenolic compounds and the appearance of melanins, which change the organoleptic properties of the products (colour, aroma, texture and nutritional quality). Therefore, the prevention of this browning reaction has always been a challenge to food scientists [89, 90].

In pome fruits the start of browning is associated with decompartmentation of intracellular structures as the vacuole, mitochondria and plastids. Tyrosinase activity and polyphenol content of pear tissue do not seem to be limiting factors during the initiation of brown core (Chapter 2).

AA is an important substance in defence mechanisms against free radicals [76]. Firstly, AA has been demonstrated to reduce oxidised tocopherol directly [77]. In the cell, GSH and AA form a redox couple, which is involved in the regeneration of AA [91]. Secondly, AA is directly involved in the removal of lipid alkoxyl ($LO^{\cdot}$) and peroxyl ($LOO^{\cdot}$) radicals [77]. Subsequently, the reaction product is either enzymatically or non-enzymatically reduced or broken down. Thirdly, AA can regenerate quinones back to precursor phenols in a coupled oxidation/reduction reaction. During this process DHA is formed [27].

It has been previously reported that the active centre of tyrosinase from mushroom and frog epidermis is capable of catalysing the oxidation of AA [33]. The aim of this study is to demonstrate the AO activity of pear PPO as well as to propose a possible implication of the 'AA/tyrosinase system' in the development of the brown core storage disorder in pears. The ability of tyrosinase to catalyse the oxidation of AA in plant kingdom has not been described so far.

4.2 Materials and methods

4.2.1 Chemicals

AA was purchased from Merck (Germany). 4-*tert*-butylcatechol (TBC) was purchased form Fluka (Sweden). Chlorogenic acid and tropolone were supplied by

Sigma (Holland). Cucumber AO (EC 1.10.3.3) (300 units mg^{-1}) was supplied by Calbiochem (Holland). Stock solutions of the different compounds were prepared freshly every day. All other reagents were of analytical grade and supplied by Merck.

4.2.2 Plant material

Pears, cultivar Conference were harvested in The Netherlands (province Gelderland). The fruits were harvested at the optimal date for CA storage predicted by firmness (September 8, 1998). Pears were stored under standard conditions in an experimental facility as described in Chapter 2. Cortex tissue was frozen in liquid nitrogen and freeze-dried. The freeze-dried samples were pulverised to powder in a mortar under liquid nitrogen.

4.2.3 Pear PPO purification

Pear PPO was extracted according to the procedure of Espín *et al.* [92] and partially purified by using two chromatographic steps. Briefly, 20 g of freeze-dried pear powder was dialysed against water overnight, and freeze-dried again. The powder was dissolved in 250 ml 50 m*M* sodium phosphate buffer pH 7 with 3% Triton X-114, and incubated for 15 min under continuous stirring at 4°C. After temperature phase-partitioning at 35°C [92], the detergent-rich phase (lower phase) which contained mainly pigments, phenols and highly hydrophobic proteins, was discarded after centrifugation at 8000 x *g* for 10 min at 25°C. The supernatant was brought to 30% saturation with solid ammoniumsulfate, and centrifuged at 20,000 x *g* at 4°C. The pellet was discarded and the supernatant was brought to 75% ammoniumsulfate. After centrifugation at 20,000 x *g* at 4°C, the supernatant was discarded and the pellet dissolved in 2 ml 50 m*M* sodium phosphate buffer pH 7. This homogenate was dialysed against water overnight. Additional ammoniumsulfate was added to give a final concentration of 1 *M* and applied into a Phenyl Sepharose High Performance™ column (Pharmacia, Sweden) (hydrophobic interaction; 32 cm length, 2.6 cm diameter) previously equilibrated with 1 *M* ammoniumsulfate in 50 m*M* sodium phosphate buffer pH 7. The column was eluted (3 ml min^{-1}) with a stepwise gradient of decreasing ammoniumsulfate concentrations (Fig. 4.1, panel A). The same

procedure was repeated two times. Fractions (6 ml) with PPO activity were pooled, dialysed overnight against water and freeze-dried.

The freeze-dried material was dissolved in 2 ml of 20 m*M* BIS-TRIS buffer pH 6.5 and applied into a DEAE-Sepharose Fast Flow™ column (Pharmacia, Sweden) (anionic exchange; length 25 cm, diameter 2.6 cm) previously equilibrated with 20 m*M* BIS-TRIS buffer pH 6.5. The column was eluted (3 ml min^{-1}) with a stepwise gradient of increasing NaCl concentrations (Fig. 4.1, panel B). Fractions (6 ml) with PPO activity were pooled, dialysed overnight against water and freeze-dried. This purification procedure yielded a single pear tyrosinase isoform with an isoelectric point of 4.5 determined by analytical isoelectric focusing (results not shown). The purification factor was 56 with an overall yield of 25%.

4.2.4 Oxymetric assays

O_2 consumption was followed with a YSI MODEL 5300 oxymeter (USA) based on the Clark electrode with Teflon membrane, and equipped with a Kipp & Zonen recorder (Holland). The system was calibrated using the TBC/tyrosinase method [93].

The standard reaction medium contained 50 m*M* sodium phosphate buffer pH 7, 10 m*M* AA and 0.1% sodium dodecyl sulphate (SDS) and 0.16 μg ml^{-1} pear PPO. The presence of SDS in the reaction was critical to fully activate pear PPO, which was isolated mainly in its latent form [94]. The O_2 concentration was 0.26 m*M* (saturation) in all the assays. The assay mixture was stirred continuous and the temperature of the mixture was controlled (25°C) using a circulating water bath. The final volume in the polarographic assays was 3 ml.

Pear PPO activity on chlorogenic acid was determined by measuring the O_2 consumption of the reaction, due to the high instability of its *o*-quinone.

Activity of commercial cucumber AO was also determined by measuring the O_2 consumption in the presence of AA. 0.1% SDS was added to maintain the same assay conditions as when pear PPO was assayed. This proportion of SDS did not affect cucumber AO.

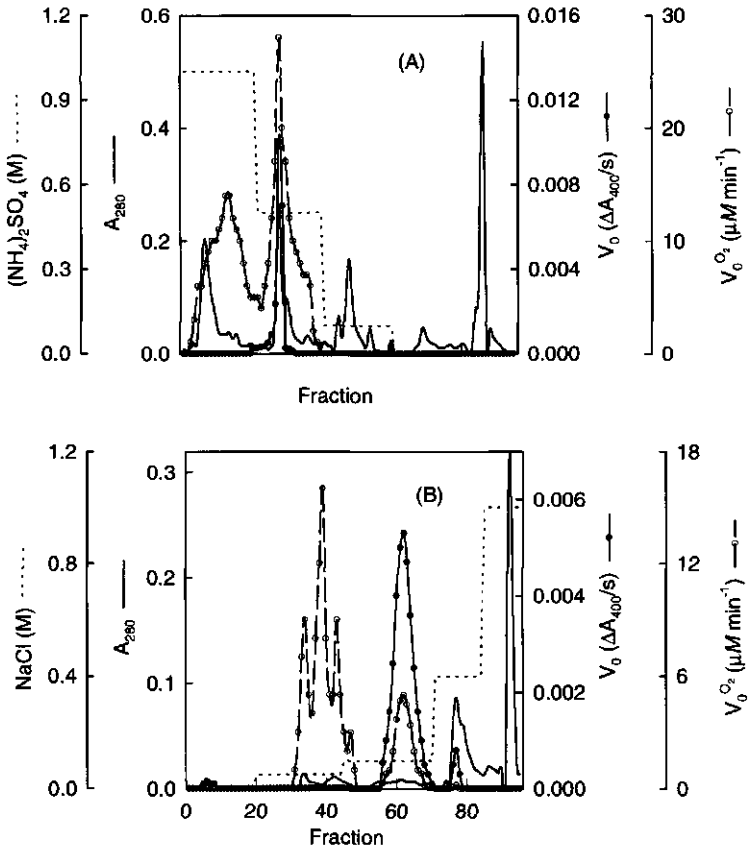


Figure 4.1. Partial purification of pear tyrosinase. Chromatographic experiments were carried out as described in the Material and Methods. Panel A: Hydrophobic interaction chromatography (Phenyl Sepharose High Performance). Panel B: Anionic exchange chromatography (DEAE-Sepharose Fast Flow). Dashed lines designate gradient of either ammonium sulphate (A) or sodium chloride (B). Solid lines (without symbols) designate A_{280} absorbing material. (●) Pear PPO activity on 2.5 mM TBC (diphenolase activity) in 50 mM sodium phosphate buffer pH 7 with 0.1% SDS (○). O_2 rate consumption on 10 mM AA (AO activity) in 50 mM sodium phosphate buffer pH 7 with 0.1% SDS.

4.2.5 Spectrophotometric Assays

PPO activity on TBC carried out by measuring the appearance of the product (4-*tert*-butylbenzoquinone) at 400 nm in a UV/Vis Perkin Elmer Lambda-2 spectrophotometer (Germany) on-line connected to a Pentium-100 microcomputer (The Netherlands). Temperature was controlled at 25°C using a circulating bath with a heater/cooler (precision of ± 0.1°C). 0.1% SDS was also added to the reaction medium. The reference cuvette contained all the components except the enzyme with a final volume of 1 ml.

4.2.6 Kinetic data analysis

The values of Km and Vmax were calculated from duplicate measurements of the rate, V_0, for each initial substrate concentration ($[S]_0$). The reciprocal of the variances of V_0 were used as weight factors to the non-linear regression fitting of V_0 vs. $[S]_0$ to the Michaelis equation [95]. The fitting was carried out by using a Gauss-Newton algorithm [96] implemented in the Sigma Plot™ 2.01 program for Windows™. Initial estimations of Km and Vmax were obtained from the Hanes-Woolf equation, a linear transformation of the Michaelis equation [95].

4.2.7 Protein determination

Protein content was determined by using the method of Bradford [97] using bovine serum albumin as standard.

4.3 Results and discussion

The ability of pear PPO to catalyse the oxidation of AA was characterised by studying the effect of pH, pear PPO and AA concentrations as well as the effect of known PPO and AO inhibitors to discriminate a possible contamination of endogenous pear AO.

4.3.1 Effect of pH

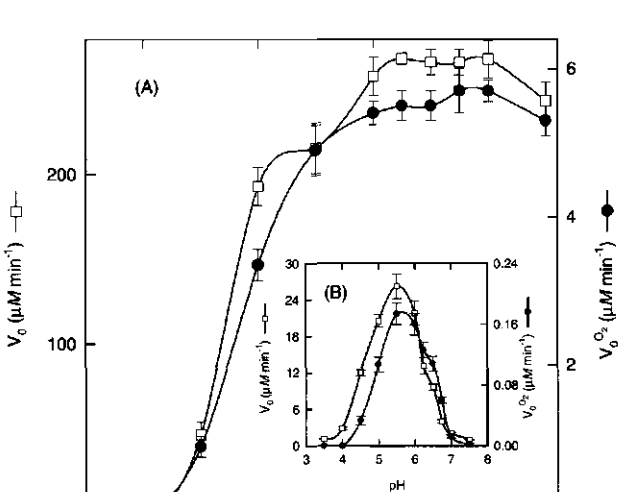


Figure 4.2. Dependence of O_2 consumption rate and o-quinone accumulation on pH in the oxidation of AA and TBC, respectively, catalysed by pear PPO. (A) Conditions were as follows: 0.1 SDS, 50 mM sodium acetate buffer (pH-3.5-5.5) or 50 mM sodium phosphate buffer (pH 6-7.5), 10 mM AA (●) or 2.5 mM TBC (□) and 0.16 µg ml^{-1} pear PPO. (B) Conditions were as in (A) but without SDS.

Both AO and diphenolase activities of pear PPO showed the same profile as a function of pH in accordance with previous reports relating to the AO of tyrosinase in other sources (Fig. 4.2) [33, 98]. In the presence of SDS, the fully active pear PPO had an optimum pH from 6 to 7. In the absence of SDS, only the active PPO already present, as well as the enzyme activated by acid shocking could be measured. In this case, the optimum pH was around 5.5. This bell shaped profile was in accordance with previous studies in which active pear PPO was determined [92]. It is known that acid shocking can activate latent tyrosinases, but with less efficiency than SDS [99]. Therefore, to measure the total PPO activity 0.1% SDS was used.

4.3.2 Effect of pear polyphenol oxidase concentration

The O_2 consumption in the presence of 10 m*M* AA increased linearly with time and proportional to pear PPO concentration (Fig. 4.3). This linear dependence demonstrated that the reaction was truly catalytic and it was not due to any artefact such as a high blank.

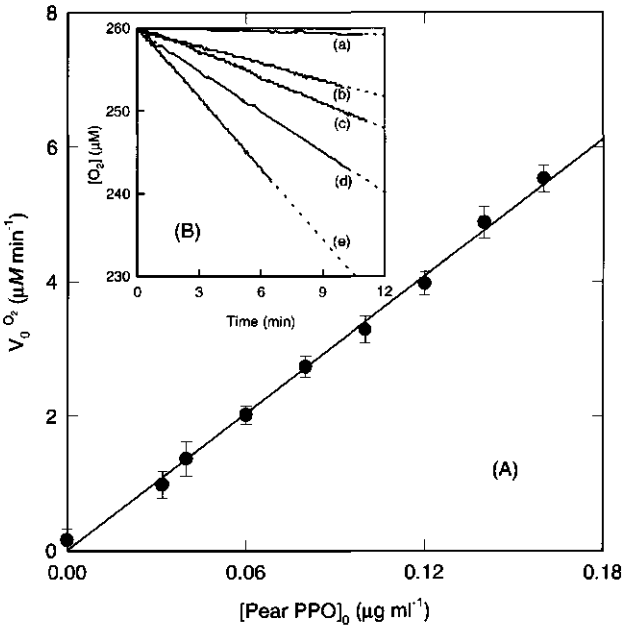


Figure 4.3. (A) Dependence of O_2 consumption rate (●) on pear PPO concentration in the oxidation of AA catalysed by pear PPO. Conditions were as follows: 50 mM sodium phosphate buffer, 10 mM AA, 0.1% SDS and (0.032-0.16) μg ml^{-1} pear PPO. (B) O_2 consumption recordings (———) at different pear PPO concentrations: (a) 0 = blank, (b) 0.04, (c) 0.06, (d) 0.1 and (e) 0.16 μg ml^{-1}. Assay conditions were as in (A). Experimental recordings were fitted using linear regression (dashed lines in panel B).

4.3.3 Effect of L-ascorbic acid concentration

The rate of O_2 consumption during the oxidation of AA catalysed by pear PPO depended hyperbolically on the AA concentration (results not shown). This saturation phenomenon was another proof to indicate that the reaction was truly catalytic.

The stoichiometry of the reaction was 1 : 2 (1 O_2 consumed : 2 AA consumed, results not shown), which agreed with previous studies on other PPO sources [33, 98].

Table 4.1. Effect of tropolone and sodium chloride on pear PPO and on cucumber AO. Conditions were: 0.1% SDS; 50 mM sodium phosphate buffer pH 7 (for tropolone assay) or 50 mM sodium acetate buffer pH 5.5 (for sodium chloride assay), AA (for AO activity), TBC 2.5 mM (for pear diphenolase activity) and 0.16 µg ml^{-1} pear PPO or 9 µg ml^{-1} cucumber AO. SDS was also used in cucumber AO experiments to maintain the same assay conditions. Conditions were: 0.1% SDS; 50 mM sodium phosphate buffer pH 7 (for tropolone assay) or 50 mM sodium acetate buffer pH 5.5 (for sodium chloride assay), AA (for AO activity), TBC 2.5 mM (for pear diphenolase activity) and 0.16 µg ml^{-1} pear PPO or 9 µg ml^{-1} cucumber AO. SDS was also used in cucumber AO experiments to maintain the same assay conditions.

		Percentage inhibition		
Inhibitor	Concentration (m*M*)	Pear PPO (Diphenolase activity)	Pear PPO (AO activity)	Cucumber AO
Control	0	0	0	0
Tropolone	1	60	40	100
	0.2	15	10	95
NaCl	15	97	100	0
	5	30	36	0

Non-linear regression fitting of V_0 of O_2 consumption on AA concentration gave a Km value of 0.55 ± 0.03 m*M* and a Vmax value of 6 ± 0.4 µ*M* min^{-1} at pH 7. The Km increased when pH decreased (Km = 0.5 m*M* at pH 7.5; Km = 0.67 m*M* at pH 6.5; Km = 0.86 m*M* at pH 6). The Vmax decreased only slightly when pH decreased (Vmax = 6 µ*M* min^{-1} at pH 7.5; Vmax = 5.8 µ*M* min^{-1} at pH 6.5; Vmax = 5.65 µ*M* min^{-1} at pH 6). It is known that AA presents pK_a values of 4.2 (pK_1) and 11.6 (pK_2) [100]. This

indicates that one hydroxyl group of the AA molecule is dissociated at pH 7. It is also known that this deprotonation is required in the reaction mechanism of PPO [101, 102]. This could explain the relatively high affinity of pear PPO for AA at pH 7 or 7.5.

Vmax and Km values for chlorogenic acid (the most abundant phenol in pears [28]) were also calculated at pH 7. A Km value of 15.4 ± 1.1 m*M* and Vmax value of 241 ± 20 μ*M* min^{-1} were determined in the same assay conditions as for AA. It is noteworthy that despite the high Vmax value for chlorogenic acid (around 40 times higher than that for AA) the Km value was around 30 times lower for AA than for chlorogenic acid, which means that pear PPO has a higher affinity for AA. The catalytic power (Vmax/Km) of pear PPO towards chlorogenic acid and AA had values of 1.56 x 10^{-2} min^{-1} and 1.1 x 10^{-2} min^{-1}, respectively. This relatively small difference could support the hypothesis that in a defined microenvironment in pear tissue, in which both pear PPO and AA are present, the oxidation of AA catalysed by pear PPO is possible.

4.3.4 Effect of tropolone and sodium chloride

AO activity was found in fractions in which pear tyrosinase was not present (Fig. 4.1). It is reasonable to think that fractions (which were discarded) with this activity contained AO. However, to corroborate that the AO activity in our assay medium was due to pear PPO activity and not to remaining traces of pear AO, the inhibitory action of tropolone (a copper chelator) and sodium chloride (a competitive PPO inhibitor) was studied (Table 4.1). For this purpose, commercial AO from cucumber as standard AO was used. A strong inhibition of this AO was found in the presence of tropolone. This inhibition was higher for cucumber AO than for both diphenolase (when TBC was used), and AO activities of pear PPO which agreed with earlier studies on mushroom and frog epidermis tyrosinases [33, 98]. To study the effect of sodium chloride the assays were performed at pH 5.5 because chloride ions show their inhibitory effect at pHs lower than 6 [33]. In the presence of sodium chloride, the AO activity of pear PPO was strongly inhibited (100% inhibition with 15 m*M* Sodium chloride), whereas cucumber AO activity was not affected (Table 4.1).

4.3.5 Brown core in pears

Brown core is a storage disorder attributed to the action of tyrosinase. In apples, polyphenols are located in the vacuoles [56], which means that in healthy tissue enzyme and substrate are physically separated by at least the tonoplast (see Chapter 2). In pears, this compartimentation has not been described so far. However, in Chapter 2 it was demonstrated that decompartmentation is likely involved in the development of the brown core in pears.

AA can be found in different compartments of the cell and its concentration depends on species and intracellular location. It has been reported that AA might be synthesised in both cytosol and mitochondria [103]. A new enzyme involved in the biosynthetic pathway of AA, L-galactose dehydrogenase, which probably clears up the last missing step in this pathway was recently reported [39].

It is known that AA is not mainly found in the vacuole [103, 104]. This organelle occupies most of the cell in fruits and vegetables, which means that AA is concentrated in the small remaining cell volume. High values of AA concentration (20-50 m*M*) have been found in both chloroplastic and cytosolic compartments from pea and spinach leaves [103]. An average AA concentration of 152 ± 12 µ*M* was found in the overall cortex of pears used in the present study. As this volume refers to the total cell volume, it can be assumed that its concentration in specific cell compartments of pears is much higher, or lower.

Ponting and Joslyn [34] stated that there is a close relation between browning of apple tissue and AA levels. Browning of the fruit flesh occurs when AA levels drop below a certain threshold. It seems that browning does not occur until all AA in the tissue is oxidised. Brown tissue does not contain AA (Chapter 3).

PPO is generally present in plastids or chloroplasts in intact cells [105], in microbodies [106], in cytosol [92, 107], etc. Extraction protocols used in the present study, with and without the detergent Triton X-114, did not differ in the amount of PPO extracted. This means that pear PPO was not mainly membrane-bound in the cortex tissue of this variety of pear fruit. The sub-cellular location of PPO for pear has not been described yet. However, a recent study [108] on the sub-cellular location of PPO

in apple demonstrates that it is mainly found near the cell walls, probably in plastids but not in the vacuole.

In summary, taking into account that:

I. Pear PPO is able to catalyse the oxidation of AA (this chapter).

II. AA concentration in pear cells is supposed to be high ([103] and own observations).

III. AA levels are critical to maintain membrane integrity (Chapter 2 and 3).

IV. Brown core disorder in Conference pears occurs when decompartmentation takes place, probably -amongst other factors- as a result of the decrease of AA level (Chapter 3).

We suggest a possible implication of pear PPO in the brown core disorder not only by acting on endogenous phenols such as chlorogenic acid, but also on AA. In this way, pear PPO could contribute in the decrease of AA levels and thus accelerate brown core in pears.

Acknowledgements

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5. Ascorbic acid and tissue browning in pears under controlled atmosphere conditions [6]

Summary

The relationships between gas composition during storage and AA levels, and between AA levels and the development of internal browning, are studied in Conference and Rocha pears (Pyrus communis L.). In both cultivars, AA levels decline under (brown core-inducing) CA conditions, i.e. lowered O_2 and enhanced CO_2 concentrations. Brown core is initiated in a cultivar-dependent manner when an AA level threshold was passed. Several models describing the relation between AA levels and browning were fitted to estimate these AA thresholds. These thresholds depend on cultivar, picking date and growing location, varying between 2 and 6 mg $100g^{-1}$ FW.

[6] This chapter is based on: Veltman RH, Kho RM, Van Schaik ACR, Sanders MG, Oosterhaven J. 2000. Ascorbic acid and tissue browning in pears (*Pyrus communis* L. cvs Rocha and Conference) under controlled atmosphere conditions. Postharvest Biology and Technology 19: 129-137.

AA losses in both cultivars exceeded 50% of the initial values. In Conference pears, most AA was lost when fruits were transferred to CA. In Rocha fruits AA was lost mainly during long-term CA.

5.1 Introduction

AA is an antioxidant, which acts against ROS in concert with other antioxidants such as GSH and α-tocopherol, in a system referred to as the AA-GSH cycle [75]. AA may also be involved in the conversion of ACC (a precursor) to ethylene [37], and in photosynthesis, where it removes hydrogen peroxide formed during photoreduction in PS-I [38].

AA is not only important during the life of plants and during storage or shelf life of fruits, but also for human health. In this regard, AA is a quality parameter of fruits, and should be kept at an appropriate level. AA levels, however, tend to decrease during storage and processing of fruits and vegetables.

Generally, AA levels in various apple cultivars are lower under ULO conditions than under air in cold storage [109]. Levels slowly decrease both under CA and during air storage in 'Boskoop' and 'Golden Delicious' [110, 111], and they slowly decline in potatoes during storage by as much as 1/6 of the original amount after 8 or 9 months [112].

As described in Chapter 3, a relationship was found between AA content and the susceptibility to browning in pears during experimental storage under various brown core-inducing conditions. Conference pears tend to develop tissue disorders, like brown core, when AA levels drop below a certain value.

In this chapter we tested and expanded on these results. Pears were monitored in a static system, and gas conditions and storage temperatures were comparable to those in commercial practice. AA levels and the susceptibility towards browning in Conference and Rocha pears were monitored during CA storage throughout the season. AA and browning values are discussed in relation to storage conditions, harvest date and growing location.

5.2 Materials and methods

5.2.1 Plant material

Conference pears (*Pyrus communis* L.) were harvested (orchard 1) in The Netherlands (province Gelderland) on 3, 10, 16, and 24 September, and 1 October, 1997, referred to as picks 1, 2, 3, 4 and 5 respectively. Pick 2 was the optimal harvest date for CA storage (based on firmness of the fruits). Rocha pears were picked on 2 August, 1997 in Portugal at two locations, Santos (orchard 2) and Picarra (orchard 3).

5.2.2 Storage and treatment

Conference pears were precooled at the storage temperature (-1°C) for one week before CA was applied. For bulk storage, Conference pears were stored in a static storage facility as described in Chapter 2 at -1°C, 2 kPa ± 0.1 O_2 and <0.7 ± 0.1 kPa CO_2 (standard CA conditions for Conference fruit) or 3 ± 0.1 kPa CO_2. Relative humidity in the containers was 97-99%.

For short-term experiments (Fig. 5.4), Conference pears were kept in the flow-through system described in Chapter 3 at 10°C to maintain a reasonable rate of metabolism, and under a series of O_2 concentrations (1 kPa, 2 kPa, 7 kPa and 21 kPa) with or without 10 ± 0.1 kPa CO_2. Before the start of the flow-through experiment the pears (pick 2) were stored for 7 months in the static system under standard CA conditions.

Rocha pears were transported to The Netherlands in 3 days by truck under cooled conditions (2-3°C). Before transportation, pears were precooled at –0.5°C. Altogether Rocha pears were subjected to cooled conditions for a period of one month between harvest and application of CA. Bulk storage of Rocha pears took place in the same static system as Conference pears at –0.5°C, under 1 ± 0.1 kPa or 3 ± 0.1 kPa O_2 combined with <0.7 kPa or 3 ± 0.1 kPa CO_2. The standard CA condition for Rocha pears was 3 kPa O_2 and <0.7 kPa CO_2. Rocha pears were also stored under control conditions (21 kPa O_2 and <0.7 kPa CO_2) in the same static system.

For every harvest date (Conference), orchard (Rocha), storage condition, and time (both cvs) 2 samples of 5 pears each were taken. From each sample internal browning (paragraph 5.2.3) and AA content (paragraph 5.2.4) were determined. Determinations for Conference pears were performed at t=0, after 100 days, and after 200 days. Rocha pears were judged at t=0, and after 60, 100, 150, 200 and 270 days after CA had been applied.

5.2.3 Brown index

The brown index was earlier described in paragraph *3.2.4.*

5.2.4 Ascorbic acid measurements

The method for determining AA in pears was earlier described in paragraph *3.2.2.* Fruits were peeled, and cut length-wise. With a corkbore (diameter 17 mm) samples were taken from the cortex tissue just above the core tissue, at the stalk side, and just adjacent to the middle vein. Samples of 5 pears were mixed.

5.2.5 Firmness

Firmness of the Rocha pears during storage was monitored with a penetrometer (plunger diameter 11 mm) placed in a drill stand, using 20 fruits per sample. Values are expressed as Newton.

5.2.6 Statistical analysis

Statistical analyses were done with the ANOVA and FITNONLINEAR directives of the computer program Genstat (Genstat 5 committee, 1993). Average values are accompanied by standard errors between brackets.

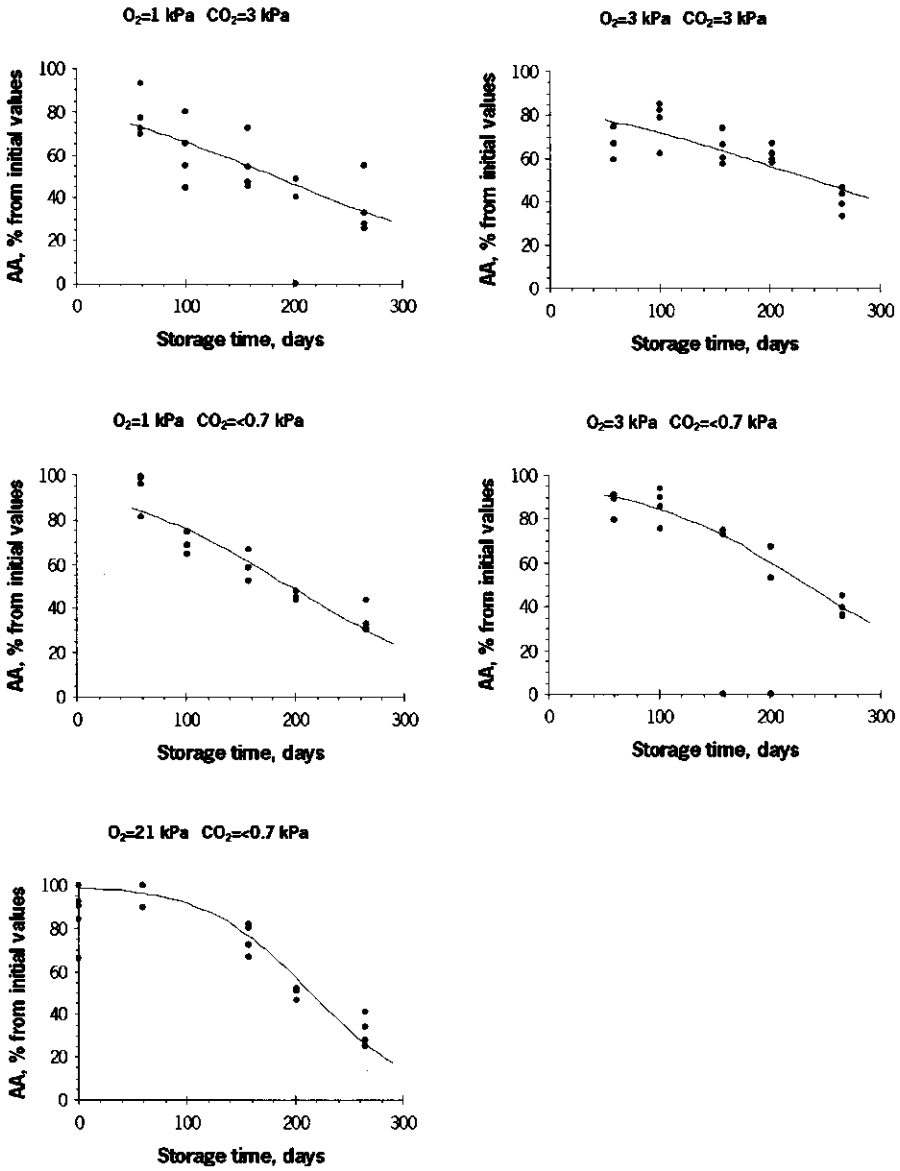

Figure 5.1. Dependence of AA in Rocha pears on storage time and storage conditions; lines fitted according to Eq 5.1 (Table 5.1). Pears were stored at 1 kPa, 3 kPa, 21 kPa O_2 combined with <0.7 kPa or 3 kPa CO_2 (Panel a-e). Every data point (●) is an average of a mixed sample of five fruits. AA losses are given as a percentage of the initial AA concentration just after harvest. The adjusted R^2 of the model (——) is 81%.

5.3 Results

The AA level of Conference pear cortex tissue was 7.21 mg $100g^{-1}$ FW on average at harvest (all picks combined). AA levels strongly declined in the period between harvest and 100 days of storage with 61%, 63%, 71%, 76%, and 73% on average (± 2%) for pick 1 to 5 respectively. On average, losses were 17% (± 1%) higher with enhanced CO_2 (3 kPa) compared to standard CA storage. The reduction in AA levels in pears of pick 1 and 2 differed significantly from pick 3, 4 and 5. After initial losses, no significant further reduction of AA levels was found in the period between 100 and 200 days storage. When pears were stored under cooled conditions, and not transferred to CA 7 days after harvest, higher AA levels were retained for more than a month (data not shown). No AA determinations were done after 1 month because the pears were ripe and had lost much water.

In Rocha pears no decrease in AA levels of more than 35% was seen after 100 days in CA. As distinct from Conference pears, where losses seem to have taken place just after harvest, AA losses in Rocha took place over the whole storage period. After 250 days in standard CA and control conditions, levels were reduced by about 60% (Fig. 5.1, panel d and e), while after 270 days some incidence of browning was seen under both conditions.

Before CA storage, just after the pears arrived in The Netherlands, the initial mean AA concentration was 8.22 (± 0.29) mg $100g^{-1}$ FW for pears from orchard 2 and 6.11 (± 0.24) mg $100g^{-1}$ FW for pears from orchard 3. In Fig. 5.1 these (initial) values are taken as 100%, and AA values from both orchards were taken together. The decrease in AA levels during storage could be described by the sigmoid function of Eq 5.1. The percentage variance accounted for (adjusted R^2) is 81%.

$$AA\% = \frac{100\%}{1 + e^{\beta_{ij} \cdot (\text{storage time} - \gamma_{ij})}} \qquad 5.1$$

Table 5.1. Fitted parameters (Eq 5.1) for Rocha pears, stored in various conditions.

CO_2	O_2		
	1 kPa	3 kPa	21 kPa
3 kPa	ß=0.0081	ß=0.0067	-
	(± 0.0014),	(± 0.0014),	-
	γ=182 (± 13)	γ=239 (± 20)	
<0.7 kPa	ß=0.0119	ß=0.0129	ß=0.0208
	(± 0.0016),	± 0.0020),	(± 0.0028),
	γ=196 (± 9)	γ=233 (± 11)	γ=215 (± 7)

AA% is the AA concentration as a percentage of its initial value, the storage time is in days, and β_{ij} and γ_{ij} are the slope parameter and inflection point respectively, both estimated for each O_2 and CO_2 combination (Table 5.1). Higher CO_2 concentrations (3 kPa, Fig. 5.1, a and b) lead to flatter slopes (β) (Table 5.1). However, at 3 kPa CO_2 the loss of AA during the first 60 days seemed higher than predicted from the line fitted through the data points according to Eq 5.1 (Fig. 5.1). The pears under 3 kPa CO_2 lost more AA during the first 60 days than pears stored under <0.7 kPa CO_2. The inflection point (γ, the middle of the symmetrical sigmoid) was earlier with 1 kPa O_2 than with 3 kPa. The effect of the O_2 concentration on the slope was not significant, nor was the effect of CO_2 on the inflection point.

The firmness of Rocha pears remained the same during the storage period, which indicates that, as with Conference pears (Chapter 3), the decrease in AA was not related to ripening. The initial firmness value was 1.2 N on average, while after 250 days values were still between 1.0 and 1.1 N. This decrease was not significant. Differences in firmness between Rocha pears from the two orchards and pears stored at the different conditions were also not significant.

Browning in Conference pears was only found when fruits were picked after the optimal CA picking date (pick 2) (Fig. 5.2), independent of the CO_2 concentration during storage. In Eq 5.2, internal browning of Conference pears is described as a

function of AA for different picking times (Fig. 5.2). The percentage variance accounted for (adjusted R^2) is 84%.

$$Browning = \frac{\alpha_i}{1 + e^{\beta(AA-\gamma_i)}} \qquad 5.2$$

Table 5.2. Fitted parameters of Eq 5.2 with standard errors for Conference pears at various dates and stored in standard CA conditions (<0.7 kPa CO_2) and under enhanced (3 kPa) CO_2; data from both storage conditions were combined. For all picking times, ß (the slope parameter) was estimated to be 3.28 (± 0.72).

Pick time	$\alpha_{i\,(\%)}$	γ_i (mg 100g FW^{-1})
1	0 (± 2.9)	-
2	0 (± 2.9)	-
3	45 (± 10.9)	1.69 (± 0.27)
4	82 (± 7.3)	1.70 (± 0.12)
5	93 (± 6.4)	1.27 (± 0.10)

In Eq 5.2 AA is given in mg $100g^{-1}$ FW, α_i is an estimated maximum browning percentage depending on picking time, β is a slope parameter, and γ_i is the point of inflection of the curve depending on picking time (Table 5.2). For all harvest dates, β was estimated to be 3.28 (± 0.72).

AA levels in pears from pick 1 and 2 were always higher than 1.1 mg $100g^{-1}$ FW (Fig. 5.2), while no internal browning was found. When pears were harvested later (pick 3, 4 and 5), the estimated maximum increased (α_i, Table 5.2). The value for the inflection point, however, decreased (γ_i, Table 5.2).

According to the browning model (Eq 5.2, Table 5.2), an AA limit can be recognised below which internal browning developed (Fig. 5.2). The AA concentration $100g^{-1}$ FW at which browning exceeded the 5% level was 2.32 (± 0.31)

for pick 3, 2.53 (± 0.24) for pick 4, and 2.15 (± 0.21) for pick 5. Pears of pick 1 and 2 didn't turn brown under the conditions applied in these experiments. However, after a longer period of storage or at conditions that induce internal disorders, these fruits showed internal browning as well (data not shown).

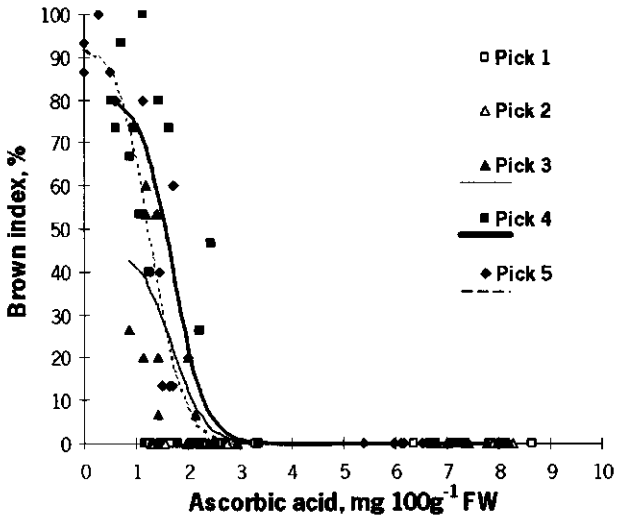

Figure 5.2. Dependence of internal browning of Conference pears on AA and harvest date. Every data point is an average of a mixed sample of five fruits. Data from pears stored in the standard CA conditions (<0.7 kPa CO_2) and under enhanced CO_2 (3 kPa) were combined in this graph. Lines were fitted according to the model formulated in Eq 5.2 with the estimated parameters as described in Table 5.2 for picks 3, 4 and 5. The adjusted R^2 of the model is 84%.

The harvest date seems to be the main factor in the relationship between AA and browning. An estimated, average threshold (seen in pick 3, 4 and 5), below which browning developed, strongly depended on the harvest date.

The first incidence of flesh browning in Rocha pears was seen after 150 days of storage at enhanced CO_2 and/or lowered O_2 concentrations. AA concentrations were generally higher in pears from orchard 2, and these concentrations were clearly more reduced by browning-inducing storage conditions (low O_2, elevated CO_2, Fig. 5.1), than in pears from orchard 3. Absolute AA levels in fruits from orchard 3 were less affected by storage conditions, although, calculated as percentage AA, losses were

comparable for both orchards. The observed browning in fruits of orchard 3 was limited after 270 days.

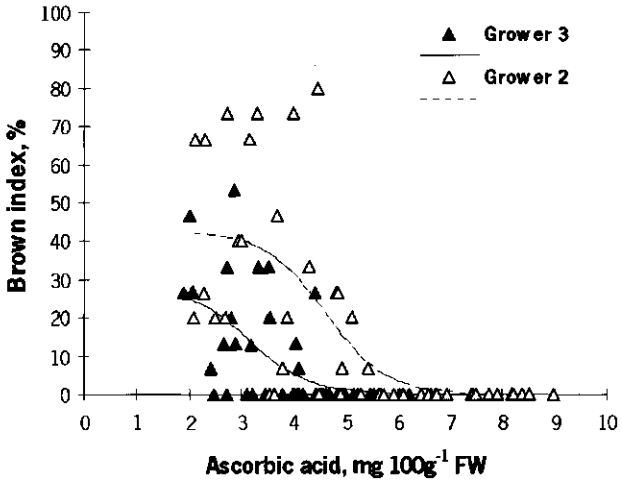

Figure 5.3. Dependence of internal browning of Rocha pears on AA and orchard. Every data point is an average of a mixed sample of five fruits. Data from pears stored in the five conditions described in paragraph 5.2 were combined in this graph. Lines were fitted according to the model formulated in Eq 5.2 with the estimated parameters as described in Table 5.3. The adjusted R^2 of the model is 46%.

In Fig. 5.3, internal browning of Rocha pears as a function of AA and growing location is described. The percentage variance accounted for (adjusted R^2), by the sigmoid given in Eq 5.2 is 46%.Both, α_i, the maximal browning percentage and γ_i, the point of inflection of the curve, depended on growing location (Table 5.3). For both locations, β was estimated to be 1.74 (± 0.75).

According to the browning model (Eq 5.2, Table 5.3), the AA concentration (mg $100g^{-1}$ FW) at which browning exceeded the 5% level was 5.77 (± 0.71) for orchard 2 and 4.04 (± 1.24) for orchard 3.

At higher temperatures, AA levels in Conference pears were affected by gas conditions over a very short term (5 days). Fig. 5.4 shows these levels in pears stored in a flow-through system at four O_2 concentrations with (10 kPa) or without (0 kPa) CO_2. At 10 kPa CO_2, AA was 0.47 (± 0.09) mg $100g^{-1}$ FW lower compared to 0

kPa CO_2. Effects of O_2 concentrations were not significant at the 5% level after five days.

Table 5.3. Fitted parameters of Eq 5.2 with standard errors for Rocha pears stored in 5 different conditions. For both growing locations, β (the slope parameter) was estimated to be 1.74 (± 0.75).

Orchard	α_i (%)	γ_i (mg 100g^{-1} FW)
2	42.6 (± 6.1)	4.61 (± 0.34)
3	28.5 (± 13.0)	3.15 (± 0.71)

5.4 Discussion

AA levels in Rocha pears are not stable during the storage period. During 200 days storage under standard CA and control conditions these levels were reduced by about 50% and 30% for orchards 2 and 3 respectively. A further postponement of the start of CA storage of Rocha pears might retain higher AA levels. In commercial practice, in The Netherlands, Conference pears are precooled for about 20 days before CA storage is started, which avoids brown core to a large extent.

In Conference pears, AA losses were around 70% in the period between harvest and after 100 days standard CA storage. It seems that the main AA decline takes place when fruits are brought under CA. Pears can lose AA in a relatively short time after this event, depending on conditions applied (Fig. 5.4). After CA has been applied, further losses are minimal. This is in contrast to with Rocha pears, where losses take place under CA, while AA differences between standard CA and control conditions were rather small.

AA losses during storage of fruits and vegetables have often been reported. In potatoes, for example, losses vary from 21% to 60% when tubers are stored for 8 months at 5-6°C, depending on the cultivar [78]. AA levels decreased more quickly in apples of 5 different apple cultivars stored under CA compared to storage under low

temperatures without CA [109]. On the other hand, vitamin C in asparagus, peas, snap beans, spinach, broccoli and Brussels sprouts was lost more quickly in air than at low O_2 concentrations, and losses were dependent on storage temperature [113]. Data on vitamin C levels during storage in modified atmosphere packaging (MAP) or CA, however, are relatively rare; a good overview is missing.

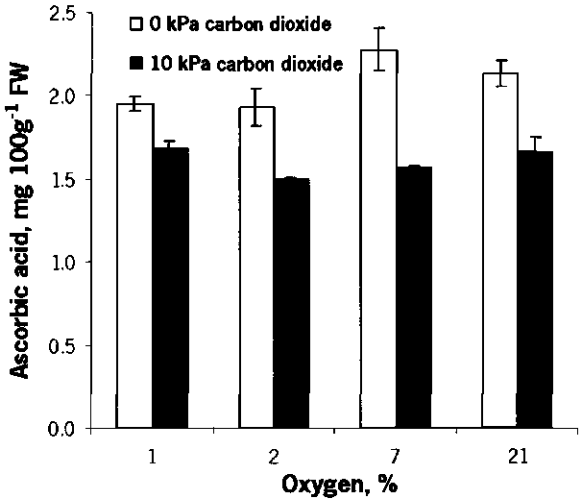

Figure 5.4. AA levels in Conference pears after 5 days of storage in a flow-through system. Fruits were stored in the following conditions: 1, 2, 7 and 21 kPa O_2 combined with 0 kPa or 10 kPa CO_2. From every condition, two mixed samples of five pears were taken. Differences between CO_2 concentrations were significant at the 5% level, those between O_2 concentrations were not. Values are expressed as mg $100g^{-1}$ FW.

In Conference pears, AA levels were reduced by CO_2, both during short-term (5 days) and long-term (storage period) experiments. In Rocha pears, AA losses are more complicated. During the beginning (first 50 days) of storage under CO_2, levels seemed to decrease faster. However, the rate of loss after 50 days was lower than in storage without CO_2. AA levels also clearly decreased under lowered O_2 concentrations during storage of Conference pears (Fig. 3.1). In both pear cultivars enhanced CO_2 and lowered O_2 concentrations induced browning as well.

Comparable results were found by Agar *et al.* [82] in several berry fruits. A rapid decrease in AA was found in strawberries, when stored in 15 kPa CO_2, and the

highest concentrations were found in strawberries stored under air. Comparable results were found in raspberries, red and black currants and blackberries. In pomegranates, AA decreased with increasing CO_2 concentrations [114].

In Chapter 3 it was shown that beneath a certain threshold of AA, pears always show browning, and that AA could be used as an indicator for this disorder. It became also clear that CO_2 lowers AA levels before brown core developed. From the results in this chapter it seems that the harvest date is a complicating factor (Fig. 5.2). Conference pears picked after the optimal date for CA storage show browning already at relatively high AA levels. From these data it seems very likely that, at a physiological level, there is no direct relation between AA and browning, but that at least a third factor is involved. The effect of this third factor is strongly dependent on the harvest date.

For Rocha pears grown in Portugal both the overall AA levels and the AA-threshold for browning were clearly higher (Fig. 5.3). In Chapter 3 it was concluded that AA levels decreased when pears were stored at brown-inducing gas conditions (lowered O_2 or elevated CO_2), and, after this, browning was induced when a certain threshold is reached. The data presented in this paper show the same trend for this cultivar.

AA is an important antioxidant in the plant cell, while browning is an oxidation reaction. However, little is known about the reason why AA concentrations are reduced by enhanced CO_2 and lowered O_2. Storage conditions, storage duration and picking date have a clear influence on AA levels in Rocha and Conference pears. AA levels can probably act as a quality parameter, being an indicator for the development of brown core depending on the picking date. These levels can give information about the quality of fruit during storage. Secondly, AA is an essential substance in the human diet, and therefore one of the reasons fruit is consumed. Suboptimal storage conditions, such as enhanced CO_2 or lowered O_2 concentrations, can result in a diminished food value. This research emphasises that gas conditions should also be optimised for AA contents.

Acknowledgements

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6. Free oxygen radicals and a limited energy availability [7]

Summary

Storage of *Pyrus communis* under hypoxia, especially in the presence of increased CO_2 concentrations, can lead to brown core, a storage disorder in pears. Brown core development, and AA and ATP levels were examined under various compositions of O_2 (0-21 kPa) and CO_2 (0 and 5 kPa) during 31 days of storage. Furthermore, the ATP production was estimated based on respiration data established during this storage experiment. Hypoxia leads to brown core, a decrease of AA and ATP levels, and a lower ATP-production. AA levels were decreased before brown core became visible. Adding CO_2 to the storage atmosphere increased the severity of brown core. CO_2 addition also decreased AA levels with about 46% at O_2 concentrations of 2.5 kPa and higher. CO_2, however, has variable affects on the generation of ATP. No internal browning was found at 0 kPa O_2, and no decrease of AA was recorded at anoxia, independently of the presence of CO_2.

[7] This chapter is based on: Veltman RH, Lenthéric I, Van der Plas LHW, Peppelenbos HW. 2002. Internal browning in *Pyrus communis* fruits is caused by a combined effect of oxygen free radicals and a limited energy availability. Submitted to Postharvest Biology and Technology.

In general, antioxidant (i.e. AA) and ATP levels and production were decreased at conditions that induce brown core. These results support the hypothesis that the initiation of internal browning is a combination of the action of oxygen free radicals, which cause damage to internal membrane structures, and a lack of maintenance energy, because less ATP is produced under hypoxia. This combination leads to decompartmentation of intracellular structures and the initiation of brown pigmentation, which is visible in pears affected by brown core.

6.1 Introduction

For commercial reasons pears are often subjected to low O_2 concentrations (i.e. CA) after harvest to maintain fruit quality for several months. Often pears are found that did not survive these conditions, showing aberrations, like brown core and necrotic tissue. Analysis of the physiological processes involved showed that brown core always precedes necrosis, but that no relation was found between the activity of tyrosinase (EC 1.14.18.1) –the enzyme involved in tissue-browning- and the occurrence of brown core (Chapter 2). Although tyrosinase activity and the total phenolic content of pear cells are crucial factors, their mere presence does not seem to be sufficient to start the process of brown core development. In the cells of healthy tissues tyrosinase and its substrate are physically separated [56]. Brown tissues will therefore only be found after cellular decompartmentation, caused by changes in membrane permeability or membrane disruption.

The research that led to this chapter focussed on the cause of decompartmentation, and the role of CO_2 being the main factor involved in the initiation of brown core. Earlier chapters gave two suggestions for the cause of cellular decompartmentation. Firstly, it can be caused by oxygen free radicals, as they induce lipid peroxidation, leading to membrane breakdown [115]. Free radical action, however, is normally neutralised by antioxidants such as AA, GSH and α-tocopherol, acting together in the so-called AA-GSH-cycle [75]. Agar *et al.* [82] and Haffner *et al.* [109] found that hypoxia can lead to decreased levels of AA in fruits. In Chapter 3 the relation between AA concentrations and the initiation of brown core was shown: brown core could be avoided to a great extent, and AA levels increased

again, when CO_2 concentrations were lowered before an AA level of 1.3 mg $100g^{-1}$ was reached. Lenthéric *et al.* [116] found a correlation between decreasing AA and GSH levels, SOD and catalase activity, and the occurrence of tissue damage in pear fruits.

Secondly, the initiation of brown core in pears can be related to a lack of energy under CA storage. Fruits need a certain maintenance energy (ME) level to survive [69]. CO_2 is known to affect respiration at several points potentially leading to a decreased ATP generation. In this way CO_2 is thought to be indirectly involved in membrane damage.

The goal of this study was to establish how hypoxia and increased CO_2 concentrations affect oxygen radical scavenging and energy generation. ATP concentrations were determined and ATP production was estimated based on respiration and fermentation rates. The dynamics of AA concentrations, as an indicator-molecule for oxygen radical scavenging, are related to energy metabolism, metabolic rates and tissue damage in pear fruits.

6.2 Materials and methods

6.2.1 Plant material

Pears (*Pyrus communis* L. cv. Conference) were harvested in Ommeren, The Netherlands (province Gelderland), on 21 September 1999, two weeks after the optimal picking date for long-term storage. Previous experiments showed that such pears have a high potential for tissue browning when stored under hypoxia and/or enhanced CO_2 concentrations (Chapter 5).

6.2.2 Storage and sampling

Pears were subjected to 0, 0.5, 1, 2.5, 6 and 21 (± 0.1) kPa O_2 combined with 0 or 5 (± 0.1) kPa CO_2 (in duplicate) at 5°C in the flow-through system earlier described in Chapter 3. Pear fruits were sampled after 0, 3, 10, 17, 24, and 31 days of storage under the conditions mentioned. Samples for AA and ATP measurements were taken

from cortex tissue. Per container (12 gas conditions in duplicate; 24 containers) two mixed samples were taken every week. Both mixed samples consisted of material from 5 pear fruits. To prepare the samples, a transverse slice was taken from the largest diameter of the fruit. An ATP sample was taken randomly with a small corkbore (Ø 5mm), halfway the radius of this slice, between the core and the peel. The upper part (stem-side) of the same fruit was peeled, and the cortex was used for the AA determination. Both, AA and ATP samples were directly frozen in liquid nitrogen, and stored in a –80°C freezer for less than a week before measurements were done.

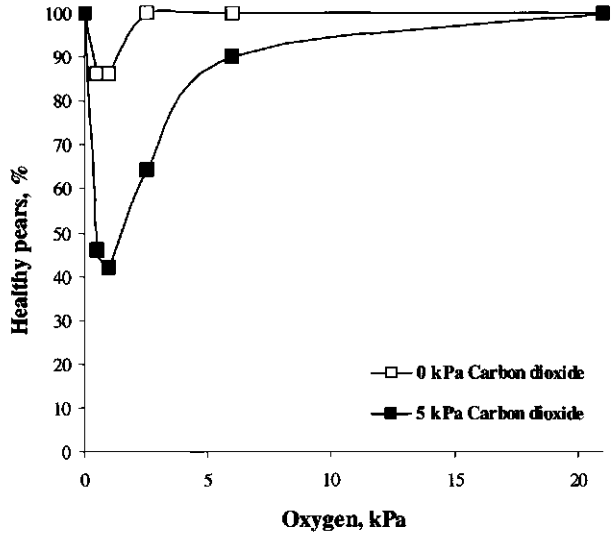

Figure 6.1. Brown core in pears after being stored at various O_2 concentrations with (■) or without (□) 5 kPa CO_2 for 31 days. Values are expressed as percentages healthy pears. The measurements shown in Fig. 6.1 and Fig. 6.2 were done on the same pears.

Gas exchange rates were measured at day 0, 2, 9, 16, 23, and 30 in cuvettes that were connected in duplicate (in series) to the outlet of each container. Ethanol samples were taken from these cuvettes at day 9, 16, 23 and 30.

6.2.3 AA and judgement of internal browning

AA was determined using the method described in Paragraph 3.2.2. Paragraph 3.2.4 describes the evaluation of internal browning of pears, and the brown core-index.

6.2.4 ATP and AEC

Approximately 1 gram of frozen tissue was homogenised in 5 ml 2% w/v tri-chloro acetic acid (TCA) in water with an Ultra-Turrax mixer. Extraction took place on ice for 35 minutes. Samples were centrifuged at 10,000 rpm for 3 min. Twenty-five μl of supernatant was diluted with 75 μl of a freshly prepared 75 *m*M phosphate buffer (pH 7.5) with 0.5 *m*M Phosphoenol Pyruvate (PEP, Sigma P-7127) and 15 *m*M magnesium chloride. For determining ATP amounts in the extracts a luciferin/luciferase kit was used (Bio-Orbit Oy, Finland). Twenty μl of sample diluted in PEP buffer was mixed with 380 μl of TRIS-acetate-EDTA-buffer and 100 μl of reagent from the kit in special tubes (Bio-Orbit Oy). The light emission by the reaction was determined with a Bio-Orbit 1250 Luminometer. Samples were diluted to such an extent that reproducible results could be obtained without TCA removal. Acidity due to TCA was sufficiently buffered by the phosphate buffer in the assay. The assay was first successfully tested with standard ATP solutions (0-100 *n*M) and pear samples combined with these standard ATP solutions.

To be able to calculate AEC values ADP and AMP were determined in the same samples. ADP was transformed to ATP by adding Pyruvate Kinase (PK, 2500 units ml^{-1}, Sigma P-9136, Eq 6.1) to the 80 μl sample left in PEP buffer. This reaction took place in a water bath during 15 minutes at 35°C. After stopping the reaction on ice, the amount of ATP in a 20 μl sample was read as described above. AMP was transformed in ADP (using ATP present in the sample) by adding Adenylate Kinase (AK or Myokinase, Sigma M-5520, 2500 units ml^{-1}, Eq 6.2) to the 40 μl of sample left. This ADP was then transformed in ATP by Pyruvate Kinase, already present in the solution. This reaction took place during 15 minutes at 35°C as well. Hereafter, the reaction was again stopped on ice, and the amount of ATP in 20 μl sample was determined as described above. The enzymatic conversion steps to retrieve ATP

were first tested using purchased ADP and AMP (Sigma). AEC values were calculated using Eq 6.3.

$$2\,ADP + 2\,PEP \xrightarrow{PK,\ Mg^{2+},\ K^{+}} 2\,ATP + 2\,Pyruvate \qquad 6.1$$

$$AMP + ATP \xrightarrow{AK,\ Mg^{2+}} 2\,ADP \qquad 6.2$$

$$AEC = \frac{(ATP + 0.5ADP)}{(ATP + ADP + AMP)} \qquad 6.3$$

6.2.5 Respiration, ethanol production, and estimation of ATP production

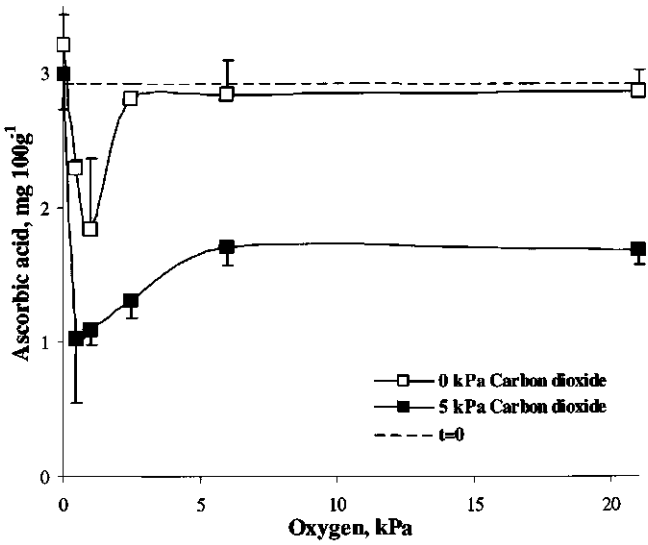

Figure 6.2. AA levels in pears after being stored at various O_2 concentrations with (■) or without (□) 5 kPa CO_2 for 31 days. For every data point AA concentrations, expressed as mg $100g^{-1}$ FW, in 10 pears were averaged. AA levels at t=0 are shown by the dashed, horizontal line. The measurements shown in Fig. 6.1 and Fig. 6.2 were done on the same pears.

Forty-eight cuvettes (1500 ml), with in each one pear enclosed, were connected to the 24 containers (duplicate) of the flow-through system. Respiration of the fruits was determined as described by De Wild *et al.* [12]. Ethanol levels in the cuvettes were determined just before and just after they had been closed for the respiration measurement (see De Wild *et al.* [12]) using a Chrompack (Varian, UK) CP9001 gas chromatograph, with a back-flush. Detector: N_2-stabilised FID. Columns: a WCOT fused silica 25m x 0.53mm ID, CP sil 5CB pre-column, and a WCOT fusef silica 50m x 0.53mm ID, CP sil 8 CB column. The carrier gas was helium (flow 30 ml min^{-1}).

Next to ATP levels ATP production was estimated. Generation of ATP was calculated by combining oxidative and fermentative processes [86]:

$$ATP = (Vo_2 \cdot 6) + (Vco_2 - Vo_2 \cdot RQ_{ox}) \qquad 6.4$$

'ATP' in Eq 6.4 represents the generation of ATP (in *n*mol kg^{-1} s^{-1}), Vo_2 and Vco_2 are the O_2 consumption and CO_2 production respectively (both in *n*mol (kg s^{-1}), and RQ_{ox} is the average respiration quotient at ambient air. Assuming that glucose is the main carbohydrate in pears used for energy generation and that the contribution of the alternative pathway is negligible, 6 ATP molecules per O_2 molecule are produced. For ethanolic fermentation $(Vco_2 - Vo_2 \cdot RQ_{ox})$ an ATP-production of 2 per mol glucose (one for each CO_2 produced) was assumed.

6.3 Results

6.3.1 Development of brown core

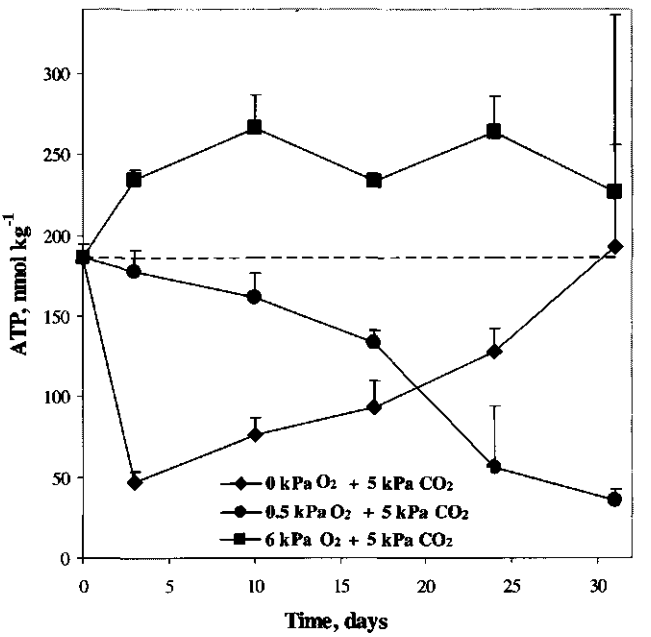

Figure 6.3. ATP concentrations in pears during storage for 31 days at 0 (●), 0.5 (◆) and 6 (■) kPa O_2 with 5 kPa CO_2 (n=4). Values are expressed as nmol kg^{-1}. ATP at t=0 is shown by the dashed, horizontal line.

Pears were stored at a range of O_2 concentrations (0-21 kPa) in an experimental set-up, to establish at which gas composition fruits were most sensitive towards brown core. Pears stored under this range of O_2 concentrations were followed for 31 days and judged regularly. When no CO_2 was added to this range of O_2 concentrations, core browning in pears was only observed at hypoxic conditions (0.5 kPa or 1 kPa, Fig. 6.1). Brown core was observed after 24 days for the first time, and after 31 days of storage at hypoxic conditions the degree of brown core reached 14%. However, brown core did not develop at 0 kPa O_2 (anoxia) during 31 days of storage.

0 kPa CO_2						
O_2	ATP Production		ATP Level		AEC	
kPa	nmol kg^{-1} s^{-1}		nmol kg^{-1}			
	Day 16	Day 30	Day 17	Day 31	Day 17	Day 31
0	15,4	15,1	116 ± 26	119 ± 23	0,72	0,59
0,5	7,5	**17,8**	127 ± 12	90 ± 9	0,87	0,91
1	27,7	**51,4**	140 ± 19	91 ± 29	0,87	0,78
2,5	51,3	113,3	226 ± 27	n.d.	0,97	0,92
6	114,2	163,0	267 ± 15	236 ± 38	0,99	0,93
21	175,7	248,1	333 ± 13	234 ± 71	0,97	0,99

5 kPa CO_2						
O_2	ATP Production		ATP Level		AEC	
kPa	nmol kg^{-1} s^{-1}		nmol kg^{-1}			
	Day 16	Day 30	Day 17	Day 31	Day 17	Day 31
0	11,1	11,4	93 ± 17	193 ± 63	0,68	0,64
0,5	**25,9**	**48,2**	133 ± 8	36 ± 7	0,91	1,00
1	**40,8**	**90,7**	151 ± 22	103 ± 32	0,92	0,97
2,5	**56,6**	**94,1**	239 ± 22	174 ± 35	0,97	n.d.
6	**46,3**	**97,8**	233 ± 2	227 ± 109	0,96	0,92
21	149,7	156,7	252 ± 22	281 ± 132	0,96	0,79

Table 6.1. ATP metabolism of pears stored at various O_2 and CO_2 compositions. The ATP production (nmol kg^{-1} s^{-1} FW) at day 16 and 30 was calculated using Eq 6.4, based on respiration data. The actual O_2 concentrations at 0 kPa were 0.19 kPa on average. ***Bold*** *values indicate pears that were affected by brown core. ATP levels on day 17 en 31 are expressed as nmol kg^{-1} FW. The ATP level at day 0 was 186 nmol kg^{-1} FW. AEC values are calculated using Eq 6.3.*

Brown core development was not only greatly enhanced when pears were subjected to an elevated CO_2 concentration (5 kPa) (Fig. 6.1), but was observed earlier as well: already after 17 days the first signs appeared at 0.5, 1, 2.5 and 6 kPa O_2. Clearly brown core was also observed at O_2 pressures where no browning occurred in the absence of CO_2. After 31 days of storage at 0.5, 1, 2.5 or 6 kPa O_2 combined with 5 kPa CO_2 the degree of brown core increased to 54, 58, 36 and 10%, respectively (Fig. 6.1). At 0 kPa and 21 kPa (normoxia), however, again no brown

core occurred. Also when pears stored at 0 kPa O_2 (with or without CO_2) were re-exposed to normoxia brown core did not develop.

6.3.2 AA levels

At various moments (every week) during the period of 31 days storage pears were judged for brown core and AA was determined in the cortex tissue of the same fruits. During 31 days of storage at 6 kPa and 21 kPa O_2 (no CO_2 added) pears retained the same AA level. However, after 31 days storage at 0.5 kPa and 1 kPa O_2 AA concentrations in pears were significantly decreased with 20% and 35%, respectively, compared to the t=0 level (2.92 mg $100g^{-1}$, Fig 6.2). This decrease in AA was already significant before brown core appeared (at day 24) under these O_2 concentrations. At day 17 the decrease of AA was 19% and 30% for 0.5 kPa and 1 kPa O_2, respectively. After 10 days this was 13% and 20%, respectively. In pears stored at 2.5 kPa O_2 and 0 kPa CO_2 AA also decreased (day 17, with 21%), but recovered after day 17, with no net decrease in AA levels and no brown core development after 31 days. Also, no significant decrease of AA concentrations in pears stored at 0 kPa O_2 was recorded.

AA levels were much more reduced at 5 kPa CO_2 compared to storage without CO_2. After 31 days at 0.5 kPa or 1 kPa O_2 with 5 kPa CO_2 AA levels were reduced with about 65% (Fig. 6.2). Also at 2.5-21 kPa O_2 AA had declined with 46% on average compared to the t=0 level. At anoxia the AA level did not change during 31 days of storage in the presence of 5 kPa CO_2.

6.3.3 ATP levels

In the same pears that were used for AA analysis ATP concentrations were determined. In pears stored at 2.5, 6 and 21 kPa O_2 (no CO_2 added) for 17 or 31 days ATP levels were retained or increased compared to the t=0 level, 186 *n*mol ATP kg^{-1} (Table 6.1). However, when pears were stored at 0.5 kPa or 1 kPa O_2 for 31 days ATP levels decreased with about 50% (Table 6.1). Also at 0 kPa O_2 the ATP concentration at day 31 was significantly decreased compared to t=0.

Five kPa CO_2 caused a larger decrease in ATP levels after 31 days at 0.5 kPa O_2 compared to the decrease without CO_2 (Table 6.1). At higher O_2 pressures, however, no significant effect of CO_2 on ATP levels was detected. At day 31 the ATP levels at 0 kPa O_2 with 5 kPa CO_2 were –in contrast to 0 kPa O_2 without CO_2- not significantly affected compared to t=0 (186 *n*mol kg^{-1} FW).

The comparison of ATP levels at t=0 and t=31 days is an oversimplification. After being kept for 3 days at 0 kPa O_2 with 5 kPa CO_2, ATP levels in pears declined with 75%. Subsequently, however, ATP levels slowly increased in time (Fig. 6.3), and after 31 days the ATP level equalled the value at t=0. When pears were stored at 0.5 kPa O_2 with 5 kPa CO_2, however, the ATP concentration gradually decreased compared to the t=0 value, resulting in a 81% decrease at day 31 (Fig. 6.3). At 6 kPa O_2 and 5 kPa CO_2 a relatively small increase compared to the value at t=0 seemed to occur that did not change during the 31 days period (Fig. 6.3).

ATP levels in pears stored at anoxia without CO_2 were decreased when day 0 and 31 were compared, while with CO_2 ATP concentrations at day 0 and 31 were equal (Table 6.1). A possible explanation for this increasing ATP level at 0 kPa O_2 with CO_2 is a stimulation of the fermentative ATP production. Indeed a significant ethanol production was observed at anoxia. However, ethanol emission rates were not stimulated during storage at anoxia when CO_2 was added (result not shown).

6.3.4 ATP levels and estimated ATP production

At all storage conditions gas exchange rates of the stored pears were determined during 31 days, and based on these rates the generation of ATP was estimated (Table 6.1). Table 6.1 indicates a large difference between the estimated ATP production at normoxia (21 kPa O_2) and hypoxia (0.5 kPa O_2), especially at storage without CO_2. At anoxia, the (fermentative) ATP production was very low and comparable with and without CO_2 (Table 6.1).

ATP production rates were high when compared to the measured ATP levels, turn-over times being around 5 sec on average. AEC values, however, were generally close to 1 at O_2 pressures of 0.5 kPa and higher, which does not suggest that ATP turn-over rates could not keep up with ATP demands. Low O_2 had a clear effect on

both ATP levels and estimated ATP productions. However, there seems to be a discrepancy when ATP levels and estimated productions at day 17 and 31 are compared at 0.5 and 1 kPa O_2 (no CO_2 added). In two weeks the estimated ATP production doubles, but ATP levels decreased with about 30%. The same trend was observed at 0.5 kPa or 1 kPa O_2 with 5 kPa CO_2. This suggests an increased demand for ATP during the period brown core was initiated.

6.4 Discussion

In The Netherlands, in commercial facilities, Conference pears are normally stored at O_2 pressures between 2 kPa and 3 kPa. Based on empirical knowledge, CO_2 is kept below 1 kPa to avoid disorders like brown core. One of the hypotheses on the cause of internal browning in pears is that ATP levels drop below maintenance levels [69]. Brown core is thought to be initiated because not enough energy is available for membrane maintenance. A direct relation between energy metabolism and membrane integrity has been demonstrated in potato cell cultures by Rawyler *et al.* [117]. Potato cells subjected to anoxia showed an ATP synthesis threshold below which membrane lipids hydrolysed. The initiation of brown core in pears on a cellular level is marked by the decompartmentation of internal structures due to membrane damage (Chapter 2). The physical barrier between tyrosinase (EC 1.14.18.1), the PPO that causes browning, and polyphenols, located in the vacuole, (partly) disappears. CO_2 was always regarded as a main factor causing brown core, next to other important factors like harvest date of the pears (Chapter 5) and the O_2 concentration during storage, (Fig. 6.1). But, the sensitivity of pears towards CO_2, causing the brown core disorder, seems to increase with decreasing O_2 concentrations. This suggests that CO_2 in a way affects respiration and energy generation. Williams and Patterson [23] and Hulme [118] already stated that increased CO_2 levels can inhibit succinic dehydrogenase activities, resulting in an accumulation of succinic acid, a toxicant for plant tissues. CO_2 might also influence other parts of the respiration process; effects on more upstream (glycolysis) and downstream (electron transport chain) parts have both been described. In Bartlett pears it was shown that fructose-6-phospate was accumulated and fructose-1,6-diphosphate was

substantially reduced under CO_2, which suggests that CO_2 has an inhibitory effect on phosphofructokinase [119]. Furthermore, Gonzàlez-Meler *et al.* [120] reported the inhibitory effect of CO_2 on cytochrome c oxidase in soybean.

It is not directly clear that ATP production or ATP levels are specifically affected by CO_2 at O_2 levels which give rise to brown core when storage in the presence and in the absence of CO_2 (i.e. 2.5 and 6 kPa O_2; Fig. 6.1) are compared. The ATP-production at 2.5 and 6 kPa O_2 was the same in the presence of 5 kPa CO_2 (Table 6.1), while at 6 kPa with 5 kPa CO_2 the degree of brown core was very limited. The ATP levels at 2.5 and 6 kPa were not clearly different from the control levels at t=0 or from each other. Therefore, no indications for ATP levels or ATP production being directly casually involved in CO_2-induced brown core could be found. Apparently ATP production, ATP concentrations and AEC are not the only factors involved in brown core initiation: at 0 kPa CO_2, the estimated ATP production and ATP levels are both low, but no internal browning occurred (Fig. 6.1, Table 6.1).

A second explanation for the membrane damage leading to decompartmentation of intracellular structures is oxygen free radical action. Numerous examples are given in literature of the relation between radicals and tissue damage. Bailly *et al.* [121], for instance, found a relation between viability loss in sunflower seeds and the content of free radical scavenging enzymes. Ageing of potato tubers is accompanied by a progressive increase in oxidative stress, as evidenced by an increased activity of the GSH-mediated free radical scavenging system [71]. The mitochondria in the cell are generally considered as the main or only source of radical formation. Not all O_2 is tetravalently reduced to water via cytochrome oxidase in the respiratory chain. A small proportion of the O_2 accepts only one electron, and superoxide is formed. Ubisemiquinone seems to serve as the primary electron-donor, responsible for 80% of the superoxide formation [122]. There are several indications that oxygen radicals are indeed involved in the development of browning in the present study. Firstly, AA levels in pear tissue decreased sharply at conditions that induce brown core, i.e. at low O_2 (0.5-1 kPa) and high CO_2 (5 kPa). Furthermore, AA levels drop before the onset of browning (AA decreases are not caused by cell death caused by brown core itself, Chapter 3), which was also shown in this investigation, and browning can be largely avoided by changing gas conditions before this onset. Thirdly, at 0 kPa O_2 no

oxygen free radicals are formed, and in agreement with this, but still unexpectedly, no brown core was observed in pears stored at anoxia during the period of 31 days (with or without CO_2).

However, the observation that AA levels at 21 kPa O_2 with 5 kPa CO_2 were lower than at 0.5 kPa O_2 without CO_2 (Fig. 6.2), while under the first condition -in contrast to the second- no internal browning was observed (Fig. 6.1) does not fit into a simple explanation, in which AA levels solely explain internal browning. It seems that CO_2 causes AA to equilibrate at a lower level in a process independent of O_2 concentrations (Fig. 6.2). Fig. 6.2 suggests that CO_2 directly affects AA levels, which are lowered by about a 46% on average (6 and 21 kPa O_2). AA levels were not affected and brown core was not observed when pears were stored at anoxia, probably because no oxygen radicals have to be neutralised. These observations only indirectly indicate the involvement of radicals in the development of brown core. Further research should elucidate the direct involvement of free radicals.

None of the determined parameters alone (AA and ATP levels, estimated ATP generation, AEC) could be correlated directly to the development of internal browning. More likely brown core is caused by a combination of these (and other) factors. In other words: by a combination of a low energy availability and the action of oxygen free radicals. Based on Fig. 6.1 and 6.2 we suggest that at hypoxia oxygen radicals are present, but the amount of available energy is somehow too low to maintain membrane integrity and regenerate antioxidants to counteract these oxygen radicals. This opens two options as a reaction; an increase in ATP generation or a decrease in energy utilisation. Under hypoxia (CA) the first option, with O_2 availability being limited for respiration, is not possible. Therefore, not all the ATP-consuming reactions, necessary for the survival of the tissue can be carried out properly. When, as a consequence, oxygen free radical scavengers are not recycled, reduced levels of AA (Fig. 6.2) can be the consequence. The combination of low levels of scavengers and ongoing radical production results in decompartmentation and the browning reaction. Only under (near) anoxia, the need for scavenging reactions has disappeared and ATP is only necessary for maintenance: apparently the fermentative ATP-production is enough for maintenance-related processes.

Although in this experiment the above hypothesis could explain the initiation of brown core under hypoxia the exact role of CO_2 is still not clear. Future research should give more insight in the effect of increased CO_2 concentrations on the antioxidant metabolism, and bring up other sites of action of CO_2, which are important for the initiation of brown core.

Acknowledgements

The authors like to thank Tjerk Lammers, Els Otma, Nadia Rascalou and Erik Schaap for technical assistance. Special thanks to Cristian Larrigaudièrre for reading the manuscript. The European Commission financially supported this work under contract FAIR-96-1803.

7. Do pears with internal browning emit ethane due to membrane peroxidation? [8]

Summary

In Chapter 3 it was suggested that lipid peroxidation can be the cause, or one of the causes, for membrane breakdown, having intracellular decompartmentation as a consequence. It was also suggested that ethane can act as an indicator for this membrane breakdown, being a waste product of certain fatty acids in the cell membranes. In this chapter the ethane emission of pears affected by brown core is further investigated. The method for measuring ethane, making use of a laser-based photoacoustic detector, did not reveal that affected pears produce ethane.

[8] This chapter is based on unpublished results from measurements performed in co-operation with Stefan T. Persijn and supervised by Frans J.M. Harren at the University of Nijmegen (Department of Molucular- and Laser Physics).

7.1 Introduction

During lipid peroxidation molecular O_2 is incorporated in unsaturated fatty acids (LH) to form lipid hydroperoxides (LOOH). A so-called 'initiator' promotes lipid peroxidation (I•, Eq 7.1).

$$I\bullet + LH \longrightarrow IH + L\bullet \qquad \textit{Initiation} \quad 7.1$$

$$L\bullet + O_2 \longrightarrow LOO\bullet \qquad \textit{O}_2\textit{-addition} \quad 7.2$$

$$LOO\bullet + LH \longrightarrow LOOH + L\bullet \qquad \textit{Propagation} \quad 7.3$$

This initiator causes hydrogen abstraction and formation of a lipid alkyl radical (L•, Eq 7.1). Lipid alkyl radicals can easily add O_2 to form lipid peroxyl radicals (LOO•, Eq 7.2). Via propagation the peroxidation reaction proceeds (Eq 7.3). Transition metals (e.g. Fe^{2+}) can substantially enhance the propagation. Fe^{2+} cleaves LOOH to highly reactive alkoxyl (LO•, Eq 7.4) radicals, which in turn abstract hydrogen from lipids to form new alkyl radicals (Eq 7.5).

$$Fe^{2+} + LOOH \longrightarrow Fe^{3+} + LO\bullet + OH^- \qquad 7.4$$

$$LO\bullet + LH \longrightarrow LOH + L\bullet \qquad 7.5$$

It is hypothesised that lipid peroxidation is initiated by OH•, formed via the **Haber-Weiss** reaction, that consists of the superoxide dependent Fe^{3+} reduction and subsequent Fe^{2+} catalysed H_2O_2 cleavage (Eqs 7.6-7-8) [123].

$O_2^- + Fe^{3+} \longrightarrow O_2 + Fe^{2+}$ 7.6

$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH\bullet + OH^-$ *Fenton reaction* 7.7

$O_2^- + H_2O_2 \longrightarrow OH\bullet + OH^- + O_2$ *Harber-Weiss reaction* 7.8

Dumelin and Tappel [124] showed that hydrocarbon gases evolve from fatty acid hydroperoxides, and not during peroxidation. The major hydrocarbon gas products are pentane from ω-6 fatty acids, and ethane or ethylene from ω-3 fatty acids, like linolenic acid.

John and Curtis [125] hypothesised that linolenic acid is the precursor for ethane. When root tissue was homogenised in a linolenic acid enriched solution the tissue liberated high levels of ethane. Lieberman and Mapson [126] confirmed this finding. They concluded that the ethane producing particulate system requires the presence of an unsaturated fatty acid.

In Chapter 3 (Fig. 3.5) it was indicated that brown pears and pears with cavities produce and emit ethane. Unless the limited set-up of the (pilot) experiment, the amount of ethane emitted could be more or less correlated to the measure of browning and cavities. In this chapter we tried to prove that ethane emission of pears is the result of peroxidation by:

I. Switching gas conditions during measuring ethane from normoxia to anoxia. No formation of oxygen radicals is to be expected under anoxia. Therefore, it was expected that the ethane emission decreases after switching to anoxia, and is restored after switching to normoxia

II. Accumulation of ethane from pears with a varying brown core and cavity incidence, to further establish the relation between severity of aberrations and the amount of ethane produced on fruit level.

III. Trapping ethylene to increase the sensitivity of ethane measurements.

IV. Examining pieces of tissue from the fruit –brown and healthy- to localise the site of ethane formation on tissue level.

7.2 Materials and methods

7.2.1 Plant material and storage

Table 7.1. Laser lines selected to measure ethane with a CO laser in the overtone infrared region. One laser line was selected for each gas to be determined. At these lines there is a strong absorption of these gases (24P8 for ethane and 24P14 for ethylene, Fig. 7.2 and Fig. 7.3). The other lines are necessary to correct for the water emission, and to calculate concentrations of gases [128].

	Laser line	**Frequency** cm^{-1}
1	22P11	309.397.998
2	22P12	30.901.499
3	23P12	30.411.499
4	24P8	3006.95
5	24P9	30.033.898
6	24P10	2999.76
7	24P14	2984.57
8	26P12	2895.04

Pears were harvested in Ommeren (The Netherlands) at the optimal harvest for CA storage, and two weeks hereafter. Late (after the optimal date for CA storage) picked pears after were susceptible for internal browning and the formation of internal necrosis (cavities) to a high extent. Pears were stored for 5 months under standard CA conditions in a static system as described in Chapter 2. With late picked pears it was attempted to show the relation between internal browning and the measure of ethane formation; healthy pears from the optimal harvest date acted as a control in this experiment.

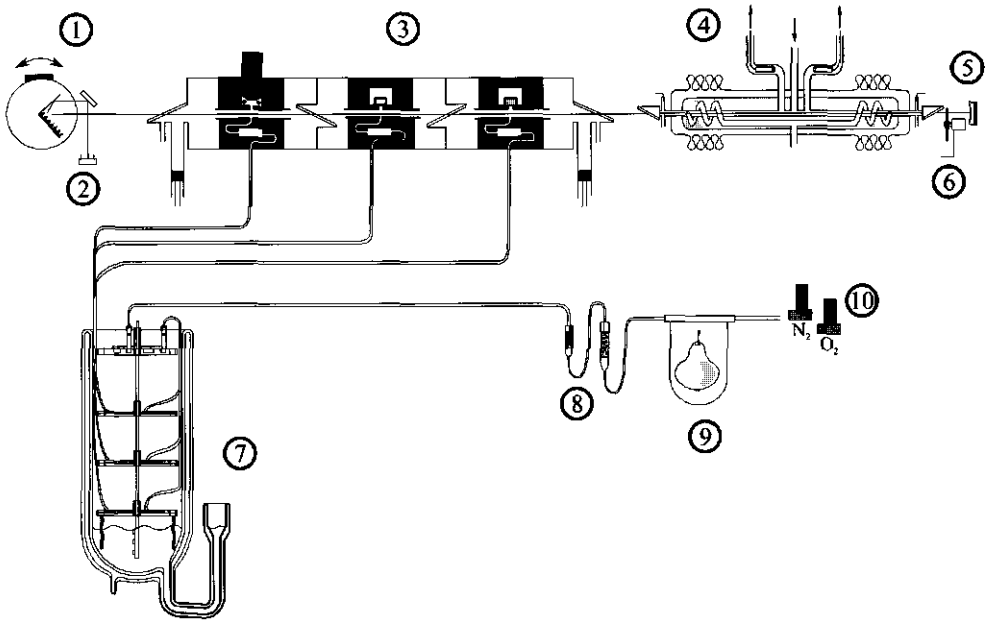

Figure 7.1. Overview of the used equipment: ① step motor, ② power meter, ③ three photoacoustic cells, ④ laser tube with liquid nitrogen cooling, ⑤ mirror, ⑥ chopper, ⑦ cooling trap to pre-clean gas samples, ⑧ Ethysorb trap to remove ethylene and a potassium carbonate trap to remove water, ⑨ 1-L cuvette with a fruit enclosed, ⑩ Mass Flow Controllers to compose gas conditions. Picture kindly provided by the department of Molecular- and Laser Physics of the University of Nijmegen.

7.2.2 Laser equipment

The ethane and ethylene releases were monitored using a CO-laser-based photoacoustic (PA) detector (Fig. 7.1). The detector consisted of three PA cells placed inside the cavity of a liquid nitrogen-cooled CO laser [127]. Three cuvettes were connected to the three PA cells. One cuvette was used to determine the background concentration of ethane and ethylene, while the two other cuvettes were used in a duplicate experiment. PA signals were determined on eight laser frequencies and the concentrations of the gases under investigation were calculated using the matrix calculation as has been developed by Meyer and Sigrist [128]. A complete measurement cycle lasted approximately five minutes, which is short with respect to the low flow rates and slowly changing gas emission of the pears.

7.2.3 Ethane and ethylene spectra

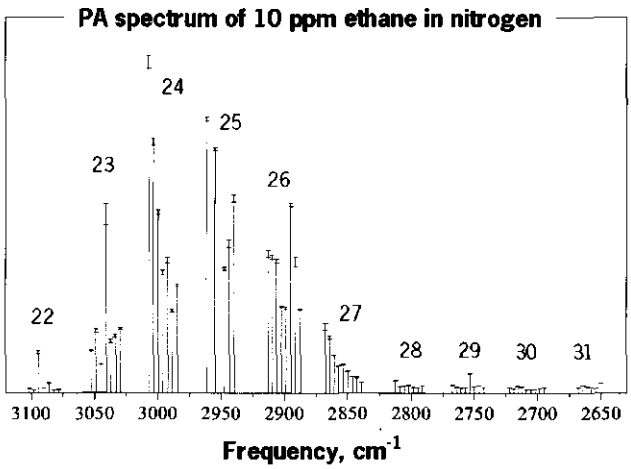

Figure 7.2. PA spectrum of 10 ppm ethane diluted in N_2. The PA spectrum shows only the strongest absorption lines in the CO overtone laser region. Numbers refer to different vibrational bands. Every vibrational band consists of several rotational transitions.

Ethane and ethylene emissions were monitored using a CO-laser-based photoacoustic (PA) spectrometer. The CO laser operated in the overtone mode (2.5-4 μm) where both molecules posses a clear fingerprint absorption pattern (Fig. 7.2 and Fig. 7.3). The spectrometer consisted of three PA cells placed inside the cavity of a liquid nitrogen-cooled CO laser [127]. Introduction of three PA cells inside the laser cavity (which means that the laser-beam is passing 4 Brewster windows) only slightly affected the laser intensity and gave therefore PA signals that were similar to a CO laser operated with only a single PA cell.

A single fruit was enclosed in a cuvette with a volume of 1 litre. A gas mixture of nitrogen and O_2 was passed over the fruit at a flow rate of 1 l $\cdot$ h^{-1}. The O_2 level in the sample flow was monitored using a Xendos 1800 O_2 analyser. A cold trap operated

at about –150°C reduced the spectral interference by water and other gases but did not affect the concentration of ethane and ethylene.

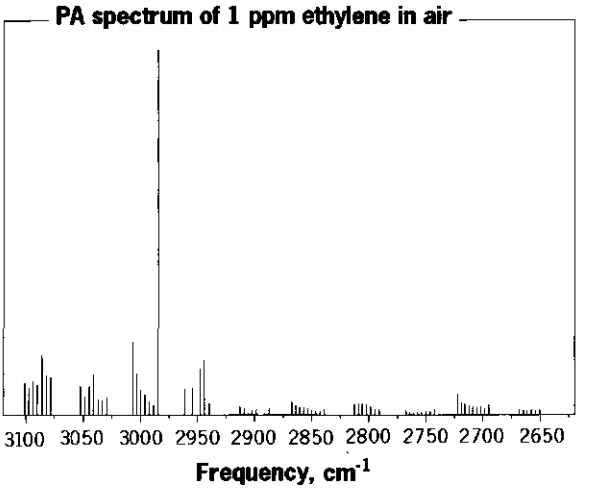

Figure 7.3. PA spectrum of 1 ppm ethylene diluted in air. The PA spectrum only shows the strongest absorption lines in the CO overtone laser region.

7.3 Results and discussion

7.3.1 High ethylene emissions

Pears produced ethylene at a much higher rate than ethane (a few hundred times), making ethane detection difficult due to spectral interference. Therefore, ethylene was removed from the flow exiting the cuvette using a potassium permanganate (Ethysorb) scrubber that oxidises alkenes to form glycols (e.g. ethylene is oxidised to ethylene glycol, Eq 7.9). As a result measurements on ethane became more reliable and less noisy.

$$3C_2H_4 + 2KMnO_4 + 4H_2O \longrightarrow 2MnO_2 + 2KOH + 3CH_2OHCH_2OH \qquad 7.9$$

The permanganate scrubber removed ethylene for 100%, while ethane from a bottle passed it for 100%. However, no ethane emission from pears could be established. Even at the lowest possible flow, 1 l h^{-1}, ethane emissions were below noise levels (0.5-1 ppb). When gas from a bottle was mixed with gas from the cuvette with the pear, no ethane was halted by the scrubber. No relation could be established between the degree of internal browning and ethane emissions in flow-through experiments. And, no relation was shown between gas conditions and ethane formation after switching from normoxia to anoxia and back.

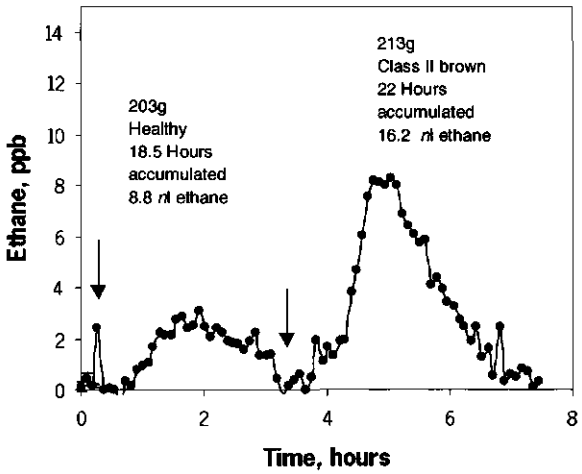

Figure 7.4. Typical results of an accumulation experiment. Average ethane releases were very small. The accumulated gas in two cuvettes with one pear each was determined one after the other.

7.3.2 Accumulation of ethane

To increase the sensitivity of the measurements ethane was accumulated in closed cuvettes for more than 15 hours at ambient air (room temperature). To decrease the effect of ethylene and CO_2 both permanganate and carbonate grains were applied at the bottom of the cuvette. After accumulation small amounts of ethane were observed. In Fig. 7.4 the gas emitted from two separate pears was determined, one healthy, the other with moderate browning (class II, for the

classification of brown core and cavities see section 2.2.3). However the ethane emissions were extremely low compared to the accumulation period.

Table 7.2. Overview of experiments on ethane detection. Ethane was determined in gas emitted by whole fruits and fruit pieces ('Flow-through'). Fruit pieces were taken from healthy tissue or completely brown tissue from the same fruit. Ethane was also determined after accumulation in a cuvette ('accumulation'). nd = not detectable.

	Description	Method	C_2H_2 release
			pl g^{-1} h^{-1}
1	Healthy	Accumulation	2,5
2	Brown class II	Accumulation	3,5
3	Healthy	Accumulation	2,3
4	Severe cavities, class I	Accumulation	1,3
5	Healthy	Flow-through	nd
6	Healthy	Flow-through	nd
7	Brown class I, cavities class I	Accumulation	nd
8	Brown class I, cavities class I	Accumulation	>3
9	Piece: healthy tissue	Flow-through	nd
10	Piece: healthy tissue	Flow-through	nd
11	Piece: brown tissue	Flow-through	nd
12	Piece: brown tissue	Flow-through	nd

After 18.5 and 22 hours respectively only 8.8 and 16.2 *n*l ethane accumulated. Furthermore, it became clear that not only internally injured fruits produce ethane, but also healthy ones, stored at standard CA conditions. Summarising, the relation between internal aberrations and the formation of ethane was not clear (Table 7.2). Even after very long accumulation periods, ethane emissions were extremely low. There are no indications that a lack of O_2 inhibited ethane formation (Fig. 7.5). O_2 levels were never lower than 15 kPa, and no inhibition of respiration are to be expected under these circumstances (Chapter 8). Respiration was estimated to be about 300 *n*mol s^{-1} kg^{-1} FW (at 22°C) at normoxia.

7.3.3 Ethane emission from pear pieces

To establish the source of ethane formation pieces of tissue were taken (with a corkbore) from fruits. In these experiments pieces of healthy tissue were compared to pieces of brown tissue. However, ethane emissions from these pieces were too small to be detected. Furthermore, the pieces produced a lot of ethylene (attributed to wounding during sampling), and they dried out very quickly, which disturbed the measurements. In earlier experiments with avocado (data not shown) exactly the same problems showed up, and it was concluded that ethane and ethylene can only be determined properly on whole fruits.

7.3.4 Conclusions

Before ethane could be determined properly the problem of interference with ethylene had to be solved. Ethylene, which is produced during ripening of the fruit, is emitted in large amounts by (pre-) climacteric pears (and other fruits). Permanganate grains removed ethylene for 100% in flow-through experiments, and for the major part during accumulation experiments. After the interference problems were solved, ethane emissions were obviously very low, also in pears with evident internal browning and/or cavity formation, which is in contradiction with the results presented in Fig. 3.5. Although it can not be excluded that this difference in ethane emission can be explained by orchard factors (year of growth, place of growth, etc), this difference is more likely a result of an artefact.

Another explanation is that in Fig. 3.5 not ethane but methane was determined. During ethane experiments (results not shown) it became clear that methane can be a disturbing factor. Methane and ethane were very hard to separate. When methane would be a disturbing factor, the origin of it in the experiments presented in Fig. 3.5 is not known, since all gases used were from bottles or were cleaned by passing a catalysator. It is not known how methane production would depend on the severity of internal browning and formation of cavities.

Recent experiments did not show a relation between gas conditions and ethane emission (data not shown). Ethane was determined while gas conditions were

switched from normoxia (21 kPa O_2) to anoxia. The idea was that during normoxia radicals are formed, which cause lipid peroxidation, and next the formation of ethane, while at anoxia no oxygen free radicals are formed, and no ethane is expected. Konze and Elstner [87] found that anaerobic conditions reduced both endogenous ethylene and ethane formation in potato. They hypothesised that ethane is formed from α-linolenic acid. Furthermore, they hypothesise that the ethane formation can be owed to peroxidation processes taking place in mitochondria or chloroplasts.

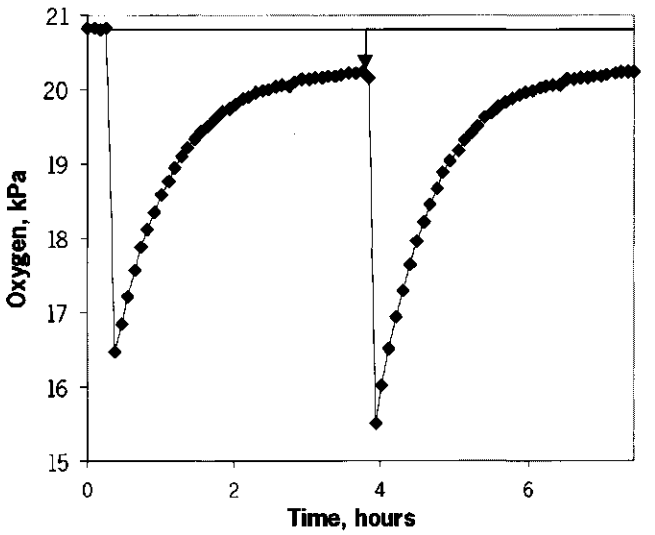

Figure 7.5. A characteristic post-accumulation O_2 response curve. After accumulation of ethane cuvettes were reconnected to the flow through system, and the O_2 exiting the cuvette was monitored. The O_2 concentration increased, however it never reached the level of 20.8 kPa as at t=0, because of fruit respiration (indicated by the arrow). Steady-state O_2 consumption is approximately 300 nmol s^{-1} kg^{-1} FW (22°C). In succession the O_2 consumption of two pears was established. There were no indications that respiration is inhibited significantly during accumulation, were O_2 levels as low as 15.5 kPa were reached).

Acknowledgements

I like thank all the personnel of the department of Molecular- and Laser Physics of the University of Nijmegen for introducing me into the world of PA-lasers, and all their help and patience with answering my questions.

8. O_2 and CO_2 skin permeances of pear [9]

Summary

To determine the skin permeance for O_2 and CO_2 in pear, often neon (Ne) is used, and O_2 and CO_2 permeances are calculated from the Ne permeance using Graham's law. In this paper O_2 and CO_2 permeances are calculated with this method, and compared with a second method using a new gas exchange model that is based on internal and external gas composition.

Permeances estimated using the new model and multivariate, multi-response regression analysis were lower for O_2 and CO_2 than those found with the Ne method, especially for O_2. The lower permeances are explained by the assumption that the Ne method only assesses the skin permeance, while the O_2 and CO_2 permeances established by using the new model represent all the barriers between mitochondria, the place where respiration occurs and the external atmosphere. The smaller CO_2

[9] This chapter is based on a paper submitted to Journal of Experimental Botany. Veltman RH, Schouten RE, De Wild HPJ, Koopen TJ, Staal MG, Tijskens LMM. Determination of O_2 and CO_2 skin permeances in pear (*Pyrus communis* L. cv Conference) using a new gas exchange model based on internal gas composition.

permeance found using the new model might be explained by the relatively high pH of the cytosol.

Besides presenting a new gas exchange model, several methodological improvements are suggested for gas extraction from fruits and the measurement of gas exchange. Also the participation of photosynthesis at different light intensities is established and furthermore a more accurate method using computer imaging for the determination of the skin surface area is proposed. To confirm the supposed absence of O_2 gradients in the aqueous phase of the pear, which is a prerequisite for using Fick's first law in our model, O_2 concentrations in the cortex tissue are determined using an O_2-electrode.

8.1 Introduction

Gas exchange of fruits is governed by the combined action of CO_2 production (respiration and fermentation) and O_2 consumption (respiration) on the one hand, and the transport of these gases from and to the inside of the cell by diffusion on the other hand. However, in literature respiration and fermentation of fruits is almost exclusively based on external gas conditions. Technical difficulties in determining internal gas levels and the destructive nature of the applied techniques are the common reasons for this omission. As a consequence, only scarce information is available on the state and level of internal gas concentrations.

Considering only external gas conditions in modelling respiration and fermentation of fruits and vegetables implies that the diffusion part of the process is formally neglected and that the diffusion oriented effects are implicitly incorporated in the parameters. Several barriers exist in fruits for the transport of gases from outside the product into the cells and in the opposite direction (e.g. the skin, flesh, cell wall, membranes and cytoplasm), each exerting its own effect on the overall gas exchange rate that can be measured experimentally.

According to Rajapakse *et al.* [129] the major pathway for O_2 and CO_2 exchange is through the lenticels of the fruit. The aqueous phase inside the fruit represents a secondary barrier especially for O_2 since the solubility of O_2 in water is considerably

lower then the solubility of CO_2. This effect may lead to additional concentration gradients with respect to O_2 concentration over the mass of the fruit flesh.

The main goal of the current research was to adapt and extend existing modelling approaches on fruit respiration by including a diffusion controlled component and to make the effects of diffusive transport more explicit. At the same time, a possible way to correct gas exchange due to occurring photosynthesis is developed and presented. To achieve these goals, materials and methods had to be improved or developed (Computer Imaging techniques, methods to determine the surface area and volume of fruits), and control experiments (O_2 gradients in the liquid phase of the fruit flesh, gas extraction control with neon (Ne)) had to be performed.

At aerobic conditions O_2 is consumed by the product in the same process as CO_2 is produced. Data on gas exchange of both gases can therefore be pooled for integral analysis as was reported by Hertog *et al.* [130]. In this study a similar approach to respiration modelling as in Hertog *et al.* [130] was used, and a diffusion component to estimate gas exchange independent of skin resistance was integrated. The diffusion component in the overall measured gas exchange can be described by Fick's first and second law. If the simplification can be made that the skin of the pear is the only or major barrier to diffusion of O_2 and CO_2 the internal concentration of the gases is at equilibrium inside the fruit flesh. Only in that case the diffusion process can be approximated with Fick's first law. Solomos [131] and Banks and Nicholson [132] showed, however, that this is not the case for fruits with a low porosity, like pears. Lammertyn *et al.* [133] indeed used Fick's second law to resolve the problem that gases can not diffuse freely in the fruit flesh, but were forced to apply numerical methods to handle the gas exchange data of Conference pears. Describing diffusion by Fick's second law is, at least for the time being, out of reach for practical applications. Although the assumption that all internal barriers and the skin can be treated as one barrier may not be completely satisfactory, it brings a practical solution one step closer to actually describe internal respiration and diffusion characteristics of O_2 and CO_2.

In this paper, also the differences in the parameter values for the permeance to O_2 and CO_2 between those determined by Ne diffusion and those estimated by statistical non-linear regression analysis of gas exchange data are explained and modelled.

8.2 Materials and methods

8.2.1 Plant material

Pears (*Pyrus communis* L. cv. Conference) were harvested in Randwijk, The Netherlands, on 18 September 2001, the optimal picking date for long-term CA storage (established by using the Streif index). The pears used were from one orchard, picked from trees standing next to each other; pears from trees at the end of the tree-rows were not included. The pears used in this study were considered as one batch with comparable ripening, diffusion and respiration characteristics. Fruits had not been under CA before experiments were performed. Until the experiments started (about one month after harvest), fruits were stored in crates at 0°C wrapped up in plastic to avoid water loss.

8.2.2 Internal gas measurements

For determination of CO_2 and O_2 partial pressures in the intercellular spaces of pear tissue the method of Saltveit [134] was used after being slightly modified. Preliminary testing revealed that after 1 min at 0.35 atm pressure the maximum amount of gas was extracted from a pear. For gas leakage testing two calibration gases were used: (i) 5 kPa O_2 and 5 kPa CO_2 with nitrogen as balance gas, and (ii) pure nitrogen gas. No gas leakage from the desiccator to the bell jar, where extracted gas is collected [134], could be established.

To check if all the gas was extracted from a pear, fruits were loaded with Ne gas overnight (7 ml of Ne was added to a 2-l cuvette with a pear enclosed). Directly after addition of Ne, the partial pressure in the cuvette was determined. After 8 h it was assumed that gas in the cuvette had reached equilibrium with gas in the pear, and internal gas was extracted from the fruits. The Ne partial pressure in the gas

extracted from the pear was determined and compared to the Ne partial pressure added to the cuvette at t=0.

8.2.3 The influence of photosynthesis on gas exchange: experiment A

In a climate chamber 12 pears (20°C, enclosed in 2-L cuvettes) were subjected to 4 subsequent light levels: 0 ('dark'), 16.4 (± 1.2, 'dim light'), 56.6 (± 3.6, 'full light') and, again, 0 μmol m^{-2} s^{-1}. The light levels were measured in the 12 cuvettes using a Skye PAR light meter (Skye Instruments Ltd, UK). After adaptation to a new light level (at least 2 hours) gas exchange of the individual fruits was determined as described by De Wild *et al.* [12], using a Chrompack CP 2002 gas chromatograph (Varian Chrompack Benelux) equipped with an automated sampling system.

8.2.4 Gas exchange and gas extraction: experiment B

Pears were subjected to 0, 0.5, 1, 2.5, 6 and 21 kPa O_2 combined with 0, 5 or 10 kPa CO_2 (20°C, 95% RH). Individual fruits were enclosed in 2-L cuvettes, which were connected to a flow-through system as described in Chapter 3. Every condition was applied in duplicate or in eight-fold (in case of 0-5, 6-5 and 21-5; O_2-CO_2). Gas exchange rates were determined after the pears had been subjected to these conditions for two days using the method of De Wild *et al.* [12]. Hereafter, the cuvettes were reconnected to the flow-through system for at least two hours (to remove accumulated CO_2) before internal gas was extracted from the same, individual pears. Finally, weight, volume, length and circumference of the fruits were determined for the calculations on fruit surface and gas resistance of the skin.

8.2.5 Diffusion resistance determined with Ne: experiment C

Sixteen cuvettes (2-L), each containing one pear, were connected to a flow-through system (Chapter 3). The O_2 partial pressure applied was 21 kPa with 0 kPa CO_2. Experiments took place at 20°C. The method described by Peppelenbos and Jeksrud [59] to determine skin resistance for O_2 and CO_2 by using Ne (7 ml per cuvette, 10 h incubation) was applied with some adaptations. Because the total

intercellular space of a pear is low (about 5%) pears were, after loading them with Ne, quickly transferred to an empty, clean cuvette. Not the decrease of the Ne partial pressure in the cuvette was determined (because it was too small to be measurable), but the increase of the Ne partial pressure in this second cuvette. Before the fruit was transferred from the first to the second cuvette the Ne partial pressure in the first cuvette was determined. After transfer to the second cuvette the Ne efflux was monitored (determined after 0, 80, 160 and 240 s) with the same gas chromatograph that was used for gas exchange experiments. Measured Ne percentages were converted to partial pressures using total pressure as determined with a Druck PDI 265 manometer. After determining the resistance for Ne, internal O_2 and CO_2 partial pressures were determined for the same pears.

8.2.6 Fruit surface measurements and Computer Imaging

The surface of a pear was calculated using two methods. The first method assumes that the shape of a pear can be simulated by a combination of half a sphere with a circular, bottomless cone on top of it. Assuming that the length of the pear is equal to the height of the cone plus the radius of the sphere, the surface of a pear can be expressed in terms of pear length (L) and pear circumference (O) (Eq 8.1):

$$A = \frac{1}{2\pi} \cdot O^2 + \frac{1}{2} O \sqrt{\frac{1}{4\pi^2} \cdot O + \left(L \cdot \frac{1}{2\pi} \cdot O \right)^2} \qquad 8.1$$

For the second method a Computer Imaging (CI) technique was developed. A pear was placed in a container with a controlled light environment. An image was acquired using a 3 CCD colour camera (JVC KY-F30 3CCD). This image was transferred to a computer with a colour frame-grabber. Next, the image was segmented in two regions: background and object. Under the assumption that a pear is rotationally symmetrical and mathematically convex, a pear can be approximated by a finite series of cylinders with an equal, small thickness and a varying radius. The

height of every cylinder was one pixel, and the diameter of it was determined in pixels. Next, for each cylinder from stem to flower the outer surface (A) was determined, and the outer surfaces of all cylinders were summed, obtaining the estimation of the fruit's surface. To obtain the surface area and width in SI-units the Computer IA method was calibrated with respect to a picture taken from an object with known dimensions (a ruler), from which a pixel per mm conversion factor was determined.

To test the accuracy of both methods, a set of 12 pears was measured using the CI method and the sphere-cone method, and afterwards the pears were peeled accurately to obtain the surface area of the entire skin. Using the CI method the surface area was overestimated by 3.5% (± 2.7%) on average. The area estimated with the sphere-cone was in the same range (2.2% underestimated), however the standard deviation was a factor 2.5 higher compared to the CI method. The CI method has, next to the accuracy enhancement, also the advantage to facilitate quick measurements of large quantities of fruits.

8.2.7 O_2-electrode measurements

O_2 profiles in the aqueous phase of the pear were determined as described in Smid *et al.* [135] with an O_2-electrode [136], which was mounted to a motor-driven micro-manipulator (Märzhäuser MM33), and stepwise, perpendicularly pierced into the belly of a pear (at the largest diameter of the fruit). O_2 profiles were determined in a cooled room, at 5°C. The electrode was calibrated using nitrogen and air saturated tap water. O_2-saturated tap water (5°C) contains 398.5 μM O_2. (Microscal Measurements, The Hague, The Netherlands).

8.2.8 The gas exchange model

Nowadays' general models describing gas exchange of fruits and vegetables are based on external gas conditions, and use a Michaelis-Menten approach [130, 137, 138]. The O_2 consumption rate (V_{O_2} in mol $kg^{-1}s^{-1}$) is formulated as (Eq 8.2):

$$V_{O_2} = \frac{Vm_{O_2} \cdot O_{2e}}{Km_{O_2} \cdot \left(1 + \frac{CO_{2e}}{Kmc_{CO_2}}\right) + O_{2e} \cdot \left(1 + \frac{CO_{2e}}{Kmu_{CO_2}}\right)} \qquad 8.2$$

where CO_{2e} and O_{2e} are the external gas conditions expressed as partial pressures (Pa), Vm_{O_2} is the maximum O_2 consumption rate (mol kg^{-1} s^{-1}), Km_{O_2} the Michaelis constant for O_2 consumption (Pa), Kmc_{CO_2}, the Michaelis constant for the competitive CO_2 inhibition of O_2 consumption (Pa), and Kmu_{CO_2} the Michaelis constant for the uncompetitive CO_2 inhibition of O_2 consumption (Pa). The CO_2 production rate (V_{CO_2} in mol $kg^{-1}s^{-1}$) is the simultaneous result of aerobic respiration and fermentation and can be described as (Eq 8.3):

$$V_{CO_2} = RQ_{ox} \cdot V_{O_2} + \frac{Vm_{CO_{2(f)}}}{\left(1 + \frac{O_{2e}}{Kmc_{O_{2(f)}}} + \frac{CO_{2e}}{Kmc_{CO_{2(f)}}}\right) \cdot Km_{CO_{2(f)}} + 1} \qquad 8.3$$

where RQ_{ox} represents the respiration quotient for oxidative respiration, $Vmco_{2(f)}$ the maximum fermentative CO_2 production rate (mol $kg^{-1}s^{-1}$), $Km_{CO_{2(f)}}$ the Michaelis constant for fermentative CO_2 production (Pa), $Kmco_{2(f)}$ the Michaelis constant for competitive inhibition of fermentative CO_2 production by O_2 (Pa) and $Kmcco_{2(f)}$ the Michaelis constant for competitive inhibition of fermentative CO_2 production by CO_2 (Pa). Units of parameters have been converted according to the unit system proposed by Banks *et al.* [139].

Modelling gas exchange with internal gas conditions may be accomplished by using the existing gas exchange model and exchanging the external for the internal gas conditions. However, the effect of diffusion resistance towards gases of the skin of the fruit should be considered in the gas exchange model when significant

differences between internal and external gas conditions are encountered. Under the assumption that there is only one barrier for transport of gasses, namely the skin of the pear, diffusion can be described with Fick's first law [140]. Fick's first law is a general equation for mass flux through a surface driven by a concentration gradient. For the case of a fixed external gas condition (C_e) and a changing gas concentration inside the pear Fick's law can be represented as (Eq 8.4):

$$\frac{\frac{d}{dt}M(t)}{A} = -P \cdot (C_e - C_i(t)) \qquad 8.4$$

where d(M(t))/dt represents the mass flux (mol s^{-1}), A the surface of the pear (m^2), P the diffusion coefficient or permeance (mol s^{-1} m^{-2} Pa^{-1}), C_e and $C_i(t)$ the external and internal gas concentrations, respectively (Pa). Eq 8.4 can be converted to the gas exchange rate (V_d in mol $kg^{-1}s^{-1}$) due to diffusion processes by converting the mass flux to the diffusion rate per kg pear (M_p). As gas exchange measurements were determined after 2.5 days it was assumed that steady state conditions were reached between the internal environment of the pear and the external gas partial pressures. Therefore, the internal gas concentration, $C_i(t)$ can be assumed to be independent of time (Eq 8.5):

$$V_d = -\frac{P \cdot (C_e - C_i) \cdot A}{M_p} \qquad 8.5$$

The measured gas exchange rates can be described as being the combined result of internal respiration and fermentation with the exchange rate for the diffusion of O_2 and CO_2 (Eq 8.6-8.7).

$$V_{O_2} = \frac{Vm_{O_2} \cdot O_{2i}}{Km_{O_2} \cdot \left(1 + \frac{CO_{2i}}{Kmc_{CO_2}}\right) + O_{2i} \cdot \left(1 + \frac{CO_{2i}}{Kmu_{CO_2}}\right)} - \frac{P_{O_2} \cdot (O_{2e} - O_{2i}) \cdot A}{M_p} \quad 8.6$$

$$V_{CO_2} = RQ_{ox} \cdot V_{o_2} + \frac{Vm_{CO_{2(f)}}}{\left(1 + \frac{O_{2i}}{Kmc_{O_{2(f)}}} + \frac{CO_{2i}}{Kmc_{CO_{2(f)}}}\right) \cdot Km_{CO_{2(f)}} + 1} - \frac{P_{CO_2} \cdot (CO_{2e} - CO_{2i}) \cdot A}{M_p} \quad 8.7$$

where CO_{2i} and O_{2i} are the internal gas partial pressures (Pa), and where P_{O_2} and P_{CO_2} (mol s^{-1} m^{-2} Pa^{-1}) are the permeances for O_2 and CO_2, respectively.

8.2.9 Statistical analysis

Experimental data on external gas exchange rates were analysed statistically using the non-linear regression routine of Genstat 5 (release 3.2, Lawes Agricultural Trust, Rothamsted Experimental Station, UK). The set of gas exchange data was analysed using either the model formulation of Eqs 8.2-8.3 or the model formulation of Eqs 8.6-8.7, for analysis 1 and analysis 2, respectively. For analysis 1 the gas exchange data were analysed simultaneously using external gas conditions (multivariate, multi-response regression analysis). For analysis 2 the gas exchange data were analysed treating measured internal and external gas partial pressure and surface values (CI method) simultaneously as independent variables, and O_2 consumption and CO_2 production rates as dependent variables (multi-response, multi-variate, non-linear regression analysis).

O_2-electrode measurements (8.2.7) were analysed using Student's t-test to examine the O_2 concentration over distance, from the skin to the core of different pears. A 5% significance level was adopted.

8.3 Results and discussion

8.3.1 Photosynthesis

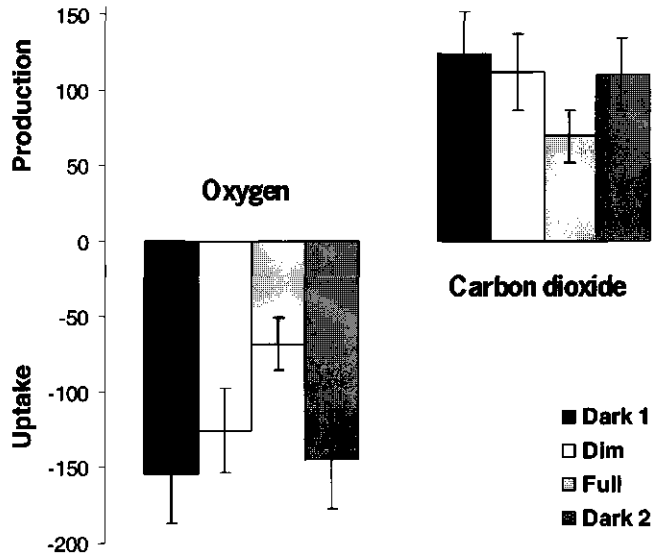

Figure 8.1. Net gas exchange was measured of pears (n=12) that went through a cycle of darkness (initial value), dim light, full light and darkness (experiment A). Gas exchange measurements were done on the same pears after adaptation to the different light-steps (see 8.2). The O_2 uptake and CO_2 production (in nmol $kg^{-1}s^{-1}$) are given during the respective first dark period ('Dark 1') dim light, full light and the second dark period ('Dark 2').

Preliminary experiments showed that photosynthesis affected gas exchange rate and gas extraction measurements. Dim strip light was already sufficient to highly affect these measurements in pears: photosynthesis significantly increased intercellular O_2 levels and decreased intercellular CO_2 levels (results not shown).

Experiment A quantified the sensitivity of gas exchange measurements to known amounts of light, and also revealed the short term in which photosynthesis affected net gas exchange (Fig. 8.1). Gas exchange was inhibited by about 20% in dim light and with over 50% under full light compared to gas exchange in the dark. Generally, it has been already known for long that photosynthesis affects respiration measurements. However, still a lot of erroneous interpretations of results can be explained by this simple fact, and still a lot of experimental set-ups do not consider this. Results shown and used in this paper are from experiments performed in almost darkness, using dimmed green light.

8.3.2 O_2 in the aqueous phase of a pear

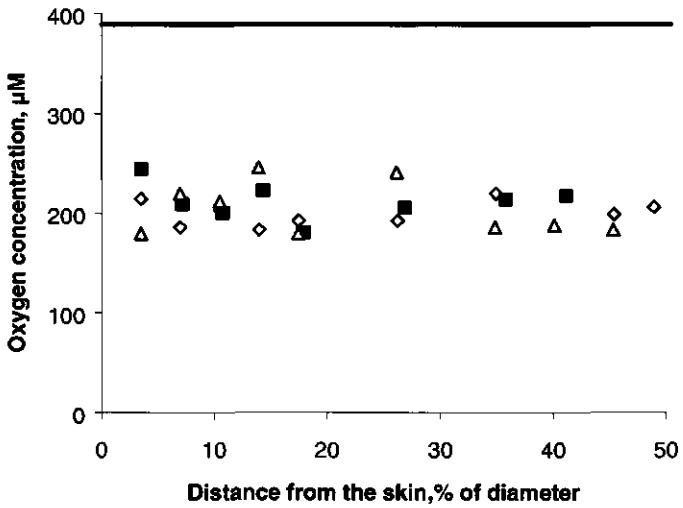

Figure 8.2. O_2 concentrations in the aqueous phase of a pear determined with an O_2 electrode as a function of the distance from the skin of the fruit. The distance between the tip of the electrode and the skin of the pear is expressed as a percentage of the total diameter (0% stands for the skin, 50% stands for the centre of the fruit). The average radius of the fruits was 57.3 mm (± 1.6). The measurements were performed at 5°C at ambient air. The horizontal line at 398.5 µM O_2 represents the maximal O_2-saturation of tap water at 5°C. Different symbols represent different pears

We assumed (like Rajapakse *et al.* [129]) that in the pear intercellular gas-filled spaces are evenly distributed, and gases like O_2 and CO_2 can be transported without

a noteworthy diffusion resistance through these spaces. Therefore, O_2 does not have to diffuse all the way from the skin to the mitochondria through the aqueous phase, which is a slow process. It is assumed that O_2 moves from the gas phase into the aqueous phase close to the cells where it is used for respiration. Our model describes the pear as a sphere in which the border (the skin of the fruit) is the only diffusion resistance for O_2 and CO_2. The model, however, does not include the diffusion of O_2 and CO_2 from the intercellular spaces to the aqueous phase and visa versa.

Although O_2 concentrations in the aqueous phase of the pear were not included in the model, the absence of an O_2 profile in the aqueous phase would also exclude a gradient in the intercellular spaces, which makes the choice of using Fick's first law plausible. To check this assumption, O_2 concentrations were determined using an O_2 electrode that was pierced into the fruit flesh of a pear. This electrode measures the O_2 concentration in the aqueous phase. The small needle-like tip (Ø 0.6 mm) crushes the cells and O_2 concentrations, on a cell level, are averaged. Fig. 8.2 gives three examples of O_2 profiles in three individual pears. Several fruits were tested and in every case statistical analyses did not show a O_2 gradient ($p<0.05$). O_2 profiles were also determined at 20°C (data not shown) showing the same result: no gradient in the aqueous phase could be recorded.

At 5°C and ambient air about 50% of the maximal O_2 solubility (in pure water) was established in the pear cortex tissue, i.e. around 200 μM. The absence of O_2 gradients indeed indicates that the assumption of free diffusion in this sphere model seems warranted. CO_2 concentrations in the aqueous phase of the fruit were not determined because no reliable CO_2 electrode is available. It was assumed that CO_2 gradients were also absent.

8.3.3 Gas extraction, experiment B

To get insight in the gas composition in the intercellular spaces, gas was extracted from pear fruits (experiment B) and analysed for O_2 and CO_2. The volume of gas extracted was compared with the volume of the individual pears, resulting in a porosity of around 5%, which corresponds to earlier porosity estimations of 5.4% [12].

This means that cells in the fruit flesh of a pear are tightly packed without much intercellular spacing. This is also shown in Fig. 8.3.

Figure 8.3. Electron-microscopic photo of pear cortex tissue (magnification 600x). The former contact surfaces between the cells are visible with groups of cell-cell junctions (plasmodesmata) (①)[141]*. Intercellular spaces, which can be seen between the contact surfaces are small (②).*

The method of gas extraction was checked using Ne. Pears were loaded with Ne and gas was extracted from the fruit. On average, the internal Ne partial pressure was 0.8% (± 0.4%, n=4) lower compared to the initial partial pressure in the cuvette. This test showed that nearly all the Ne gas was extracted from a pear, and that the method of gas extraction was valid.

The internal O_2 and CO_2 partial pressures in pears stored under various conditions were determined. Fig. 8.4 shows the internal versus the external O_2 an CO_2 partial pressures. The general trend is that O_{2i} was always lower than O_{2e}, and that for pears stored at increasing O_{2e} the difference between internal and external partial pressure increased. The same trend was found by Dadzie *et al.* [83] for two apple cultivars. A difference with these experiments with apples, however, is that some pears showed almost no difference between internal and external gas conditions over a range of O_2 partial pressures. For pears preserved in the presence (5 and 10 kPa) or in the absence of CO_2, the accumulation of CO_2 internally was considerable, and the CO_{2i} partial pressure was about 2 kPa higher than CO_{2e} (Fig. 8.4B, dashed line), independent of CO_{2e}. In general the variation in the accumulation of CO_{2i} in Fig. 8.4B

was large and may be explained by different respiration and fermentation rates at varying O_{2i}, which were not taken into account. It is, however, clear that diffusion characteristics for O_2 and CO_2 are quite different.

8.3.4 Results on gas exchange and application of the gas exchange model

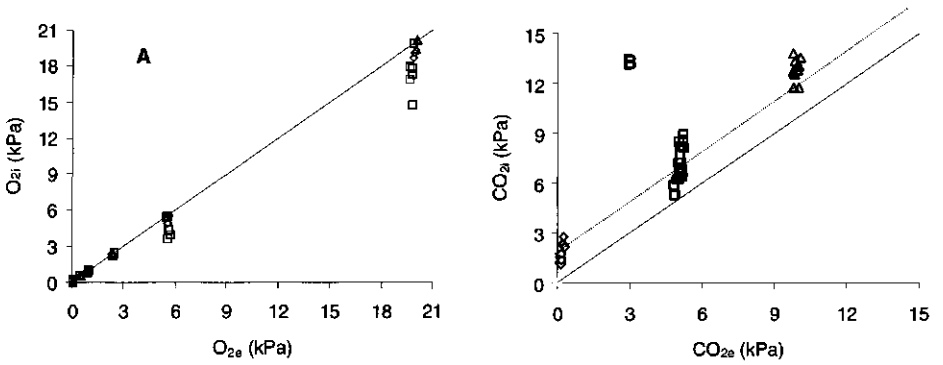

Figure 8.4. Internal (O_{2i}) versus external (O_{2e}) measurements of the O_2 partial pressure (Panel A) and internal (CO_{2i}) and external (CO_{2e}) measurements of the CO_2 partial pressure (Panel B) for externally applied CO_2 partial pressures of 0 (◊), 5 (□) and 10 (Δ) kPa. The line represents x=y, the dashed line an offset of this line of 2%.

In the gas exchange model the RQ_{ox} parameter is assumed to be a constant factor independent of partial pressures. In literature it is shown that this value is often estimated smaller than, but close to 1 [22, 130]. This implies that the CO_2 production is partly originating from glucose and partly from other sources resulting in less CO_2 production or that due to an artefact a relatively smaller CO_2 production was measured. It is known that in pome fruits next to glucose, malate [142] is important as substrate for CO_2 production. During storage of apples not more that 10% of the initial sugar level is consumed, while the acid level may fall by as much as 50%, malate being the prominent organic acid [143]. Malate will generate more CO_2 per O_2 compared to glucose (1.33 instead of 1.00), resulting in a value of RQ_{ox} higher than 1 [144]. De Wild and Peppelenbos [145] stated that during gas exchange measurements the CO_2 accumulation in the cuvette could decrease the exchange rate of CO_2 itself, because of CO_2 accumulation in intercellular spaces and in the aqueous phase.

Using the model formulation of Eq 8.2-8.3 the value of RQ_{ox} would then be underestimated. Likely, the RQ_{ox} value must be higher than the estimated values in literature. Here, we assumed the value for the RQ_{ox} in the model formulation of Eq 8.7 being 1. With this assumption possible effects of CO_2 on the decrease of RQ, potentially caused by, among others, the effect of CO_2 on the malate metabolism [142], were neglected.

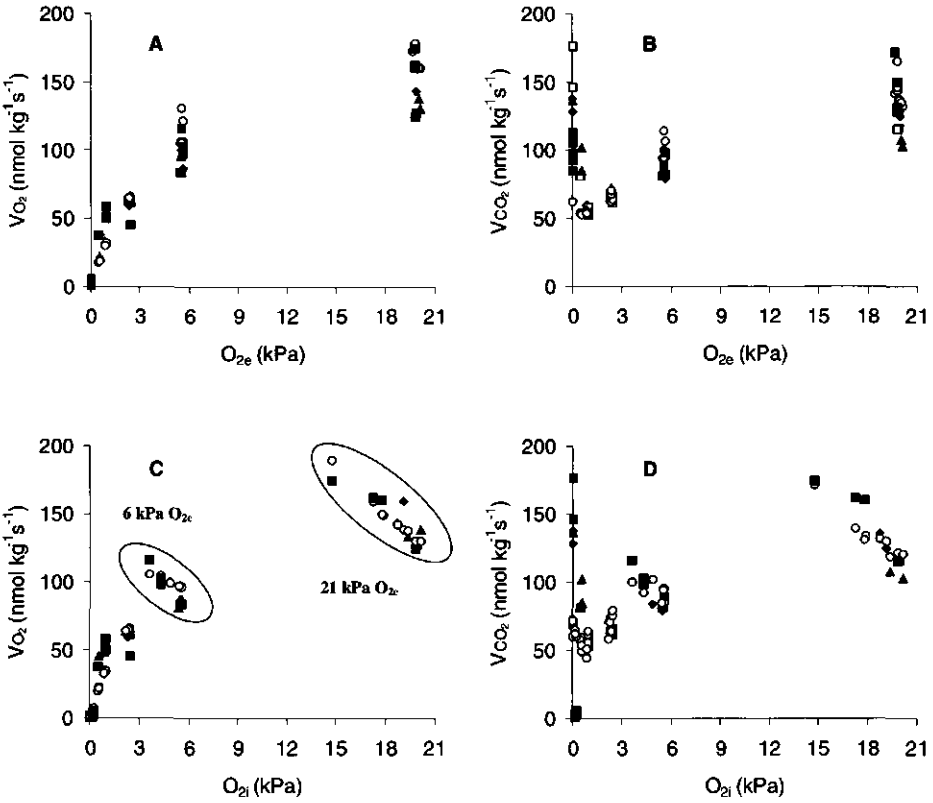

Figure 8.5. O_2 consumption (Panels A and C) and CO_2 production measurements (Panel B and D) for 0 (♦), 5 (■) and 10 (▲) kPa externally applied CO_2 partial pressures for Conference pears as function of external O_2 (O_{2e}, Panel A and B) or internal O_2 (O_{2i}, Panels C and D). Model simulations, applying the parameters from Table 8.1, are shown in panel A and B (analysis 1), and in the panel C and D (analysis 2) as open dots.

Fig. 8.5 shows experimental data on O_2 and CO_2 gas exchange for various externally applied CO_2 partial pressures as function of measured internally and

externally O_2 partial pressures (closed symbols). The upper panels, where the gas exchange is shown as function of O_{2e} partial pressure, generally follow Michaelis-Menten kinetics. Variation in gas exchange data apparently increased when plotted against O_{2i} (Fig. 8.5, panel C and Panel D), because now not only variation was present in the gas exchange measurements but also in O_{2i}. Looking at Fig. 8.5 (Panel C) it appears that at O_{2e} of 21 kPa (upper right dots), $O_{2i} \cong O_{2e}$ at relatively low respiration values (~125) and $O_{2i} < O_{2e}$ at relatively higher respiration rates (~200). The same trend can be seen at 6 kPa O_{2e}

Table 1. Results of the non-linear regression analysis of the gas exchange data for pears (cv Conference) using external (Analysis 1) or internal gas conditions including diffusion (Analysis 2) using O_2 and CO_2 simultaneously as independent variables. [a] *s.e.= standard error of estimation.* [b] *n.e.= not estimated.* [c] *n = number of data points.*

Parameter		Analysis 1		Analysis 2	
		estimate	s.e.[a]	estimate	s.e.[a]
Parameters describing respiration					
Vm_{O_2}	(nmol $kg^{-1}s^{-1}$)	199.58	9.95	150.6	6.5
Km_{O_2}	(kPa)	4.89	0.67	3.11	0.38
Km_{nCO_2}	(kPa)	8		8	
Parameters describing fermentation					
RQ_{ox}		0.806	0.036	1	
$Vm_{CO_{2fr}}$	(nmol $kg^{-1}s^{-1}$)	126.14	6.03	169.33	8.06
$Km_{CO_{2fr}}$	(kPa)	0.427	0.098	0.281	0.05
Parameters describing diffusion					
P_{O_2}	(pmol s^{-1} m^{-2} Pa^{-1})	n.e.[b]		-164.2	20.1
P_{CO_2}	(pmol s^{-1} m^{-2} Pa^{-1})	n.e.[b]		-85.7	17.3
R^2_{adj} (%)		86.6		90.5	
n[c]		102		102	

Table 8.1 shows the results of the multi-response multivariate non-linear regression analysis using only external gas conditions (analysis 1) and using simultaneously internal and external gas conditions together with measured surface as explaining variables (analysis 2) in one optimisation. During the iterative process of non-linear regression for both analyses the parameter $Kmcco_{2(f)}$ has been fixed at

the value 1 as the fermentative CO_2 production is over-parameterised [138]. Both $Kmcco_2$ and $Kmuco_2$, the Michaelis constants for the inhibition of the O_2 consumption by CO_2, tended towards extremely large values in the analyses. Both parameters were replaced with one parameter, $Kmnco_2$, which results in non-competitive inhibition by CO_2 of the O_2 consumption [138]. This formulation was also used for Conference pears by De Wild *et al.* [12] and Lammertyn *et al.* [146]. However, also for $Kmnco_2$ an infinite value was estimated (Table 8.1), indicating a small or absent inhibitory effect of CO_2.

The estimated values for the parameters of analysis 1 are comparable with those found by Hertog *et al.* [130] for apple and chicory. Simulated data, applying the parameters used from Table 8.1 in the model formulations, are shown in Fig. 8.5 as open dots. Both models are capable of explaining the gas exchange data, with percentages accounted for (R^2_{adj}) of 87 and 91% for analysis 1 and analysis 2, respectively.

The estimated parameters describing respiration and fermentation by the two models are significantly different. Gas exchange according to analysis 2 can be described having a lower Vmo_2 and a lower Kmo_2 than respiration according to analysis 1 (Table 8.1). Comparing panel A and panel C (Fig. 8.5) shows that the lower estimated Kmo_2 for analysis 2 is a result of O_{2i} values being lower than externally applied O_{2e}, which is shown by a shift to the left in panel C. This observation is especially visible around 6 and 21 kPa O_{2e}. Lower values for Kmo_2 and $Kmco_{2(f)}$, the O_2 concentration at which half the maximal CO_2 production due to fermentation is reached, (Table 8.1) in analysis 2 compared to analysis 1 might point to an increased affinity for O_2 when respiration and fermentation are described using internal concentrations of O_2. The inclusion of O_{2i} and CO_{2i} in analysis 2 lead to the lower Vmo_2. The lower value for Vmo_2 is accompanied by a higher value for $Vmco_{2(f)}$ when analysis 2 is compared to analysis 1. This might point to a shift in CO_2 production rate, indicating less oxidative and more fermentative CO_2 production when internal gas measurements were considered.

8.3.5 Results on permeance measurements, experiment C

In experiment C the resistance to Ne was determined by establishing the molar Ne efflux rate. Only 4 data points were used per pear as the emission of Ne slows down quickly after 4 minutes. Variations in the resistance measurements per pear were negligible, but not between pears. Resistance to Ne was converted to permeance for O_2 and CO_2 using Graham's law, following the method described in Peppelenbos and Jeksrud [59]. The Ne-derived permeance for the batch of Conference pears was 722 ± 118 *p*mol s^{-1} m^{-2} Pa^{-1} for O_2 and 615 ± 100 *p*mol s^{-1} m^{-2} Pa^{-1} for CO_2 (n=16). Although literature on permeance measurements of pears is scarce, the measurements reported by Amarante *et al.* [147] using O_2 instead of Ne, are within the range found for this batch of Conference pears. Amarante *et al.* [147] reported O_2 and CO_2 permeance measurements, expressed as *n*mol s^{-1} m^{-2} Pa^{-1}, for Bartlett (0.60, 0.58), Packham's Triumph (0.25, 0.15) and Doyenne du Comice (0.35, 0.35) pears.

Permeances for O_2 and CO_2 for the batch of Conference pears obtained from analysis 2 (Table 8.1, 164 and 86 pmol s^{-1} Pa^{-1} respectively) are considerable different from Ne-derived O_2 and CO_2 permeances. This difference may be explained partly by the inert nature of Ne. Ne measurements may be regarded as indicator of only the permeance due to physical properties of the pear skin. The O_2 and CO_2 permeances, as established by analysis 2, may be regarded as the permeance due to all the barriers between mitochondria, the place where respiration occurs, and the external atmosphere [129]. This means that O_2 and CO_2 have to pass more barriers than Ne. Analysis 2 estimated a lower permeance for CO_2 than for O_2. This result is in contradiction with permeance measurements of pepper fruit by Banks and Nicholson [132], who found values of 244 and 24 mol $s^{-1}m^{-2}Pa^{-1}$ for CO_2 and O_2, respectively. Banks and Nicholson explained their results with the suggestion that for waxed fruit, O_2 is mainly diffusing through stomata, while CO_2 also moves through the skin of the fruit [148]. However, when determining the CO_2 permeance, the solubility of CO_2 in the cytoplasm must be considered. The pH of the cytosol of pears is normally between 7 and 7.5 [84]. At this pH the majority of the CO_2, (80-90%) [149] is present in the bicarbonate form. Bicarbonate not only diffuses much slower because

it is a bigger molecule, it is also a charged molecule which makes it difficult to pass membranes.

8.3.6 General concluding remarks

For this study all experiments were carried out with the same batch of pears with comparable respiration and ripening characteristics. This experimental set-up was chosen to make respiration, internal gas and diffusion measurements as comparable as possible, because for technical reasons it is not possible to do all the experiments on the same pears. The purpose of our study was to make a gas exchange/diffusion model as an instrument in future research. Solid information on the dependency of CO_2 and O_2 diffusion resistance on commodity, cultivar, harvest date, growing location (orchard, climate), ripening stage and how gas exchange and diffusion parameters vary during the storage season, and during ripening, is still scanty, and it is very likely that, even when the same cultivar is taken into account, diffusion resistances and gas exchange rates are variable. Research will be necessary to get insight in the relative importance of these factors. Summarising, our model is tentative and it can be improved and extended on several points, e.g. by including temperature dependency in the model (like in Hertog *et al.* [130]).

The internal accumulation of CO_2 as described by our model may have several consequences. Firstly, the suggestion that considerable CO_2 accumulation may take place in pears might be advantageous, because there may be a considerable need for recycled internal CO_2 as substrate for photosynthesis during growth [150]. Secondly, various pear cultivars (for example the Conference pears used in this research) can be extremely sensitive towards internal browning which is generally thought to be a CO_2 disorder. External measurement of the CO_2 partial pressure is not sufficient for estimations of the internal CO_2 partial pressure (Fig. 8.4), which might, in part, explain the sometimes unexpected occurrence of internal browning.

The pears that were used in this investigation have not been under CA, and respiration of these pears was not inhibited by CO_2, indicated by the value for Km_{CO_2}, which was considered infinite (Table 8.1), although literature citing values for Km_{CO_2} for Conference pears exists [12, 133, 151]. When pears are stored for several

weeks under CA with the addition of CO_2, an inhibitory effect on respiration emerges, which seems to coincide with the appearance of internal browning [151] (H.P.J. de Wild, personal communication). Future research should confirm this observation and should further clarify the relation between CO_2 and internal browning in (Conference) pears.

Acknowledgements

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9. General discussion [10]

9.1 Introduction

9.1.1 Effect of CO_2 on respiration and fermentation

CO_2 can influence the respiratory metabolism of several non-photosynthetic plant tissues. It is extensively applied in CA to inhibit respiration and to extend the postharvest life of several commodities. However, increased CO_2 concentrations often lead to injury in fruits and loss of quality in vegetables. An example of such an injury in pears is brown core, which is often accompanied by the formation of cavities later during storage (Fig. 9.1). Among various factors that accelerate brown core (like for instance low O_2 levels [152], storage temperature and ripening stage), application of enhanced CO_2 levels is the most important. However, the biochemical and physiological background of core browning, and the mechanism behind its development is still not well understood.

[10] This chapter is based on a proceeding written for the CA2001 conference held in Rotterdam 11-15 July 2001. Veltman RH, Peppelenbos HW. A proposed mechanism behind the development of internal browning in pears (*Pyrus communis* cv Conference). Submitted to Acta Horticulturae.

In 1917 Kidd [3] already demonstrated the inhibition of fruit respiration by CO_2. This was recorded by measuring O_2 uptake and CO_2 production of the fruit. For long it was not evident how CO_2 causes this depression, and what its site(s) of action are. It was suggested [65, 118] that increased CO_2 levels inhibit succinic dehydrogenase activities, resulting in an accumulation of succinic acid, a toxicant for plant tissues. However, this does not unambiguously explain CO_2 injury. In lettuce, for instance, CO_2 injuries were more severe at temperatures below 10°C, while at 10 and 15°C the accumulation of succinic acid was more obvious [153]. CO_2 might also influence other parts of the respiration process; effects on more upstream (glycolysis) and downstream (electron transport chain) parts have both been described. In Bartlett pears it was shown that fructose-6-phospate was accumulated and fructose-1,6-diphosphate was substantially reduced under CO_2, which suggests that CO_2 has an inhibitory effect on phosphofructokinase [119]. Furthermore, Bendall *et al.* [154] reported the inhibitory effect of CO_2 on cytochrome c oxidase in *Ricinus* mitochondria and Gonzàlez-Meler [120] in soybean. Gonzalez-Meler *et al.* [120] suggested that a reversible carbamylation by CO_2 [155] may be involved in the inhibition of cytochrome c oxidase, although from their study it could not be concluded which chemical species of inorganic carbon inhibited it. Palet *et al.* [156, 157] showed that the inhibition of cytochrome c oxidase from carnation callus and pea leaf mitochondria depended on the concentration of free dissolved CO_2 in the reaction medium.

Less is known about the possible interaction of CO_2 and the process of fermentation. The presence of acetaldehyde and ethanol, end-products of fermentation, seems to be part of the natural pear ripening process [18], but ethanol emissions of pears are significantly enhanced by high CO_2 levels (80 kPa) in preclimacteric Bartlett pears stored at hypoxia [18]. This ethanol emission can probably be explained by a shift from respiration to fermentation, because –as stated above- CO_2 can inhibit normal respiration, and thus more energy must be obtained from the fermentation pathway. Under anoxia, however, during on-line experiments (data not shown) the addition of CO_2 (10 kPa) did not enhance the ethanol emission from the fruit. This does not exclude that fermentation rates are higher under these conditions, because ethanol *emission* do not necessarily have to equal ethanol *production* because ethanol can be accumulated in the fruit. However, CO_2 (5 kPa) did not have

a significant influence on the amount of ethanol in the cortex tissue after 9 days storage at various conditions. Under anoxia ethanol values were 32.3 μM (± 6.4), while under 0.5 kPa O_2 this value was 0.84 μM (± 0.11), and at normoxia 75 nM (± 41).

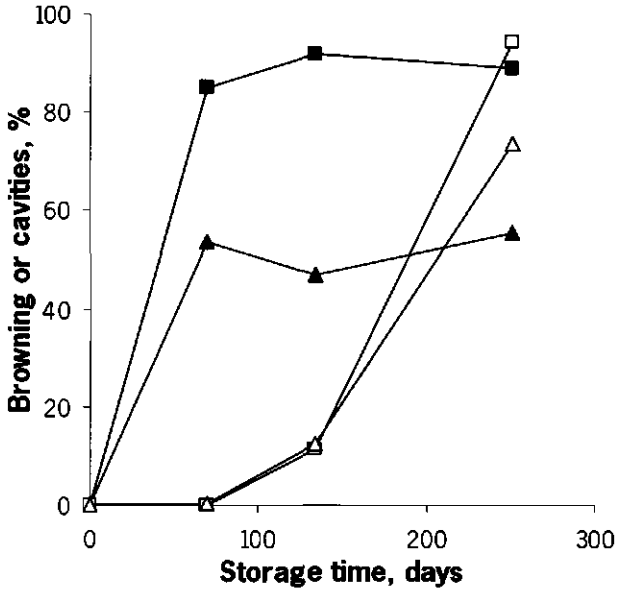

Figure 9.1. An example of how internal browning (filled symbols) and cavities (open symbols) in pears from two growing locations develops during storage (squares and triangles). Both are expressed as percentages affected fruits. Pears were stored in the experimental small-scale static storage system described in Chapter 2 at standard conditions, enriched with enhanced CO_2 (3 kPa). Brown core clearly begins during the first 2 month of storage, while later in the storage season cavities are formed.

Fermentation is a constitutive process in plant tissues (some ethanol is found at normoxia), but is of increasing importance when O_2 concentrations are lowered [158]. In transgenic tobacco Pyruvate decarboxylase (PDC) was described being the limiting factor for ethanol production during short-term anoxia, whilst alcohol dehydrogenase (ADH) activity was not limiting the fermentation process [159]. Elevated CO_2 and reduced O_2 concentrations slightly increased PDC activity in strawberry and pear [18, 160], but ADH activity was only increased at hypoxic concentrations (0.25 kPa). PDC activity was found to be 2 to 3 times higher in postclimacteric pears

compared to preclimacteric pears [18]. Probably, PDC is not the only factor controlling or initiating fermentation. Increased NADH levels due to reduced oxidative phosphorylation are also important in the control of the fermentation route [18].

9.2 Polyphenol oxidase and decompartmentation

Internal browning in pears is a direct result of the action of PPO (PPO; EC 1.10.3.1). More specific browning begins with tyrosinase activity (EC 1.14.18.1) (no significant laccase, EC 1.10.3.2, or peroxidase, EC 1.11.1.1-2, activity was recorded in pears used in this investigation). Because high CO_2 levels are associated with brown core development the question rose whether CO_2 changes tyrosinase activity during storage. Tyrosinase is present in a latent and an active form. Perhaps CO_2 activates tyrosinase directly or indirectly, or, perhaps non-latent tyrosinase becomes active due to a pH shift (Fig. 9.2).

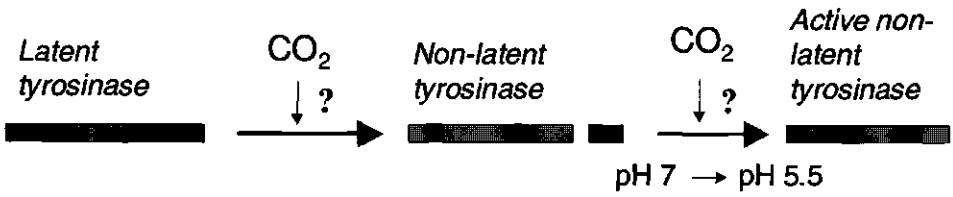

Figure 9.2. Overview of the possible effect of enhanced CO_2 on tyrosinase functioning.

CO_2 has the potential to change pH values of tissue and cell sap [153]. In normal air the cytoplasmic pH of Bartlett pears was estimated 7.4. However, when O_2 was reduced to 0.25 kPa this value decreased to approximately 7 [84], and under elevated CO_2 the cytoplasmic pH dropped to 6.6. Non-latent pear tyrosinase is active at pH values ranging from 4 to 7 (Chapter 4). Below pH 7 the activity of tyrosinase increases dramatically and at pH 6.6 this activity would be about 50-60% of Vmax, indicating that the pH-shift occurring at high CO_2 concentrations and low O_2 concentrations could potentially promote browning. However, tyrosinase is normally not cytosolic.

In higher plants, PPO is a plastidal enzyme in both photosynthetic and non-photosynthetic tissues [46]. In non-senescent tissues it is mainly located in the

thylakoid membrane of chloroplasts and in vesicles or other bodies in non-green plastid types [55, 67]. It has been detected in root plastids, potato amyloplasts, leucoplasts, etioplasts and chromoplasts of different commodities [46, 161]. While trichomal PPO in leucoplasts is freely soluble upon cell disruption, leaf chloroplast PPO's are tightly membrane associated [162]. In pear, chloroplasts can be found all over the cortex tissue (results not shown); however, probably PPO is mainly located in other plastid types considering the fact that it was found to be 100% non membrane-bound in pear cortex tissue (Chapter 4).

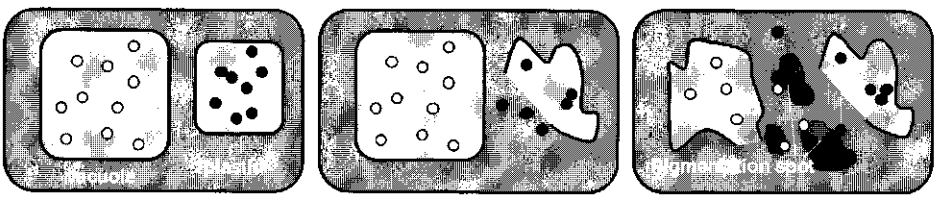

Figure 9.3. Schematic representation of the process of decompartmentation. Dots represent phenolic substrates located in the vacuole (white dots) and tyrosinase in the plastids (black dots). Pigmentation spots are the places were PPO formed quinones, which polymerised into melanins.

Because tyrosinase is localised in plastids and polyphenol substrates in the vacuole of the cell [56] internal browning is not likely caused by a simple direct pH-effect due to CO_2. More likely, (Chapter 2; Frenkel *et al.* [68]) brown core is a consequence of decompartmentation of cellular compartments, caused by membrane disintegration (Fig. 9.3). Decompartmentation is accompanied by a strongly decreased cellular pH, because the vacuolar pH is low (3.8-4.4 [84]). On average the pH of the cell after decompartmentation, estimating the vacuole to occupy 80-90% of the cell's volume, is about 5, which means that non-latent tyrosinase would be activated for about 80% (Chapter 4).

The decompartmentation hypothesis brings about two questions:

I. What are the causes of decompartmentation?

II. What happens during and after decompartmentation?

9.3 What causes decompartmentation?

9.3.1 Gas gradients in the fruit

Because brown core –as the name suggests- starts in the core of the pear, it was always assumed that O_2 and CO_2 gradients play a key role in this storage disorder. Addition of 3-5 kPa CO_2 to the storage atmosphere initiates internal browning within 10 weeks, depending on the time that brown core induction experiments were performed during the season (Fig. 9.1), and even within 4 weeks at higher temperatures (5°C, Chapter 3). Lammertyn *et al.* [133], however showed that the general idea that browning spreads concentrically through the fruit flesh, beginning in the core, is probably not correct. Using Magnetic Resonance Imaging they showed that from the initial stage internal browning affects a certain area of the core and the cortex tissue around the core. But, although the intensity of browning can increase, the area affected by brown core did not. Another common thought is that during storage a gradient of O_2 exists from the core to the skin. Chapter 8 showed that this is not the case, not in the aqueous phase, nor in the intercellular spaces.

One of the ideas behind the effect of CO_2 was its capacity to inhibit respiration, leading to a decreased ATP production [120]. Internal CO_2 concentrations are higher than external concentrations (Chapter 8), and theoretically highest in the core where internal browning usually starts. Taking the above into account, internal browning might be explained by an energy lack, caused by elevated CO_2 concentrations.

Much attention is given in literature to the passage of gases through the skin of fruits. Indeed, skin resistance for gases might be an important factor during the development of internal browning in pears. However, there is no proof that this is *the* factor. CO_2 might be able to pass the skin of the fruit easier compared to O_2, but probably, CO_2 has more difficulties to pass membranes in the pear. CO_2 dissolves very well in water, but at cytosolic pH the major part of the gas is in the bicarbonate form. Bicarbonate has probably difficulties to pass membranes, because it is a relatively large, charged molecule. Further research should elucidate if the membrane barrier is important in the development of brown core.

9.3.2 Energy shortage

CO_2 does not always inhibit respiration. Just after harvest, for example, internal browning can be observed when pears are stored at enhanced levels of CO_2, but at that time respiration is not inhibited. This is an indication that the inhibitory effect of CO_2 on fruit respiration is not the only cause of internal browning in pears. Pears stored at anoxia, which showed the lowest ATP production, unexpectedly did not show internal browning (Chapter 6). Experiments in Chapter 6 showed that when pears were stored without CO_2, disorders only emerged under a small range of O_2 concentrations (0.5-1 kPa, hypoxia) at a relatively high temperature (5°C). There was also no direct link between a decreased ATP level and/or production and the development of internal browning at certain gas conditions. Therefore, it is suggested that the inhibitory effect of CO_2 on respiration is not the most important factor in explaining the development of internal browning.

The conditions under which browning appeared also depended on the ripening stage. Ripening pears are coping with increasing ATP-sinks (ripening related processes consume ATP) and they raise their respiration, showing the so-called climacteric peak. Furthermore, the filling of intercellular spaces with water released during cell-wall breakdown might hinder diffusion. Later harvested pears (1 week after the optimal picking date for CA storage) are much more susceptible to internal browning, and probably differences in brown core susceptibility can be (partly) explained by differences in developmental stages of the fruit (Chapter 5).

The fact that pears stored at anoxia are free of internal disorders makes it unlikely that an energy shortage is the *only* explanation for disorders in hypoxic pears. An additional explanation is that pears at hypoxia suffer from radical damage, while the low energy availability hinders proper regeneration of antioxidants. Radical action might lead to lipid peroxidation and the destruction of membranes.

9.3.2 Peroxidation

Free radical formation and peroxidation of fatty acids has been described as one of the major processes destroying membranes. Mitochondria are regarded as the

main or only source of radical formation. Not all O_2 is tetravalently reduced to water via cytochrome oxidase in the respiratory chain. A small proportion of the O_2 accepts only one electron, and superoxide is formed. Ubisemiquinone seems to serve as the primary electron-donor, responsible for 80% of the superoxide formation [122]. Under normal circumstances superoxide will be oxidised to hydrogen peroxide by Mn-superoxide dismutase (Mn-SOD) and other scavenging enzymes in the mitochondria. Hydrogen peroxide can be further broken down by glutathione peroxidase (GSH-PX). When rapid oxidation of the superoxide is not possible, the remaining superoxide can attack the various cell membranes.

There are several indications that peroxidation of membranes is a factor of importance in brown core development. Firstly, AA levels in pear tissue –an important antioxidant that scavenges free radicals [103]- decreased sharply at conditions that induce brown core, i.e. at low O_2 (0.5-1 pKa) and high CO_2 (5 pKa) (Chapter 3 and 6). AA levels dropped in healthy tissue before the onset of browning at these conditions, indicating that this decrease in AA was not the consequence of the browning process itself (Chapter 3 and 6). Secondly, no browning was observed when pears were stored at anoxia (Chapter 6), and here AA levels were not affected. At anoxia there are no oxygen radicals, because the respiratory chain in the mitochondria –the expected place of radical formation- is out of order.

Ethanol can serve as an antioxidant at extremely low O_2 levels. Halliwell and Gutteridge [163] described that ethanol can react with the OH-radical with a high rate constant [164]. Because internal browning was already observed at 0.5 kPa O_2 and ethanol concentrations dropped steeply between 0 and 0.5 kPa O_2 (results not shown), ethanol can only be an effective antioxidant within this O_2 range.

9.3.3 Antioxidant interactions

Vitamin E (α-tocopherol) functions as the major lipophilic antioxidant in biological systems of its ability to react with lipid peroxyl radicals to terminate the peroxidative process. One of the reaction products is the tocopheryl radical, which is further oxidised to non-radical products, like quinones and tocopherones. As a part of the important cellular defence mechanism against irreversible oxidation one would

expect α-tocopherol to be present at relatively high levels. However, in most tissues the ratio α-tocopherol : polyunsaturated fatty acids is in the order of 1:1000.

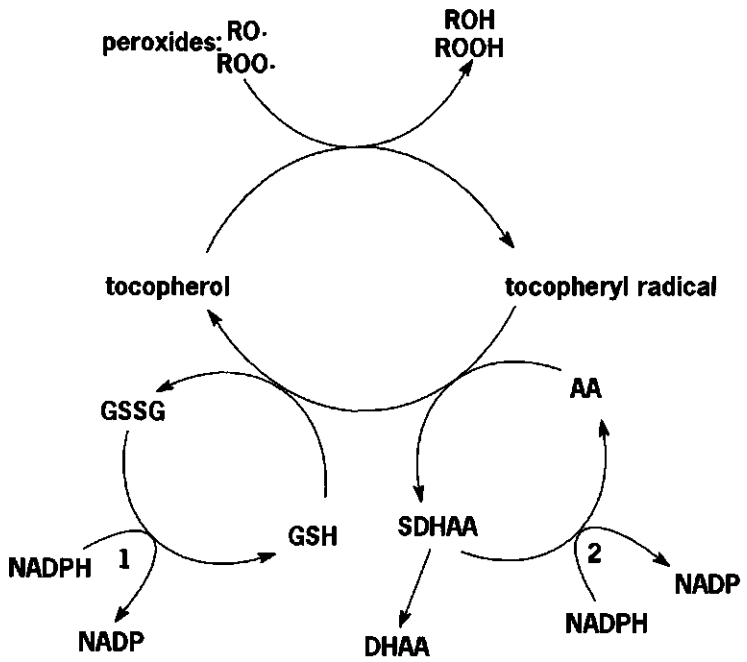

Figure 9.4. Interaction between the three most important antioxidants. GSH: reduced form of glutathione; GSSG: oxidised form of glutathione; SDHAA: semi-dehydroascorbic acid. Oxidised glutathione and semi dehydroascorbic acid (SDHAA) are regenerated by GSH reductase (1) and AA reductase (2) respectively. R = fatty acid. RO• and ROO• = alkoxyl and peroxyl radicals (Chapter 7)

For this reason, it is hypothesised that other reductants are able to interact with partly oxidised α-tocopherol, to increase the protecting capacity of the cell. In other words, cells can dispose of a much larger reduction pool to participate in reactions ending lipid peroxidation. *In vitro* experiments suggested that α-tocopherol can be regenerated through interactions with GSH or AA via both enzymatic and non-enzymatic pathways [165]. AA has been demonstrated to be able to reduce the tocopheryl radical directly. This regeneration is held responsible for the synergistic effects of these two antioxidants found in lipid peroxidase systems. Protection against lipid peroxidation has also been shown by GSH in microsomes, mitochondria

and nuclei. A possible explanation for this protection was the mechanism of enzymatic recycling of α-tocopherol (Fig. 9.4).

Decreased AA levels as seen in pears indicate that there are difficulties with the protection against ROS under conditions that cause internal browning. When AA levels drop, probably the entire antioxidant cascade is malfunctioning, which will therefore lead to membrane damage.

9.4 What happens during and after decompartmentation?

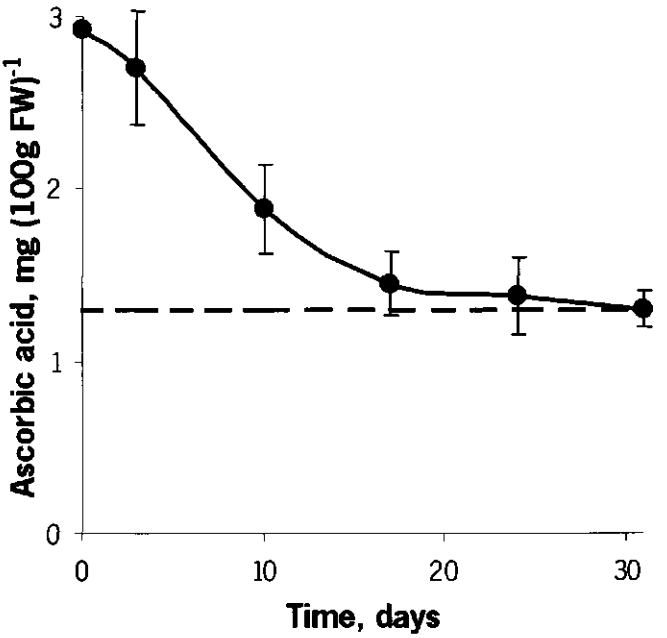

Figure 9.5. Decrease of AA in pears during storage under enhanced CO_2 levels (n=4). Pears were stored at 5°C, 2 kPa O_2 and 5 kPa CO_2. The dashed line gives the suggested level of AA in the cytosol of the cell.

AA can be found in different compartments of the cell and its concentration depends on the plant species and intracellular location. It is synthesised in both cytosol and mitochondria [103], and it is not abundant in the cell's vacuole [103, 104]. Because vacuoles occupy the major part of the plant cell volume, it can be

speculated that AA concentrations in the non-vacuolar fraction are much higher than the average concentration of about 150 μM that was found in the overall cortex of pears (Chapter 4). Indeed, high concentrations of AA (20-50 mM) are found in both chloroplastic and cytosolic compartments of pea and spinach leaves [103].

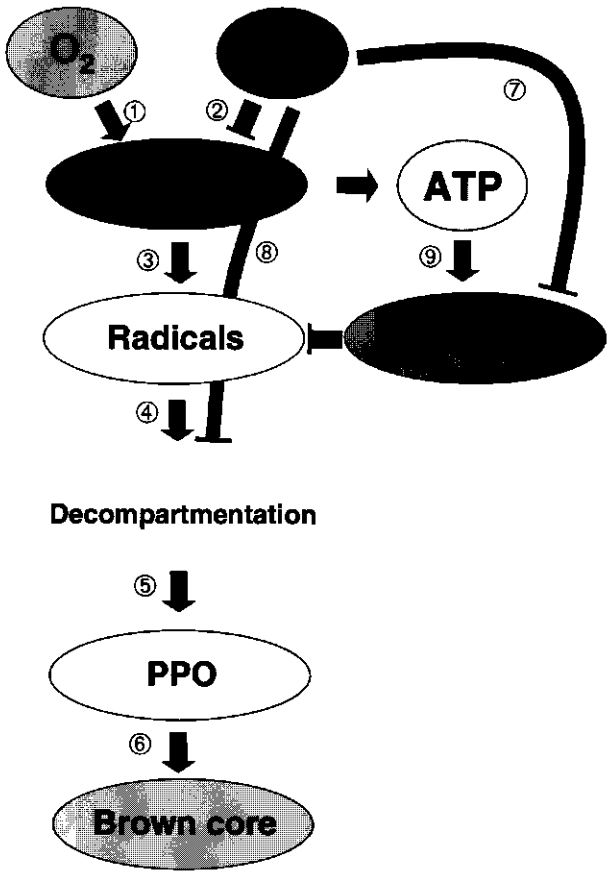

Figure 9.6. Overview of the factors involved in the development of brown core in pears.① Fruit respiration is dependent on the O_2 concentration; low O_2 concentrations limit respiration. ② Inhibition of respiration by CO_2. ③ Formation of radicals during respiration. ④ Radicals destroy (intracellular) membranes. ⑤ Decompartmentation initiates PPO action. ⑥ Brown core develops through PPO action. ⑦ Elevated CO_2 levels directly affect antioxidant levels. ⑧ CO_2 hypothetically directly negatively affects membranes. ⑨ Antioxidants can be regenerated at the expense of ATP. ⑩ Inactivation of oxygen free radicals by antioxidants.

Because AA levels quickly decrease in pears subjected to high CO_2, without the direct appearance of internal browning, it was speculated that chloroplast

membranes are disrupted before the tonoplast is damaged (Fig. 9.3, the midst picture). AA is associated with chloroplasts (removal of hydroperoxides by APX). A decrease of AA could be explained by the disruption of chloroplasts, with the consequence that AA levels would parallel cytosolic levels (Fig. 9.3 and Fig. 9.5).

Observations indirectly indicated the involvement of radicals in the development of brown core. However, disorders might as well be explained by an effect of energy shortage under certain storage conditions. Are internal membranes damaged by such an energy shortage (additionally, damage could be caused by radicals), or are membranes destroyed by radicals (also because antioxidants can not be regenerated properly due to an energy shortage)? None of the determined parameters alone (AA and ATP levels, calculated ATP generation) could be correlated directly to the development of internal browning (Chapter 6). More likely disorders are caused by a combination of these (and other) factors (Fig. 9.6, ② and ⑨).

9.5 General conclusions

The main goal of this study was to get insight in the mechanism of brown core development in pears. It is already established empirically decades ago that CO_2 is the major cause for internal browning (Chapter 1). However, it proved to be difficult to map the site(s) of CO_2 action related to this disorder.

Tyrosinase activity was not increased by enhanced CO_2 levels (Chapter 2), and therefore a direct effect on this crucial enzyme can be excluded. CO_2 is known to affect several enzymes in the cell. For instance, cytochrome c oxidase is inhibited in several species (Fig 9.6, ②). In pears respiration is inhibited by CO_2, but not during the first weeks of CA storage, directly after harvest. Later in the storage season (unpublished results) the inhibitory effect of CO_2 on respiration emerges (Chapter 8). Indeed browning only occurred after several weeks of CA storage at elevated CO_2 concentrations. However, results in Chapter 6 indicate that the inhibitory effect of CO_2 on fruit respiration is not the only factor leading to brown core, nor the effect on ATP production rates and levels.

An interesting question is why apples (cultivar Elstar) can be stored at enhanced levels of CO_2 (2.5 kPa is the storage advice) and under extreme low O_2 concentrations (<1 kPa O_2 [166, 167]) without the development of internal browning. An explanation for brown core in pears, proposed in Chapter 6, is that pears stored between 0.5 and 1 kPa O_2 are exposed to oxygen free radicals, while energy levels to regenerate antioxidants and maintain cell viability are low. In pear the production of ethanol due to fermentation was nearly or completely absent at 0.5 kPa O_2, indicating that fermentation is only initiated at extremely low O_2 concentrations, close to anoxia. In Elstar apple fermentation already starts at higher O_2 partial pressures, which probably indicates that apples are more adaptive to hypoxia and dispose over a larger amount of ATP produced by fermentation at these low O_2 concentrations.

Bean plants grown in nutrient cultures in which pH and bicarbonate concentrations were varied developed chlorosis at high bicarbonate levels regardless of the pH of these solutions [168]. Miller and Evans [168] found that respiration in plants that are not susceptible towards chlorosis is only slightly affected by elevated bicarbonate levels, and that respiration is significantly inhibited by elevated bicarbonate in plants that are chlorosis-sensitive. This suggests that chlorophyll breakdown is somehow related to inhibition of respiration. In pear there are strong indications that chloroplasts in the fruit flesh are broken down in the cortex tissue under elevated levels of CO_2. The cortex tissue of pears becomes slightly yellowish during storage at elevated levels of CO_2 and AA concentrations (AA is associated with peroxide removal in the chloroplast) drop steeply (Fig. 9.5). Rao and De Kok [169] showed that addition of CO_2 to wheat plants and the subsequent increased rate of photosynthesis also increased the level of AA and its redox state. In pears, however, being stored in the dark, photosynthesis does not take place, and at elevated CO_2 AA levels were lowered quite drastically (Chapter 3 and 6). However, the mechanism leading to this decrease is not known and subject of future study (Fig 9.6, ⑦).

Other, more direct effects of CO_2 can not be excluded based on this investigation. The ultrastructure of membranes as a possible site of CO_2 action is supported by a study showing that bicarbonate ions in equilibrium with gaseous CO_2 can change the interfacial tension of a water-lipid interface [170], which could impair the ability to maintain structural membrane continuity, leading to a membrane collapse (Fig. 9.6,

⑧). Another, speculative, direct effect of CO_2 is the formation of insoluble $CaCO_3$ salts due to high CO_2 pressures, thus rendering Ca unavailable for membrane structure maintenance, as in calcium deficient plants [171]. A 100-minute treatment of CO_2 lead to a granulation and swelling of mitochondria [172]. Besides, an increase in vacuolisation of the plasma takes place, as was also recorded by Frenkel and Patterson [68]. Koncalova [172] suggested that factors, which in any way disturb the delicate process of oxidative phosphorylation, might disturb the morphology of the mitochondria as well. Both the proposed direct effects of CO_2, like for example on membranes and enzymes and the indirect effects, like the decrease of AA, support the idea that decompartmentation is the ultimate cause for initiation of brown core development.

Considering nearly 100 years of scientific research on fruit storage under changed atmospheres (starting with Kidd and West at the beginning of the 20th century) there is still much to learn. Frequently, products are stored at conditions of which the consequences for the fruit physiology are not clear. Ongoing physiological research should further improve storage technology and avoid risks and losses.

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10. Summary

Brown discoloration as a result of polyphenoloxidase (PPO) is a well-known problem during food preservation and –processing, which already has been established long ago. Large quantities of fruits and vegetables are lost every year because of loss of taste or because the product looks unattractive to customers. Internal browning or brown core in pears is an example of this. Because affected fruits normally appear healthy from the outside, they may be put on the market, with all the negative consequences that can be expected.

This research aimed to explain the mechanisms behind the development of brown core. The research was focussed on pears, however it obviously has a relation with similar disorders in general. The pear functions as a model, and this research has a potential spin-off to other products.

10.1 Polyphenoloxidase

The name PPO is used for a number of enzymes involved in the oxidation of several polyphenols, like chlorogenic acid and (-)-epicatechin. Two groups of PPO's can be distinguished. In this research no laccase activity in pears was established, therefore the PPO activity that was found was attributed to tyrosinase. Browning develops when tyrosinase converts polyphenols into *o*-quinones. These quinones further polymerise by reacting with a variety of cellular components, and this leads to the brown pigmentation that can be seen with the naked eye. In literature it is often

supposed that the activation of tyrosinase initiates the process of brown pigmentation. However, in this research it could not be shown that PPO is activated before or during the initiation of brown core development, or under conditions which cause internal browning (like elevated CO_2 concentrations).

In a healthy cell tyrosinase and phenolic substrates are found at different locations. Tyrosinase can be found in plastids in the cell. In chloroplasts tyrosinase is membrane-bound, while as far as known tyrosinase is not membrane-bound in other plastids. Tyrosinase exists mainly as a latent form in the cell. The ratio active : latent (around 5% : 95%) does not change clearly at the border between healthy and affected tissue, or under storage conditions that have the potential to initiate the development of brown core. In the cortex tissue of the pear tyrosinase is not membrane-bound.

Polyphenols are present in the vacuole. A membrane surrounds both, plastids and vacuoles, and PPO and its substrates do not come into contact in healthy tissue. This led to the hypothesis that the initiation of brown core development is caused by (partial) disruption of intracellular membranes and decompartmentation. A new question rose in this investigation, namely: how and under which circumstances are these membranes damaged?

10.2 Vitamin C

One of the hypotheses is that membranes are damaged by oxygen free radicals, which are produced during fruit respiration (mitochondria) and photosynthesis (chloroplast). In healthy tissue these radicals are neutralised by antioxidants. With the appearance of aerobic respiration in evolution, cells developed a protective system against radicals in which antioxidants and several enzymes, like catalase, superoxide dismutase and peroxidase play a role. One of the most abundant antioxidants in fruits (besides GSH and vitamin E), and the main reason why humans consume fruits, is vitamin C or ascorbic acid (AA). A person with 'scorbutic' is suffering from scurvy, a well-known illness in naval history, which can be overcome by the consumption of AA.

AA is an efficient quencher of oxygen free radicals. AA can directly neutralise radicals by reacting with them, but AA can also regenerate vitamin E in membranes. Vitamin E protects membranes against oxygen free radicals. Additionally, AA is necessary for ascorbate peroxidase (APX) functioning. APX neutralises peroxide in the chloroplast, where catalase is absent. Furthermore, AA can regenerate *o*-quinones to form precursor polyphenols *in vitro*, and so directly avoid browning.

AA levels in pears decrease to low levels under conditions that induce brown core. Lowered O_2 reduces AA levels compared to standard CA conditions. The addition of CO_2 to the storage atmosphere decreases AA levels even more. By monitoring AA levels in pears it is possible to predict the development of browning. AA levels increase again and brown core is largely avoided when browning-inducing storage conditions (with elevated CO_2) are changed to standard CA conditions (without CO_2) just before the moment brown core is initiated. However, the AA level is not the only factor explaining brown core development. During storage at 21 kPa O_2 with 5 kPa CO_2 for example, the AA level is lower than at 0.5 kPa O_2 without CO_2, while only under the latter condition brown core develops. The conclusion is that other factors must be involved

Under CA conditions fruit respiration is limited to a high extent. This brings along that also the energy production (ATP) is limited. A cell needs a certain energy maintenance level to survive. This maintenance energy is for example necessary for protein turnover, membrane maintenance and antioxidant regeneration. Lowering O_2 concentrations decreases both ATP levels and the rate of ATP production. Addition of CO_2 to the storage atmosphere increases the chance that brown core will develop and can inhibit respiration and ATP production. Still, CO_2 does not inhibit respiration in every case. Directly after harvest of pears CO_2 does not show an inhibitory effect on pear respiration. Future research should demonstrate if an emerging inhibitory effect on respiration during the beginning of CA storage coincides with the initiation of brown core development.

Summarising, it appears that brown core is the result of a combination of factors, like a (temporarily) shortage of available energy and an insufficient capacity to

protect the cell against oxygen free radicals caused by decreased antioxidant concentrations.

10.3 Diffusion resistance

Another important subject of the present research is the establishment of the diffusion resistance of the peel of an apple or pear. In literature it is often suggested that that the cause of internal browning can be found in the low porosity of pear tissue. This research showed that this suggestion is unlikely. O_2 concentrations in the pear are indeed slightly decreased, but not at low external applied O_2 concentrations under which browning develops. No O_2 gradients were found in the cortex tissue of a pear, which does not point at an evident O_2 diffusion resistance. An accumulation of CO_2 in pears under a variety of conditions was clearly established. The average internal CO_2 concentration was around 2 kPa higher than the externally applied concentration (at 20°C). There is a strong indication that CO_2 can accumulate in the cell in the form of bicarbonate.

10.4 Summarising

The relation between elevated CO_2 concentrations and brown core in pears is not new. However, it is still difficult to indicate the exact mechanisms by which CO_2 leads to this disorder.

CO_2 has no clear effect on PPO activity. This enzyme does not appear to be activated by CO_2 or bicarbonate. CO_2 has a clear effect on AA levels in pear tissue, which are decreased. Yet, this decrease can not be marked as *the* cause for decompartmentation. CO_2 can inhibit respiration of pears, but this inhibition is not consistent. ATP levels are not unambiguously decreased by CO_2. It is more likely that brown core is caused by a combination of these factors. Furthermore, lowered pH values caused by CO_2 and direct effects of CO_2 on membranes and enzymes may play a role.

It is clear that apples and pears can not be blindly compared. Apples are often stored at enhanced CO_2 levels (up to 4 kPa). Furthermore, it was recently found that

apples can be commercially stored at extremely low O_2 concentrations (as low as 0.4 kPa) during so-called Dynamic Control storage (DCS). It seems that this new type of storage is not suitable for storage of Conference pears. Future research should demonstrate the differences between apples and pears, which lead to the large difference in adaptation capacity to CA conditions. A profound knowledge on fruit physiology may lead to decreased risks and losses and may yield improved (interactive) storage systems, like DCS.

11. Samenvatting

Bruinverkleuring ten gevolge van polyfenoloxidase (PPO) is een oud en bekend probleem binnen de voedselbewaring en –verwerking. Grote hoeveelheden groenten en fruit gaan jaarlijks verloren door verlies van smaak en/of omdat deze producten er niet aantrekkelijk uitzien voor de consument. Interne bruinverkleuring in peren is een voorbeeld hiervan. Bij deze bewaarafwijking vindt de verkleuring plaats in het vruchtvlees binnen in de vrucht. Omdat er doorgaans aan de buitenzijde niets te zien is van de kwaal kan het zijn dat aangetaste vruchten op de markt verschijnen, met alle gevolgen van dien.

Dit onderzoek had als doel te verklaren waar interne bruinverkleuring door ontstaat. Het onderzoek is toegespitst op peren, maar heeft natuurlijk eveneens betrekking op soortgelijke afwijkingen in het algemeen. De peer fungeert slechts als een model, en dit onderzoek heeft een potentiële spin-off naar ander producten.

11.1 Polyfenoloxidase

PPO is een gezamenlijke naam voor een aantal enzymen die verschillende polyfenolen, waaronder chlorogeenzuur en (-)-epicatechine kunnen oxideren. Er zijn twee groepen PPO's te onderscheiden. In dit onderzoek werd geen laccase activiteit aangetoond in peer. Daarom is er vanuit gegaan dat de aanwezige PPO-activiteit verklaard kan worden door de aanwezigheid van tyrosinase. Bruinverkleuring ontstaat doordat tyrosinase in de cel polyfenolen omzet in *o*-quinonen. Deze stoffen

kunnen polymeriseren door een secundaire reactie aan te gaan met allerlei verbindingen in de cel, en hierdoor ontstaan de bruine pigmenten die bij aangetaste vruchten zichtbaar zijn. In veel beschikbare literatuur wordt aangenomen dat de activering van tyrosinase het bruinwordingsproces initieert. Echter, in dit onderzoek kon niet aangetoond worden dat er een activering van het enzym plaatsvindt vlak voor of tijdens de initiatie van bruining of onder condities die bruinverkleuring veroorzaken (bijvoorbeeld verhoogd CO_2).

In de gezonde cel bevinden tyrosinase en fenolische substraten zich op gescheiden locaties. Tyrosinase bevindt zich in de plastiden van de cel. In chloroplasten is tyrosinase membraangebonden. In andere plastiden is tyrosinase voor zover bekend niet membraangebonden. Tyrosinase komt voornamelijk in een latente vorm voor in de cel. De verhouding actief : latent tyrosinase (bij benadering 5% : 95%) verandert niet aantoonbaar op de grens tussen bruin en gezond weefsel, in later geplukte peren (die gevoeliger zijn voor de afwijking), of onder bewaarcondities die bruinverkleuring initiëren. In het vruchtvlees van een peer is tyrosinase niet membraan gebonden.

Polyfenolen worden aangetroffen in de vacuole. Zowel de plastiden als de vacuole wordt omgeven door een membraan. Enzym en substraat komen in gezond weefsel dus niet met elkaar in aanraking. Zo is de hypothese ontstaan dat de initiatie van bruining volgt op de beschadiging van interne membranen en decompartimentalisatie. De nieuwe vraag binnen het onderzoek werd hoe en onder welke omstandigheden deze interne membranen beschadigd raken.

11.2 Vitamine C

Eén van de hypotheses is dat membranen beschadigd raken door zuurstof radicalen die ontstaat tijdens de ademhaling van een vrucht. In gezond weefsel worden deze radicalen (die ook tijdens de fotosynthese gemaakt worden) geneutraliseerd door antioxidanten. Met het ontstaan van de aërobe ademhaling in de evolutie heeft de cel een systeem ontwikkeld waar deze antioxidanten en een aantal enzymen zoals catalase, superoxide dismutase en peroxidase deel vanuit maken. Een van de bekendste antioxidanten in fruit (naast vitamine E en GSH), en

een van de belangrijkste redenen waarom wij mensen fruit eten, is vitamine C, ofwel ascorbinezuur (AA). 'Scorbutic' is de Engelse benaming voor een persoon die aan scheurbuik lijdt, een uit de geschiedenis van de zeevaart bekende ziekte, die door AA voorkomen wordt.

AA is een zeer efficiënte wegvanger van vrije zuurstof radicalen. AA is in staat om zelf met deze radicalen te reageren, maar het kan ook vitamine E in de membranen regenereren (reduceren). Vitamine E beschermt membranen tegen vrije zuurstof radicalen. Verder is AA nodig voor het functioneren van ascorbaat peroxidase (APX). APX neutraliseert peroxide in de chloroplast, waar geen catalase voorkomt. AA kan *o*-quinonen tevens reduceren tot hun precursor polyfenolen, en hiermee de tyrosinase reactie *in vitro* direct tegengaan.

AA gehalten worden in belangrijke mate verlaagd in fruit dat bewaard wordt onder condities die bruinverkleuring kunnen induceren. Verlaagd O_2 ten opzichte van standaard CA condities reduceert het AA gehalte. Toevoeging van CO_2 aan de bewaaratmosfeer verlaagt dit gehalte nog verder. Door AA gehalten te volgen in de tijd kan het ontstaan van bruining zelfs voorspeld worden. AA gehalten stijgen weer en bruining wordt voorkomen als net voor het punt van initiatie ongunstige bewaarcondities veranderd worden in veilige gas condities (weglating van verhoogd CO_2). Echter, AA is niet de enige verklarende factor. Bij bewaring onder 21 kPa O_2 met 5 kPa CO_2 bijvoorbeeld, is het AA gehalte in peren lager dan onder 0,5 kPa O_2 zonder CO_2, terwijl in het eerste geval geen bruining in de peren ontstaat en in het tweede geval wel. Er moeten dus andere factoren zijn, die tevens een rol spelen.

Onder CA condities wordt de ademhaling van fruit in hoge mate beperkt. Dit betekent tevens dat de energie productie (ATP-productie) drastisch beperkt wordt. Een cel heeft een bepaalde hoeveelheid energie nodig om in leven te blijven. Deze 'onderhoudsenergie' is nodig voor de turn-over van eiwitten, het onderhouden van membranen en het regenereren van antioxidanten. Verlaging van O_2 verlaagt niet alleen het ATP niveau in de cel maar tevens de snelheid van ATP productie. Toevoeging van CO_2 aan de bewaaratmosfeer verhoogt de kans op bruining in hoge mate en kan de ademhaling en dus de ATP productie verder remmen. Toch gebeurt dit niet altijd. Meteen na de oogst lijkt CO_2 de ademhaling niet te remmen.

Toekomstig onderzoek zal moeten uitwijzen of het opkomende, remmende effect van CO_2 op de ademhaling tijdens CA bewaring samenvalt met de initiatie van interne bruinverkleuring. Het lijkt er echter op dat interne bruinverkleuring ontstaat als gevolg van een combinatie van factoren waaronder de geringe hoeveelheid beschikbare energie en daarmee samenhangend het zich onvoldoende kunnen beschermen tegen vrije zuurstofradicalen door lagere antioxidant niveaus.

11.3 Diffusieweerstand

Een belangrijk ander punt binnen dit onderzoek is de bepaling van de diffusieweerstand van de schil van een appel of peer. In de literatuur wordt vaker vermeld dat de oorzaak voor bruining mogelijk gezocht moet worden in de lage porositeit van perenweefsel. Dit onderzoek toonde de waarschijnlijkheid hiervan niet onomstotelijk aan. O_2 is inderdaad in lichte mate verlaagd in het interne gasvolume van de peer. Maar bij lage O_2 concentraties (waaronder bruining ontstaat) is het verschil tussen interne en externe gasconcentraties miniem. Verder worden er geen O_2 gradiënten gevonden, hetgeen niet duidt op een gebrek aan O_2 door een hoge diffusieweerstand. Er is wel een duidelijke ophoping van CO_2 in de peer waar te nemen. Gemiddeld is de CO_2 concentratie in de gasfase van de peer ongeveer 2 kPa hoger dan de extern opgelegde waarde. Verder zijn er aanwijzingen dat CO_2 zich in de vorm van bicarbonaat kan ophopen binnen de cel.

11.4 Samenvattend

De relatie tussen bruinverkleuring in peren en verhoogde CO_2 concentraties is reeds lang bekend. Nog steeds is het moeilijk exact aan te geven wat nu precies het belangrijkste effect is van CO_2 dat leidt tot deze CA afwijking.

CO_2 heeft geen duidelijk direct effect op PPO. Dit enzym lijkt niet geactiveerd te worden door bicarbonaat of CO_2. CO_2 heeft een duidelijk verlagend effect op vitamine C gehalten. Toch is duidelijk dat deze verlaging niet *de* oorzaak is voor decompartimentalisatie. CO_2 kan de ademhaling remmen, maar CO_2 doet dit niet altijd. ATP niveaus worden niet eenduidig verlaagd door CO_2. Waarschijnlijker is dat

bruining ontstaat door een combinatie van deze factoren. Daarbij kunnen mogelijk ook pH-efecten als gevolg van CO_2 spelen en directe effecten van CO_2 op enzymen en membranen.

Duidelijk is dat, niet alleen spreekwoordelijk, appels en peren niet vergeleken kunnen worden. Appels worden vaak bewaard onder verhoogde CO_2 concentraties (tot 4 kPa). Daarbij is onlangs gebleken dat appels tijdens de zogenaamde Dynamische Controle bewaring (DCS) bij zeer lage O_2 waarden (tot 0,4 kPa) bewaard kunnen worden. Het ziet er niet naar uit dat dit nieuwe bewaarsysteem in de huidige vorm zonder meer geschikt is voor bewaring van peren. Toekomstig onderzoek zal moeten aantonen waar exact de verschillen liggen tussen appels en peren die leiden tot de grote verschillen in adaptatievermogen aan CA condities. Een verdieping van de fysiologische kennis zal kunnen leiden tot een verdere vermindering van risico's en verliezen en zal verbeterde bewaarsystemen zoals DCS kunnen voortbrengen.

Nawoord

In mijn meer dan 6 jaar bij het ATO heb ik met veel mensen samengewerkt en van velen hulp gehad. Het zou ondoenlijk zijn om iedereen hier te noemen. Toch wil ik een aantal mensen persoonlijk bedanken, omdat zij rechtstreeks te maken hebben met het werk dat vooraf ging aan dit boekje.

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Koos werd later opgevolgd door Herman Peppelenbos als afdelingshoofd. Herman leek altijd veel vertrouwen te hebben in mijn onderzoek, wat mijn eigen twijfels geregeld wegnam. Herman kan plannen als geen ander. Dit is erg belangrijk als wetenschap gecombineerd moet worden met meer commerciële activiteiten. Ik dank Herman voor zijn scherpe analyses van wetenschappelijke problemen en de financiële ruimte die hij maakte om dit werk af te ronden.

Uiteraard ben ik Linus van der Plas zeer dankbaar voor zijn promotorschap. Linus is erg kritisch (en dat is maar goed ook) wat niet altijd even makkelijk was. Sommige stukken moesten zelfs verschillende malen herschreven worden. Maar alle artikelen die Linus goed achtte, zijn later zonder veel problemen geaccepteerd.

Verder noem ik twee paranimfen, Jan Verschoor en Rob Schouten. We hebben lange tijd een kamer met zijn drieën gedeeld. In deze tijd hebben wij veel kattenkwaad uitgehaald en lol gehad. Ik herinner mij onder andere onze verzamelingen (soepkoppen, koffiebekertjes, berichten van Wageningen UR, nieuwe woorden en woordspelingen) en de fundamentele aanpassingen van elkaars computers (dit geheel tegen de richtlijnen van systeembeheer in). Het was daarom wel even wennen toen Rob vertrok naar Tuinbouw Productieketens. Rob heeft al mijn artikelen doorgelezen en we hebben er ook samen een gemaakt. Jan is een zeer sterke analyticus en is inmiddels consultant. Veel planningen voor proeven (en

projecten) heb ik eerst uitvoerig besproken met Jan en Rob waardoor vaak nieuwe en/of aanvullende ideeën ontstonden.

Alex van Schaik heeft mij geïntroduceerd in de wereld van de fruitbewaring. Ik denk dat Alex binnen onze groep één van de belangrijkste schakels is tussen wetenschap en praktijk. Dit maakt hem geregeld tot een vraagbaak. Samen met Alex maakte ik de eerste proefopzetten, maar nog steeds heb ik geregeld zijn advies nodig. Meer recentelijk is het enorme netwerk van Alex (en van Jan en sinds kort van Frank van de Geijn) van groot belang bij nieuw op te zetten projecten en acquisities.

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Curriculum vitae

Ik werd geboren op 5 maart 1969 te Apeldoorn. Ik heb echter maar heel kort in deze stad gewoond. Al snel verhuisde ik naar Groningen, hierna naar Maarssenbroek, vervolgens naar Amsterdam en weer daarna naar Nijmegen. Mijn schooltijd was door dit rondreizen wat chaotisch. De lagere school deed ik in Amsterdam (klas 1 en 2), Maarssenbroek (klas 3 en 6) en Nijmegen (klas 4 en 5). Mijn vervolgopleiding aan het VWO genoot ik aan het Thorbecke College te Utrecht (klas 1, 2 en 3), aan de Nijmeegse Scholengemeenschap (klas 4, nog een keer 4 en 5) en aan Craneveldt Volwassenenonderwijs (5 en 6 in een jaar).

In 1988 begon ik met mijn studie Biologie aan de Katholieke Universiteit Nijmegen (KUN). In 1990 koos ik binnen deze studie voor de Ecologische richting. Ik had moeite met deze keuze, omdat ik geen specifieke interesse had binnen Biologie. In 1992 begon ik mijn hoofdvakstage bij de vakgroep microbiologie. Dit was niet alleen een heel erg leuke tijd, het was ook erg leerzaam. Ik heb me er bezig gehouden met de kweek van anaerobe protozoën, en het opzetten van een c-DNA bank van *Psalteriomonas lanterna*. De ecologie raakte wat meer naar de achtergrond en ik vervolgde mijn studie met een bijvakstage bij de vakgroep Celbiologie, waar ik gewerkt heb aan de bestudering van de groei van gekweekte menselijke cellen onder invloed van cadmium en aluminium. In 1994 studeerde ik af.

Midden jaren '90 was de arbeidsmarkt voor Biologen bedroevend slecht. Het duurde dan ook een vol jaar, dat ik doorbracht met fabriekswerk, tijdelijk werk in de horeca en vrijwilligerswerk aan de KUN, voordat ik een baan accepteerde bij het toenmalige ATO-DLO. De afgelopen 6 jaar heb ik hier, met een onderbreking van 6 maanden bij de vakgroep Molecule- en Laserfysica (KUN), gewerkt. Ik was verbluft van de professionele manier van werken bij het ATO. Er was materiaal in overvloed en geld leek helemaal geen probleem. Nog veel belangrijker was dat mijn collega's driftig discussieerden over wetenschap en zonder twijfel bereid waren hun inzichten en resultaten met een ieder te delen. Mede gesteund door dit enthousiasme startte ik in circa 1996 met mijn promotietraject, waarvan hier het eindresultaat.