A Proposed Mechanism Behind the Development of Internal Browning in Pears (*Pyrus Communis* cv Conference)

Rob H. Veltman and Herman W. Peppelenbos
ATO B.V., Bornsesteeg 59,
P.O. box 17, NL-6700 AA, Wageningen,
The Netherlands
Fax: ++31 317 475347
E-mail: h.w.peppelenbos@ato.wag-ur.nl

Abstract

Storage of pears under low oxygen levels (0.5-1.0 kPa) leads to decreased ascorbic acid and ATP levels, a lower ATP-production, and to internal browning, a storage disorder in pears. Addition of 5 kPa carbon dioxide to the storage atmosphere increased the severity of this disorder. Experiments showed that anoxia can result in off-flavours, but not in internal browning. Internal browning is caused by brown pigments (melanins), which are formed due to oxidation of vacuolar polyphenols under the influence of tyrosinase (EC 1.14.18.1). We hypothesise that internal browning is initiated by a combination of oxygen radical action and a lack of maintenance energy for, amongst others, the regeneration of antioxidants. The two factors together lead to decompartmentation, bringing tyrosinase from the plastids and substrates from the vacuole together.

INTRODUCTION

Carbon dioxide can influence the respiratory metabolism of several non-photosynthetic plant tissues. It is applied extensively in controlled atmospheres to inhibit respiration and to extend the postharvest life of several commodities. However, increased carbon dioxide 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 (Figure 1). Among various factors that accelerate brown core, for instance low oxygen levels (Bertolini et al., 1997), storage temperature and ripening stage, application of enhanced carbon dioxide levels is the most important. The biochemical and physiological background of core browning, and the mechanism behind its development is still not well understood. This paper explains brown core as a result of physiological processes after combining literature data with own experiments.

As early as 1917, Kidd (Kidd, 1917) showed that carbon dioxide can inhibit respiration. The depression in respiration has been recorded by measuring oxygen uptake (Kerbel et al., 1988) as well as carbon dioxide production by the fruit. For a long time the mechanism by which carbon dioxide depresses respiration and its site(s) of action were not evident. Hulme, and William and Patterson (Hulme, 1956; Williams and Patterson, 1964) suggested that elevated carbon dioxide levels inhibit succinic dehydrogenase activity, resulting in an accumulation of succinic acid, which may be toxic to plant tissues. However, this hypothesis does not unambiguously explain carbon dioxide injury. In lettuce, for instance, carbon dioxide injuries were more severe at temperatures below 10°C, while at 10 and 15°C the accumulation of succinic acid was greater (Siriphanich and Kader, 1986). Carbon dioxide might also influence other parts of the respiratory 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 accumulated and fructose-1,6-diphosphate was substantially reduced under carbon dioxide, which suggests that carbon dioxide inhibits phosphofructokinase (Kerbel, Kader et al., 1988). Furthermore, Gonzàlez-Meler (Gonzàlez-Meler et al., 1996) reported an inhibition of cytochrome c oxidase in soybean by carbon dioxide.

Less is known about the influences of carbon dioxide on fermentation. Acetaldehyde and ethanol production seem to be part of the pear natural ripening process (Ke et al., 1994), but ethanol emissions from pears are significantly enhanced by high carbon dioxide levels (80 kPa) in preclimacteric Bartlett pears stored under hypoxia (Ke, Yahia et al., 1994). This ethanol emission could be explained by a shift from respiration to fermentation, because carbon dioxide inhibits normal respiration, and thus more energy must be obtained from the fermentation pathway. During on-line experiments under anoxia, however, the addition of carbon dioxide (10 kPa) did not enhance the ethanol emission from the fruit (data not shown). This does not exclude that fermentation rates are higher under these conditions, because ethanol *emissions* do not necessarily have to equal ethanol *productions*, and ethanol can accumulate in the fruit. However, carbon dioxide (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. Under 0.5 kPa oxygen this value was 0.84 µM ±0.11, and at normoxia 75 nM ±41.

POLYPHENOL OXIDASE AND DECOMPARTMENTATION

Internal browning in pears is a direct result of the action of polyphenol oxidase (PPO) (EC 1.10.3.1). More specific browning begins with tyrosinase (EC 1.14.18.1). No significant laccase, EC 1.10.3.2, or peroxidase, EC 1.11.1.1-2, activity was found in pear tissue. Because high carbon dioxide levels are associated with brown core development the first question is whether carbon dioxide changes tyrosinase activity. Since tyrosinase has a latent stage and an active stage, carbon dioxide may activate tyrosinase directly or indirectly. Non-latent tyrosinase may become active due to a pH shift (Figure 2). Carbon dioxide has the potential to change the pH of tissue and cell sap (Siriphanich and Kader, 1986). In normal air the cytoplasmic pH of Bartlett pears was estimated to be 7.4; however, when oxygen was reduced to 0.25 kPa this value decreased to approximately 7 (Nanos and Kader, 1993), and under elevated carbon dioxide the cytoplasmic pH dropped to 6.6. Non-latent pear tyrosinase is active at pH values ranging from 4 to 7 (Espín et al., 2000). Below pH 7 the activity of tyrosinase increases dramatically and at pH 6.6 this activity is about 50-60% of V_{Max}, indicating that the pH-shift occurring at high carbon dioxide concentrations and low oxygen concentrations could potentially promote browning. However, tyrosinase is not normally in the cytosol.

In higher plants, polyphenol oxidase is a plastidal enzyme in both photosynthetic and non-photosynthetic tissues (Steffens et al., 1994). 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 (Mayer and Harel, 1979; Mayer, 1987). It has been detected in root plastids, potato amyloplasts, leucoplasts, etioplasts and chromoplasts of different commodities (Mayer, 1964; Steffens, Harel et al., 1994). whereas trichomal polyphenol oxidase in leucoplasts is freely soluble upon cell disruption, leaf chloroplast polyphenol oxidases are tightly membrane-associated (Kowalski et al., 1992). Chloroplasts can be found all through the cortical tissue of pears (results not shown); however, polyphenol oxidase probably is located mainly in other plastid types considering the fact that it is 100% non membrane-bound in pear cortical tissue (Espín, Veltman et al., 2000).

Because tyrosinase is localised in plastids and polyphenol substrates in the vacuole of the cell (Yamaki, 1984) internal browning is not likely caused by the direct action of carbon dioxide. On the basis of observations described by Veltman et al. (Veltman et al., 1999) and Frenkel and Patterson (Frenkel and Patterson, 1973) it is hypothesised that brown core results from the decompartmentation of cellular compartments, caused by membrane disintegration (Figure 3). Decompartmentation is accompanied by a large decrease in cellular pH, since the vacuolar pH (the vacuole occupies a large part of the cell's volume) is low (3.8-4.4 (Nanos and Kader, 1993)). The average pH of the cell after decompartmentation was estimated to be about 5, which means an activation of non-latent tyrosinase of 80% (Espín, Veltman et al., 2000).
The decompartmentation hypothesis brings about two questions:
I.   What causes decompartmentation?

II.   What happens at the moment of decompartmentation?

WHAT CAUSES DECOMPARTMENTATION?

Energy Shortage

Because brown core, as the name suggests, begins in the core of the pear, it makes sense to assume that an oxygen gradient may play a role in this storage disorder. Although low oxygen may play a key role in the development of internal browning, carbon dioxide is even more important. Addition of 3-5 kPa carbon dioxide to the storage atmosphere initiates internal browning within 10 weeks (Figure 1), and even within 4 weeks at higher temperatures (5°C (Veltman et al., 1999)). Carbon dioxide further reduces respiration and therefore further decreases ATP production (Gonzàlez-Meler, Ribas-Carbó et al., 1996). Internal carbon dioxide concentrations are higher than external concentrations (Williams and Patterson, 1962), and theoretically highest in the core where internal browning usually begins. Taking the above into account, internal browning might be explained by a lack of cellular energy, caused by low oxygen and high carbon dioxide concentrations. However, pears stored under anoxia with the lowest ATP production, unexpectedly did not show internal browning (Veltman et al., 2001), even after re-exposure to normoxia. Disorders emerged only under a small range of oxygen concentrations from about 0.5 to 1 kPa (hypoxia) during short-term storage experiments at 5°C. Carbon dioxide broadened this range to over 2.5 kPa oxygen; however, pears stored under anoxia combined with carbon dioxide were again spared the disorder. The conditions under which browning appeared also depended on the stage of ripening. Ripening pears are coping with increasing ATP-sinks (ripening related processes consume ATP) and their respiration increases, exhibiting the so-called climacteric peak. Furthermore, the filling of intercellular spaces with water released during cell-wall breakdown hinders diffusion. Since pears harvested one week after the optimal picking date for CA storage are much more susceptible to internal browning, differences in brown core susceptibility can be partially be explained by differences in developmental stages of the fruit (Veltman et al., 2000).

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.

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 oxygen is tetravalently reduced to water via cytochrome oxidase in the respiratory chain. A small proportion of the oxygen accepts only one electron, and superoxide is formed. Ubisemiquinone seems to serve as the primary electron-donor, responsible for 80% of the superoxide formation (Liu, 1997). 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) and catalase. 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 in brown core development. First, levels of ascorbic acid in pear tissue, an important antioxidant that scavenges free radicals (Noctor and Foyer, 1998), decreased sharply at conditions that induce brown core, i.e. at low oxygen (0.5-1 pKa) and high carbon dioxide (5 pKa) (Veltman, Sanders et al., 1999; Veltman, Lenthéric et al., 2001). Ascorbic acid levels dropped in healthy tissue before the onset of browning at these conditions, indicating that the decrease in ascorbic acid was not the consequence of the browning process itself

(Veltman, Sanders et al., 1999). Second, no browning was observed when pears were stored at anoxia (Veltman, Lenthéric et al., 2001), and here ascorbic acid levels were not affected. There are no oxygen radicals at anoxia, because the respiratory chain in the mitochondria, the expected place of radical formation, is not active.

Ethanol can possibly serve as an antioxidant at extreme low oxygen levels. Since internal browning was observed at 0.5 kPa oxygen, and ethanol concentrations dropped steeply between 0 and 0.5 kPa oxygen, ethanol could only be an effective antioxidant within this oxygen range. Halliwell and Gutteridge (Halliwell and Gutteridge, 1989) described how ethanol can react with the OH-radical with a high rate constant (Anbar and Neta, 1967).

Antioxidant Interactions

Vitamin E (α-tocopherol) functions as the major lipophilic antioxidant in biological systems due to its ability to react with lipid peroxyl radicals to terminate the peroxidative process. One of the reaction products is the tocopherol radical, which is further oxidised to non-radical products, such as 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 α-tocopherol : polyunsaturated fatty acids ratio is in the order of 1:1000. 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 that terminate lipid peroxidation. In vitro experiments suggested that α-tocopherol can be regenerated through interactions with glutathione (GSH, L-γ-glutamyl-L-cysteinyl-glycine) or ascorbic acid via both enzymatic and non-enzymatic pathways (Thomas et al., 1992). Ascorbic acid also has been found to reduce the tocopheroxyl radical directly. This regeneration is considered to be 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 (Figure 4).

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

WHAT HAPPENS DURING AND AFTER DECOMPARTMENTATION?

Ascorbic acid can be found in different compartments of the cell and its concentration depends on the plant and intracellular location. It is synthesised in both cytosol and mitochondria (Noctor and Foyer, 1998), and it is not abundant in the cell's vacuole (Rautenkranz et al., 1994; Noctor and Foyer, 1998). Because vacuoles occupy the major part of the plant cell volume, it can be speculated that ascorbic acid concentrations in the non-vacuolar fraction are much higher than the average concentration of about 150 μM that was found in the overall cortical tissue of pears (Veltman, Kho et al., 2000). Indeed, high concentrations of ascorbic acid (20-50 mM) are found in both chloroplastic and cytosolic compartments of pea and spinach leaves (Noctor and Foyer, 1998).

Because ascorbic acid levels quickly decrease in pears subjected to high carbon dioxide, without the direct appearance of internal browning, it was speculated that chloroplast membranes are disrupted before the tonoplast is damaged (Figure 3, the middle picture). Ascorbic acid is associated with removal of hydroperoxides by APX in chloroplasts. A decrease of ascorbic acid could be explained by the disruption of chloroplasts, with the consequence that ascorbic acid levels parallel the cytosolic level (Figure 3 and 5).

Our observations indirectly indicate 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

an energy shortage (with additional damage caused by radicals), or are membranes destroyed by radicals (also because antioxidants cannot be regenerated properly due to an energy shortage). A combination of both is more likely. None of the determined parameters alone (ascorbic acid and ATP levels, calculated ATP generation) could be correlated directly to the development of internal browning. More likely disorders are caused by a combination of these (and other) factors (Figure 6). A descriptive model could combine these factors to describe the development of internal browning in pears (Veltman, Lenthéric et al., 2001).

ACKNOWLEDGEMENTS

Literature Cited
Anbar, M. and Neta, P. 1967. Intl.J.Appl.Radiat. and Isotopes 18: 493-523.
Bertolini, P., Bottardi, S., Rosa, M.D. and Folchi, A. 1997. Effect of controlled atmosphere storage on the physiological disorders and quality of Conference pears. Italian Journal of Food Science 4(9): 303-312.
Espín, J.C., Veltman, R.H. and Wichers, H. J. 2000. The oxidation of L-ascorbic acid catalysed by pear tyrosinase. Physiol.Plant 109: 1-6.
Frenkel, C. and Patterson, M.E. 1973. Effects of CO_2 on ultrastructure of Bartlett pears. HortScience. 9(4): 338-340.
Gonzàlez-Meler, M.A., Ribas-Carbó, M., Siedow, J.N. and Drake, B.G. 1996. Direct inhibition of plant mitochondrial respiration by CO_2. Plant Physiology 112: 1349-1355.
Halliwell, B. and Gutteridge, J.M.C. 1989. Free radicals in biology and medicine. Oxford, Clarendon Press.
Hulme, A.C. 1956. Carbon dioxide injury and the presence of succinic acid in apples. Nature 178: 218-219.
Ke, D., Yahia, E., Mateos, M. and Kader, A.A. 1994. Ethanolic fermentation of 'Bartlett' pears as influenced by ripening stage and atmospheric composition. Journal of the Amererican Society for Horticultural Science 119(5): 976-982.
Kerbel, E.L., Kader, A.A. and Romani, R.J. 1988. Effects of elevated CO_2 concentrations on glycolysis in intact Bartlett pear fruit. Plant Physiology 86: 1205-1209.
Kidd, F. 1917. The controlling influence of carbon dioxide. Part III - The retarding effect of carbon dioxide on respiration. Proceedings of the Royal Society London B, 87: 136-156.
Kowalski, S.P., Eannetta, N.T., Hirzel, A.T. and Steffens, J.C. 1992. Purification and characterisation of polyphenol oxidase from glandular trichomes of *Solanum berthaultii*. Plant Physiology 100: 677-684.
Liu, S. 1997. Generating, partitioning, targeting and functioning of superoxide in mitochondria. Bioscience Reports 17(3): 259-272.
Mayer, A.M. 1964. Factors controlling activity of phenolase in chloroplasts from sugar beet. Israel Journal of Botany 13: 74-81.
Mayer, A.M. 1987. Polyphenol oxidases in plants-Recent progress. Phytochemistry 26(1): 11-20.
Mayer, A.M. and Harel, E. 1979. Polyphenol oxidases in plant. Phytochemistry 18: 193-215.
Nanos, G.D. and Kader, A.A. 1993. Low O_2-induced changes in pH and energy charge in pear fruit tissue. Postharvest Biology and Technology 3: 285-291.
Noctor, G. and Foyer, C.H. 1998. Ascorbate and glutathione: keeping active oxygen under control. Annual Review of Plant Physiology and Plant Molecular Biology 49: 249-279.
Rautenkranz, A.A.F., Li, L., Mächler, F., Märtinoia, E. and Oertli, J. 1994. Transport of

ascorbic and dehydroascorbic acid across protoplast and vacuole membranes isolated from barley (*Hordeum vulgare* L. cv Gerbel) leaves. Plant Physiology 106: 187-193.

Siriphanich, J. and Kader, A.A. 1986. Change in cytoplasmic and vacuolar pH in harvested lettuce tissue as influenced by CO_2. Journal of the American Society for Horticultural Science 111(1): 73-77.

Steffens, J.C., Harel, E. and Hunt, M.D. 1994. Phenol oxidase. Genetic engineering of plant secondary metabolism. Ellis, B. E., Kuroki, G. W. and Stafford, H. A. New York/London. ISBN 0-306-44804-1, Plenum press. 28: 275-311.

Thomas, C.E., McLean, L.R., Parker, R.A. and Ohlweiler, D.F. 1992. Ascorbate and phenolic antioxidant interactions in prevention of liposomal oxidation. Lipids 27(7): 543-549.

Veltman, R.H., Kho, R.M., Schaik, v., A.C.R., Sanders, M.G. and Oosterhaven, J. 2000. Ascorbic acid and tissue browning in pears (*Pyrus communis* L. cvs Rocha and Conference) under controlled atmosphere conditions. Postharv.Biol.Technol. 19: 129-137.

Veltman, R.H., Larrigaudiere, C., Wichers, H. J., Schaik, v., A.C.R., Plas, v. d., L.H.W. and Oosterhaven, J. 1999. PPO activity and polyphenol content are not limiting factors during brown core development in pears (*Pyrus communis* L. cv. Conference). J.Plant Physiol. 154: 697-702.

Veltman, R.H., Lenthéric, I., Peppelenbos, H.W. and Van der Plas, L.H.W. 2001. Internal browning in *Pyrus communis* fruits is caused by a combined effect of free oxygen radicals and limited energy availability. Physiologia Plantarum (submitted).

Veltman, R.H., Sanders, M.G., Persijn, S. T., Peppelenbos, H.W. and Oosterhaven, J. 1999. Decreased ascorbic acid levels and brown core development in pears (Pyrus communis cv Conference). Physiol. Plant 107: 39-45.

Williams, M.W. and Patterson, M.E. 1962. Internal atmospheres in Bartlett pears stored in controlled atmospheres. American Society for Horticultural Science 40: 129-136.

Williams, M.W. and Patterson, M.E. 1964. Non-volatile organic acids and core breakdown of Bartlett pears. Agricultural and Food Chemistry 12(1): 80-83.

Yamaki, S. 1984. Isolation of vacuoles from immature apple fruit flesh and compartmentation of sugars, organic acids, phenolic compounds and amino acids. Plant and Cell Physiology 25(1): 151-166.
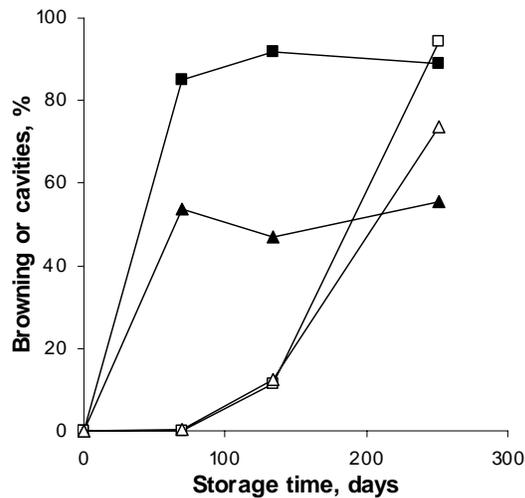
Fig. 1. Development of internal browning (filled symbols) and cavities (open symbols) in pears from two growing locations (squares and triangles). Both are expressed as percentages affected fruits. Pears were stored in an experimental small-scale static storage system at standard conditions, enriched with enhanced carbon dioxide (3 kPa). Aberrations clearly begin after 2 months, while later in the storage season cavities are formed.
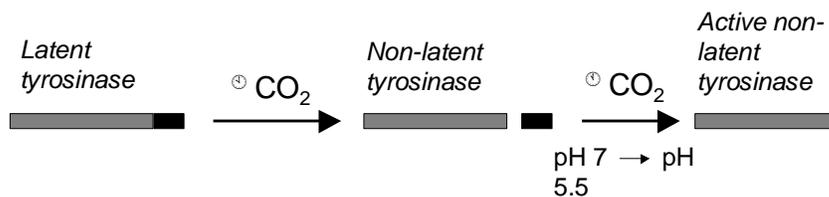

Fig. 2. Overview of the possible effect of enhanced carbon dioxide on tyrosinase functioning.
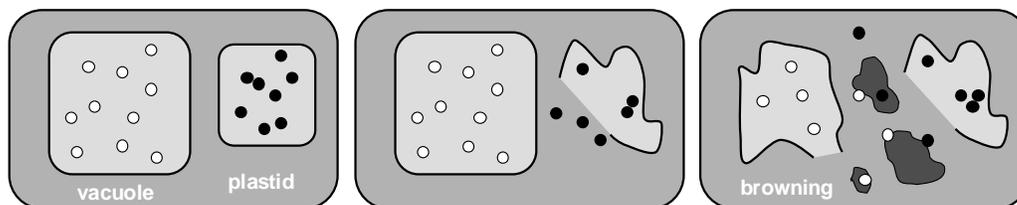
Fig. 3. Schematic representation of the process of decompartmentation. Dots represent phenolic substrates located in the vacuole (white dots) and tyrosinase in the plastids (black dots). Pigmentation spots are the places were PPO formed quinones, which polymerised into melanins.
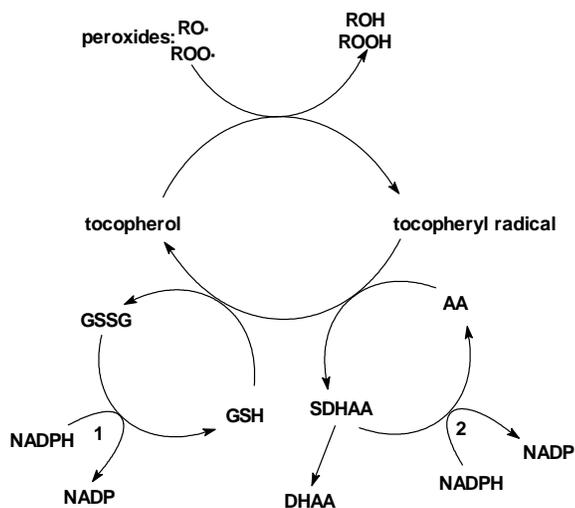
Fig. 4. Interaction between the three most important antioxidants. GSH: reduced form of glutathione; GSSG: oxidised form of glutathione; AA: ascorbic acid; SDHAA: semi-dehydroascorbic acid; DHAA: dehydroascorbic acid. Oxidised glutathione and ascorbic acid (SDHAA) are regenerated by GSH reductase (1) and AA reductase (2) respectively.
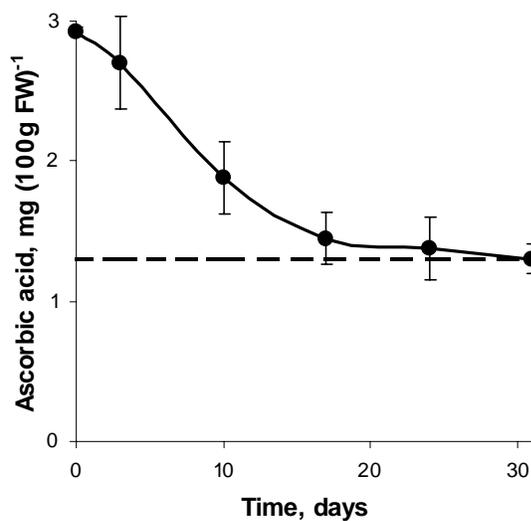

Fig. 5. Decrease of ascorbic acid in pears during storage under enhanced carbon dioxide levels (n=4). Pears were stored at 5°C, 2 kPa oxygen and 5 kPa carbon dioxide. The dashed line gives the suggested level of ascorbic acid in the cytosol of the cell.
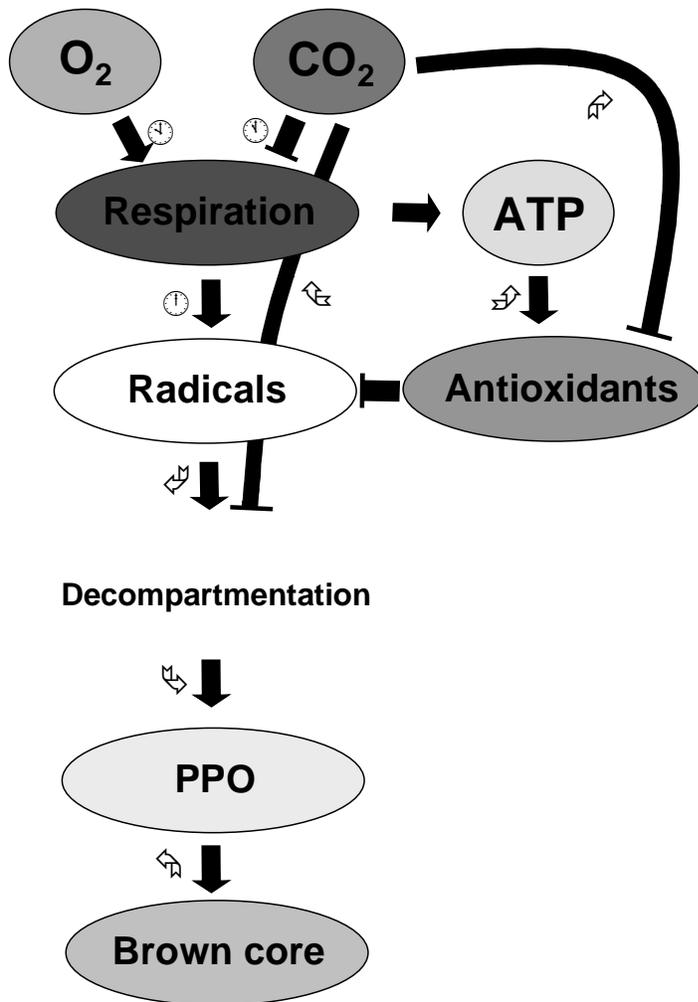
254

Fig. 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.