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Fabio Rodrigo Thewes

MÉTODOS DE ATMOSFERA CONTROLADA DINÂMICA × 1-MCP: METABOLISMO E QUALIDADE DE MAÇÃS ARMAZENADAS

Santa Maria, RS
2019

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Tese apresentada ao Curso de Pós-Graduação em Agronomia, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do título de **Doutor em Agronomia.**

Orientador: Prof. Dr. Auri Brackmann

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Aprovado em 18 de Dezembro de 2019:

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Roger Wagner, Prof. Dr. (UFSM)

Santa Maria, 18 de Dezembro de 2019.

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em especial à minha esposa Lilian Osmari Uhlmann, aos meus pais Roque e Marieta Thewes
e ao meu irmão Flavio Roberto Thewes pelo companheirismo e ajuda durante esse período.

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RESUMO

MÉTODOS DE ATMOSFERA CONTROLADA DINÂMICA × 1-MCP: METABOLISMO E QUALIDADE DE MAÇÃS ARMAZENADAS

AUTOR: Fabio Rodrigo Thewes
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A redução da pressão parcial de O_2 a níveis extremamente baixos (< 0,5 kPa) durante armazenamento em atmosfera controlada (AC) está sendo cada vez mais empregado comercialmente. Contudo, pressões parciais de O_2 excessivamente baixas podem induzir o metabolismo anaeróbico, com produção de acetaldeído e etanol, os quais em excesso podem causar distúrbios fisiológicos e *off-flavours*. Assim, para reduzir a pressão parcial de O_2 de maneira segura durante o armazenamento, é necessário o monitoramento do limite mínimo de O_2 (LMO) em tempo real para adequar a pressão parcial de O_2 ao metabolismo das frutas. Esse sistema de armazenamento é conhecido como atmosfera controlada dinâmica (ACD). Atualmente, estão disponíveis comercialmente três métodos de ACD, baseados no etanol (ACD – EtOH), fluorescência de clorofilas (ACD – FC) e quociente respiratório (ACD – QR). Nesse sentido, no presente trabalho foram desenvolvidos 5 artigos científicos com os objetivos de: [1] avaliar o efeito do armazenamento em AC, ACD – FC, ACD – QR com dois estresses múltiplos de baixo O_2 por semana e sua interação com 1-MCP sobre a qualidade, perfil volátil e expressão de enzimas relacionadas à síntese de compostos voláteis; [2] estudar o efeito do armazenamento em ACD – QR sobre a dinâmica do metabolismo anaeróbico e indução de açúcares-álcoois, como sorbitol e glicerol, e sua relação com a permeabilidade da membrana celular em maçãs e [3] desenvolver, calibrar e aplicar um novo método de ACD, baseado na produção de CO_2 das frutas (ACD – DC), para estimar o LMO, visando manter a qualidade, avaliar a atividade de enzimas, metabolismo de açúcares e de ácidos e o perfil volátil de maçãs armazenadas em ACD – DC, além disso, comparar o armazenamento em ACD-DC com os métodos de ACD – FC, ACD – QR e aplicação de 1-MCP. O armazenamento de maçãs em ACD – QR 1,5 com dois estresses de baixo O_2 por semana resulta em frutas com menor produção de etileno, maior qualidade físico-química, especialmente maior emissão de ésteres. Isso é resultado da maior expressão de genes codificadores da enzima AAT (*MdAAT1*), mesmo em frutas tratadas com 1-MCP, evidenciando que a expressão dos genes que codificam a enzima AAT (*MdAAT1*) não são dependentes de etileno quando as frutas são armazenadas em ACD – QR 1,5. Maçãs armazenadas ACD – FC tem menor acúmulo de voláteis em função da menor produção de precursores e expressão de enzimas importantes para a síntese destes compostos (*MdAAT1*), pois reduz a níveis mínimos o metabolismo aeróbico sem induzir o metabolismo anaeróbico. O armazenamento de maçãs em ACD – QR resulta na indução do metabolismo anaeróbico, acumulando acetaldeído e etanol, porém, também induz o acúmulo de sorbitol, reduzindo a permeabilidade de membrana mesmo em condições de estresse por baixo O_2. A determinação do LMO pode ser realizada de maneira precisa e em tempo real durante todo o período de armazenamento apenas pela determinação da produção de CO_2 (ACD – DC). O que permite a determinação do *set point* de O_2 da câmara de maneira dinâmica para várias cultivares de maçãs, locais de cultivo, com ou sem aplicação de 1-MCP, estádio de maturação e temperatura de armazenamento. Maçãs armazenadas em ACD – DC resultam em *set points* de O_2 e manutenção da qualidade similar àquelas armazenadas em ACD – QR e qualidade superior àquelas armazenadas em AC, AC + 1-MCP e ACD – FC, em função da redução da incidência de podridões e distúrbios fisiológicos, manutenção de maior firmeza de polpa e proporção de frutos sadios. Frutas armazenadas em ACD com concentrações extremamente baixas de O_2 também apresentam maior concentração de ésteres importantes para o aroma de maçãs, como acetato de butila, acetato de 2-metilbutila e acetato de hexila. As condições de AC e ACD não apresentam efeito no acúmulo de malato, sendo a sua concentração mais afetada pelo tempo de armazenamento. Os ácidos minoritários do ciclo de Krebs são os mais influenciados pelo armazenamento em ACD, sendo a sua concentração reduzida quando as maçãs são armazenadas nas menores pressões parciais de O2 (ACD – QR 1,5 e ACD – DC 1,3). De uma maneira geral, as melhores condições de armazenamento de maçãs por longos períodos segue a seguinte ordem: ACD – DC = ACD – QR > ACD – FC = AC + 1-MCP > AC.

Palavras-chave: *Malus domestica*. Novo método de ACD. Desordens fisiológicas. Firmeza de polpa. Metabolismo anaeróbico. Expressão gênica. Metabolismo de ácidos. Sorbitol.

ABSTRACT

DYNAMIC CONTROLLED ATMOSPHERE METHODS × 1-MCP: METABOLISM AND QUALITY OF STORED APPLES

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Lowering the O_2 partial pressures to extremely low levels (< 0.5 kPa) during controlled atmosphere (CA) storage is becoming more and more usually in commercial rooms. However, lowering to much the O_2 partial pressure can induce the anaerobic metabolism, with acetaldehyde and ethanol production, compounds that can induce physiological disorders and off-flavours, if in too high concentrations. Thus, to decrease the O_2 partial pressure in a save way, it is necessary monitor the lowest O_2 limit (LOL) in real time over the storage period, in order to set the O_2 partial pressure according to fruit metabolism. This storage system in known as dynamic controlled atmosphere (DCA). Nowadays, there are three DCA methods available commercially: based on ethanol (DCA – EtOH), chlorophyll fluorescence (DCA – CF) and respiratory quotient (DCA – RQ). In this sense, at the present study were developed 5 papers aiming at: [1] evaluate the effect of CA, DCA – CF, DCA – RQ with two low oxygen stresses a week and it's interaction with 1-MCP on the overall quality, volatile profile and expression of enzymes involved on volatile compounds synthesis; [2] study the effect of DCA – RQ storage on the dynamics of anaerobic metabolism and the induction of sugar-alcohols, such as sorbitol and glycerol, and its relationship with the membrane permeability of apples; [3] develop, calibrate and apply a novel DCA method based on CO_2 production of fruit (DCA – CD) to estimate the LOL, aiming at maintain overall quality, enzyme activity, sugars, acids metabolism and the volatile compounds profile under DCA – CD. Furthermore, compare the storage under DCA – CD with DCA – CF, DCA – RQ and 1-MCP treatment. The storage of apples under DCA – RQ 1.5 with two low oxygen stresses a week resulted in fruit with lower ethylene production, higher physical and chemical quality, especially higher esters emission. This is a result of higher level of AAT enzymes genes expression (*MdAAT1*), even when fruit were treated with 1-MCP, showing that the expression of *MdAAT1* genes are not ethylene dependent in fruit stored under DCA – RQ 1.5. Apple stored under DCA – CF had lower volatile accumulation due to lower precursors concentration and expression of enzymes involved in esters (*MdAAT1*), because reduces the aerobic respiration to a minimum level without the induction of anaerobic metabolism. The storage of apples under DCA – RQ resulted in anaerobic metabolism, accumulating acetaldehyde, ethanol and inducing sorbitol accumulation, decreasing the membrane permeability even under low O_2 stress condition. The LOL determination can be performed, in real time, over the storage period by the CO_2 production only (DCA – CD). This method allows the O_2 set point determination in a dynamic way for several apple cultivars, orchards, without and with 1-MCP treatment, harvest maturity and storage temperature. Apples storage under DCA – CD resulted in similar O_2 set points and quality maintenance as compared to DCA – RQ and higher as compared to CA, CA + 1-MCP and DCA – CF, because reduced decay and physiological disorders, maintained higher firmness and healthy fruit amount. Fruit stored under DCA with extremely low oxygen had higher main ester concentration, such as butyl acetate, 2-methylbutyl acetate and hexyl acetate. CA and DCA had no effect on malate concentration, being its concentration more affected by storage time. The Krebs cycle minority acids are significantly affected by the DCA conditions, being its concentration reduced by the storage under low oxygen storage (DCA – RQ 1.5 and DCA – CD 1.3). In general terms, the best long-term apple storage conditions follow this order: DCA – CD = DCA – RQ > DCA – CF = CA + 1-MCP > CA.

Keywords: *Malus domestica*. New DCA method. Physiological disorders. Flesh firmness. Anaerobic metabolism. Gene expression. Acids metabolism. Sorbitol.

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LISTA DE ABREVIATURAS

%	Porcentagem
μg	Micrograma
μL L^{-1}	Microlitro por litro
1-MCP	1-metilciclopropeno/1-methylcylclopropene
2meta	2-methylpropyl acetate
2metp	2-methylpropanol
2-OG	2-Oxoglutarate
AA	Acetic acid
AAT	Álcool Acetil Transferase
AC	Atmosfera controlada
ACC	Ácido 1-aminociclopropano-1-carboxílico
ACC oxidase	Ácido 1-aminociclopropano-1-carboxílico oxidase
ACC sintase	Ácido 1-aminociclopropano-1-carboxílico sintase
ACD	Atmosfera controlada dinâmica
ACD – DC	Atmosfera controlada dinâmica monitorada pela produção de CO_2
ACD – EtOH	Atmosfera controlada dinâmica monitorada pelo etanol
ACD-FC	Atmosfera controlada dinâmica monitorada pela fluorescência de clorofilas
ACD-QR	Atmosfera controlada dinâmica monitorada pelo quociente respiratório
Ace	Acetaldehyde
ACP	Anaerobic compensation point
ACR	Advanced Control Respiration
ADH	Álcool desidrogenase
AMF	Anaerobic metabolism factor
ANOVA	Análise de Variância
AR	Armazenamento refrigerado
ATP	Adenosina trifosfato
AVG	Aminoethoxyvinylglycine
BCAT	Branched-chain amino acid aminotransferase
BIAS	BIAS index
C_2H_4	Fórmula molecular do Etileno
CA	Controlled atmosphere

Cit	Citrate
cDNA	Complementary DNA
CIA	Solution containing chloroform and isoamylic alcohol in a proportion 24:1
CO_2	Dióxido de carbono (gás carbônico)
CR	Carbon release
DCA	Dynamic controlled atmosphere
DCA-CD	Dynamic controlled atmosphere monitored by CO_2 release
DCA-CF	Dynamic controlled atmosphere – chlorophyll fluorescence
DCA-RQ	Dynamic controlled atmosphere – respiratory quotient
DCS	Dynamic Control System
DNA	Deoxyribonucleic acid
DTT	dithiothreitol
dw	Agreement index
EtAC	Ethyl acetate
EtOH	Ethanol
FA	Fumarate
FAD^+/FADH	Flavina adenina dinucleotídeo oxidada/ reduzida
FID	Flame ionization detector
FIRM	Fluorescence Interactive Response Monitor
FMA	Fator de metabolismo anaeróbico
Fru	Fructose
FS	Fotossistema
GA	Glutamate
GC-FID	Cromatógrafo a gás com detector por ionização em chama
GC-MS	Cromatógrafo a gás acoplado à espectrômetro de massa
Glu	Glucose
H_2SO_4	Sulfuric acid
HPLC	High performance liquid chromatography
HS-SPME	Solid phase microextraction
IEC	Internal Ethylene Concentration
ILOs	Initila Low Oxygen Stress
kg	Quilograma
KOB	Competence Centre for Fruit Growing at Lake Constance

kPa	Kilopascal
L	Litro
LED	Light Emitting Diode
LMO/LOL	Limite mínimo de oxigênio/Lower oxygen limit
LOS	Low oxygen stress
LRI	Linear Retention index
LSD	Fisher's lowest significant difference
M	Molar
m^3	Metro cúbico
MA	Malate
MdAAT1/2	Gene para enzima Álcool Acetil Transferase em maçã
MdACO1	Gene para ACC oxidase em maçã
MdACS1	Gene para ACC sintase em maçã
MdADH1	Gene para enzima Álcool desidrogenasse em maçã
MdERS1/MdERS2	Genes para receptores de etileno em maçã
MdH1	Genes para *HISTONE 1*
MdLOX1	Gene para enzima lipoxigenase em maçã
MdPDC2	Genes para enzima piruvato descarboxilase em maçã
MdUBC	Genes para *UBIQUITIN- CONJUGATING ENZYME E2*
mEq	Mili-equivalentes
MES	2-(N-morpholino) ethane-sulfonic acid
mg	Miligrama
$MgCl_2$	Cloreto de magnésio
mL	Mililitro
mm	Milímetro
N	Newton ou Normal
N_2	Nitrogênio
Na	Sódio
NAA	Naphthaleneacetic acid
NaCl	Cloreto de sódio
NAD^+/ NADH	Nicotinamida adenina dinucleotídeo oxidada/ reduzida
NADPH	Nicotinamida adenina dinucleotídeo fosfato reduzida
NaOH	Hidróxido de sódio

ng	Nano grama
NIST	National Institute of Standards and Technology
NPP – UFSM	Núcleo de Pesquisa em Pós-Colheita da Universidade Federal de Santa Maria
NTC	No template control
O_2	Oxigênio
OA	Oxalate
OAA	Oxaloacetate
ºC	Temperatura em graus Celsius
PC	Principal Component
PCA	Ponto de compensação anaeróbica e Principal Component Analysis
pCO_2	Pressão parcial de gás carbônico
PCR	Polymerase Chain Reaction
PDC	Enzima piruvato descarboxilase
PDH	Piruvato desidrogenase
pH	Potencial hidrogeniônico
Pir	Pyruvate
pO_2	Pressão parcial de oxigênio
ppm	Partes por milhão
PQ	Plastoquinona
PVPP	Polyvinyl polypyrrolidone
qPCR	Quantitative PCR
QR	Quociente respiratório
RH	Relative humidity
RMSE	Root mean square error
RNA	Ribonucleic acid
RQ	Respiratory quotient
RT-qPCR	Real-time reverse transcription polymerase chain reaction
s	Segundo
SA	Succiante
SD	Standard deviation
Sor	Sorbitol
SPI	Starch pattern index

Suc	Sucrose
TCA	Tricarboxylic acids cycle
U	Unidades arbitrárias de absorbância
ULO	Ultralow oxygen (ultrabaixo oxigênio)
UR	Umidade relativa

SUMÁRIO

1. INTRODUÇÃO E JUSTIFICATIVA

A maçã é uma das frutas mais cultivadas apresentando grande importância no Brasil e no mundo. No Brasil a estimativa de safra varia de 1 - 1,1 milhões de toneladas anuais (KIST et al., 2018). Deste montante, em torno de 70% é da cultivar Gala e seus mutantes (AGAPOMI, 2018). Em contrapartida, na Europa o cultivo de maçãs é muito diversificado, baseado em vários cultivares e seus mutantes, porém a colheita é restrita a poucos meses do ano (WAPA, 2018). A sazonalidade da colheita dessa fruta resulta em períodos de alta e baixa oferta. Essa característica da espécie, faz com que o armazenamento seja importante na oferta de frutas de qualidade durante o ano todo a preços acessíveis aos consumidores.

Atualmente, o armazenamento de maçãs é realizado, na sua grande maioria, em sistemas de atmosfera controlada (AC), com níveis de oxigênio e gás carbônico definidos pela pesquisa experimental e mantidos constantes durante o armazenamento (ARGENTA; FAN; MATTHEIS, 2000; BRACKMANN et al., 2008, 2013; CORRÊA et al., 2010; KITTEMANN; NEUWALD; STREIF, 2015; LUMPKIN et al., 2014, 2015; WEBER et al., 2011). Essas condições de AC são definidas baseando-se em vários anos de pesquisa a fim de determinar uma condição "segura" de O_2 e CO_2 para a manutenção da qualidade das diferentes cultivares com diferentes sensibilidades ao baixo O_2 e alto CO_2. Entretanto, nesse método de armazenamento não é levado em consideração a variação do metabolismo das frutas em função da estação de cultivo, do período de armazenamento, da temperatura, uso de reguladores de crescimento, entre outros fatores que influenciam o metabolismo das frutas (WRIGHT et al., 2015). Assim, mesmo com o armazenamento de maçãs em AC, ocorrem perdas decorrentes de distúrbios fisiológicos, podridões, perda de firmeza de polpa, redução na emissão de compostos voláteis do aroma e da acidez.

A ocorrência desses problemas durante o armazenamento em AC pode estar relacionada com a pressão parcial de O_2 adotada, que pode estar acima do limite mínimo de O_2 (LMO) tolerado pelas frutas, ou seja, a respiração aeróbica das frutas não é reduzida ao máximo em função da adoção de uma pressão parcial de O_2 considerada segura, resultando em redução mais acentuada na qualidade das frutas durante o armazenamento. Em função disso, há aproximadamente 15 anos, o armazenamento de maçãs em atmosfera controlada dinâmica (ACD) tem ganhado espaço em nível comercial. A grande vantagem da ACD, em comparação à AC, é a possibilidade do monitoramento do LMO durante todo período de armazenamento, através de métodos não destrutivos, sendo possível ajustar a pressão parcial de O_2 na câmara de armazenamento em função do metabolismo das frutas, ou seja, o armazenador pode fornecer

apenas a quantidade mínima de O_2 para sobrevivência das células da fruta, reduzindo assim ao máximo a respiração sem prejuízo à sua qualidade (BESSEMANS et al., 2016; BRACKMANN; WEBER; BOTH, 2015; GASSER et al., 2008; PRANGE et al., 2015; THEWES et al., 2015a; VAN SCHAIK et al., 2015; VELTMAN; VERSCHOOR; VAN DUGTEREN, 2003, 2003; WEBER et al., 2015; WRIGHT et al., 2012).

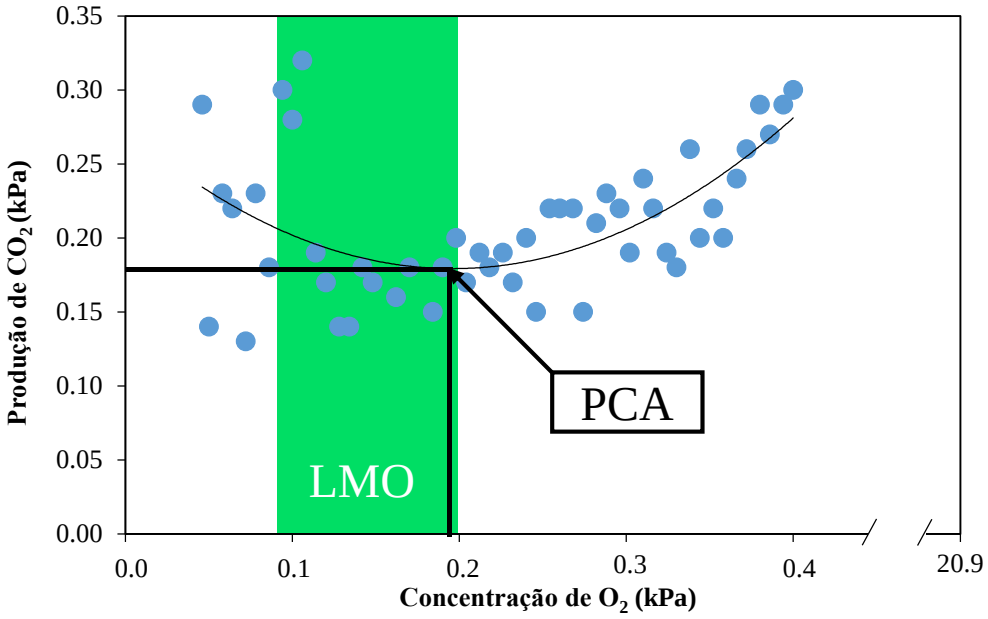
A adoção da ACD em nível comercial está ocorrendo de forma gradual, especialmente em função dos altos custos dos equipamentos necessários à sua implantação, como sensores/safepods, estanqueidade das câmaras e sistemas de absorção de CO_2 eficientes. Em nível mundial, existem três técnicas de ACD utilizadas comercialmente: baseada na produção de etanol pelas frutas (DEUCHANDE et al., 2016; SCHOUTEN et al., 1998; VELTMAN; VERSCHOOR; VAN DUGTEREN, 2003), na fluorescência de clorofilas (ACD – FC) (PRANGE et al., 2003, 2015) e no quociente respiratório, que é a relação entre a produção de CO_2 e o consumo de O_2 (ACD – QR) (BESSEMANS et al., 2016; BRACKMANN; WEBER; BOTH, 2015; WEBER et al., 2015). O armazenamento em ACD monitorada pelo etanol apresenta dificuldade de medição no interior da câmara comercial ou no suco das frutas, pois o mesmo é continuamente convertido em outros compostos voláteis, como acetato de etila, butanoato de etila, hexanoato de etila e outros compostos. A ACD – FC é a tecnologia mais empregada em nível comercial, enquanto que o armazenamento em ACD – QR está em plena expansão (BRACKMANN; WEBER; BOTH, 2015; WEBER et al., 2015). Durante o armazenamento em ACD – QR, a pressão parcial de O_2 é reduzida ao mínimo tolerado pelas frutas (geralmente $< 0,3$ kPa), o que reduz drasticamente a respiração e, consequentemente, ocorre a manutenção da qualidade das frutas, porém, ainda não é sabido se esses benefícios ocorrem em função da redução da atividade enzimática ou pela redução na expressão gênica dessas enzimas, causado pelo etanol produzido no metabolismo anaeróbico.

Como mencionado acima, um dos maiores entraves na adoção da ACD é a necessidade de equipamentos adicionais nas câmaras de AC e a falta de estanqueidade das mesmas, o que resulta na entrada de O_2, dificultando o armazenamento em pressões parciais de O_2 extremamente baixas ($< 0,5$ kPa). Assim, há dificuldade na determinação do LMO pela fluorescência de clorofilas e problemas na determinação do QR, quando o mesmo é avaliado diretamente na câmara comercial. Em função disso, quando se adota a tecnologia ACD – QR, é necessária a instalação de uma minicâmara especial no interior da câmara comercial para determinação do QR, sem que haja interferência da entrada de O_2 na câmara (BRACKMANN, 2015) ou o emprego de modelos matemáticos que corrigem a entrada de O_2 na câmara em função da não estanqueidade (BESSEMANS et al., 2018). Desta maneira, é necessário o

desenvolvimento de outras técnicas de ACD que facilitem a adoção desta tecnologia em nível comercial. Nesse sentido, um método de ACD baseado na produção de CO_2 poderia ser promissor em nível experimental e comercial.

Com a redução do O_2 a níveis extremamente baixos ocorre a indução do metabolismo anaeróbico, que faz com que a produção de CO_2 seja maior que o consumo de O_2, resultando em um QR acima de 1,0. Sendo assim, o metabolismo anaeróbico das frutas poderia ser monitorado pela respiração (produção de CO_2), uma vez que a produção de CO_2 pelas frutas diminui com a redução da pressão parcial de O_2 na câmara, até um determinado nível de O_2, quando a produção de CO_2 tende a aumentar pelo metabolismo anaeróbico (Figura 1), ponto esse conhecido como ponto de compensação anaeróbico (PCA) (BOERSIG; KADER; ROMANI, 1988). Assim, poder-se-ia desenvolver um método de ACD baseado unicamente na produção de CO_2 das maçãs para estabelecer dinamicamente o LMO. A grande vantagem da medição de CO_2, em comparação com a de O_2, é a pouca influência do ambiente externo sobre a sua determinação (BESSEMANS et al., 2016, 2018). Desta maneira, o desenvolvimento de um método de ACD baseado na produção de CO_2 das frutas poderá facilitar a adoção dessa tecnologia em nível comercial, uma vez que, a determinação da produção de CO_2 pode ser realizada em câmaras comerciais sem maiores problemas e sem a necessidade da instalação de equipamentos adicionais, como são necessários nas tecnologias disponíveis atualmente.

Figura 1 – Determinação do ponto de compensação anaeróbico (PCA) e limite mínimo de O_2 (LMO) em maçãs ‘Galaxy’ baseado apenas na produção de CO_2 das frutas.



Fonte: o Autor.

Atualmente, está bem definida que a faixa de QR ideal para o armazenamento de maçãs ‘Gala’ e ‘Fuji’ está entre 1,3 e 1,5 (BRACKMANN; WEBER; BOTH, 2015; ANESE et al., 2019; THEWES et al., 2017a; WEBER et al., 2017) porém, para cultivares de maçãs exploradas na Europa, o QR ideal ainda não está definido. Geralmente, o armazenamento em QR 1,3 resulta em melhor manutenção da qualidade física de maçãs (DE OLIVEIRA ANESE et al., 2019; THEWES et al., 2017a) e o QR 1,5 em maior emissão de compostos voláteis (BOTH et al., 2017; DONADEL et al., 2019; THEWES et al., 2017b, 2017c). Estes estudos demonstram que o armazenamento em ACD – QR é promissor na oferta de maçãs com maior qualidade geral e compostos relacionados ao aroma das frutas, o que é bem visto pelo consumidor. Contudo, estes trabalhos não explicam se a maior síntese de compostos voláteis pelas frutas armazenadas em ACD – QR é função da maior disponibilidade de precursores e/ou função da maior expressão gênica de enzimas responsáveis pela síntese destes compostos. Na literatura clássica é mencionado que, em AC convencional, a redução das pressões parciais de O_2 na câmara de armazenamento reduz o acúmulo de ésteres em função da redução da ação/produção de etileno e beta-oxidação (BANGERTH; SONG; STREIF, 2012; BRACKMANN; STREIF; BANGERTH, 1993; ECHEVERRÍA et al., 2008). Contudo, um estudo recente verificou que a produção de etileno não apresenta correlação com o acúmulo de voláteis em maçãs ‘Galaxy’ armazenadas em QR 1,5, quando ocorre indução do metabolismo anaeróbico (THEWES et al., 2017b).

Nesse sentido, é importante realizar estudos avaliando técnicas complementares a AC, como a aplicação de 1-metilciclopropeno (1-MCP), e comparar com as novas técnicas de ACD, especialmente as baseadas no QR (ACD – QR) e na produção de CO_2 (ACD – DC) que é o método de ACD desenvolvido nesta tese. O 1-MCP inibe a ação do etileno, retardando o amadurecimento das frutas e os eventos fisiológicos dependentes do etileno (BLANKENSHIP; DOLE, 2003; SISLER; SEREK, 1997; WATKINS, 2006). A ACD inibe a síntese de etileno de maneira similar a aplicação de 1-MCP (BOTH et al., 2018; THEWES et al., 2017b), entretanto, resulta no acúmulo de composto do metabolismo anaeróbico, como o etanol, que possui diversos efeitos no metabolismo das frutas (ASODA et al., 2009; JIN et al., 2013; LIU et al., 2012; WEBER et al., 2020), inclusive aumentando a emissão de ésteres. Assim, o desenvolvimento de estudos testando a nova técnica de ACD – DC e sua comparação com outros métodos de ACD, como ACD – FC e ACD – QR, e a aplicação de 1-MCP é de fundamental importância para o avanço da ciência pós-colheita e a facilitação da adoção da ACD em nível comercial reduzindo as perdas pós-colheita. O desenvolvimento de tecnologias

alternativas ao 1-MCP também é de grande importância em função do aumento da produção de frutas no sistema de cultivo orgânico, onde o uso do 1-MCP em pós-colheita é proibido (GABIOUD REBEAUD; GASSER, 2015).

A modificação da atmosfera do ambiente de armazenamento causa diversas modificações metabólicas nas frutas, como o acúmulo de succinato em condições de alto CO_2 (BEKELE et al., 2016; MATHOOKO, 1996) e modificação no metabolismo dos açúcares (BUSATTO et al., 2018; THEWES; BRACKMANN; NEUWALD, 2019) e aminoácidos quando tratadas com 1-MCP (BEKELE et al., 2015; LEE et al., 2012). Todavia, há poucas informações na literatura avaliando o metabolismo dos açúcares e ácidos do ciclo de Krebs em frutas armazenadas em condições de ACD e os efeitos da ação do etileno no metabolismo de ácidos e açúcares durante o armazenamento. Assim, o estudo do metabolismo dos açúcares e ácidos do ciclo de Krebs é de fundamental importância para explicar como as baixas pressões parciais de O_2 interferem no metabolismo das frutas a fim de explicar fisiologicamente como atuam as três técnicas de ACD (ACD – FC, ACD – QR e ACD – DC) e qual o efeito do etileno em condições de O_2 extremamente baixas (< 0,3 kPa).

Assim, pretende-se desenvolver, calibrar e validar um método de ACD inovador, baseado exclusivamente na produção de CO_2 das frutas, a fim de facilitar a medição do LMO em nível comercial. Também pretende-se estudar melhor os efeitos do uso de diferentes métodos de ACD, com estresses por baixo O_2, no metabolismo aeróbico, anaeróbico e volátil de maçãs após longos períodos de armazenamento a fim de entender o porquê da redução na emissão de compostos voláteis quando as frutas são armazenadas em ACD – FC e o aumento quando as mesmas são armazenadas em ACD – QR (AUBERT; MATHIEU-HURTIGER; VAYSSE, 2015; BOTH et al., 2017; THEWES et al., 2017c). Outro ponto importante é a comparação do novo método ACD – DC com os já utilizados comercialmente a fim de comprovar sua eficácia na determinação do LMO em tempo real durante o armazenamento e verificar sua aplicabilidade em câmaras comerciais.

1.1. HIPÓTESES

O armazenamento de maçãs em ACD – QR 1,5 com dois estresses de baixo O_2 por semana resulta em maior concentração de ésteres, maior expressão da enzima Álcool Acetil Transferase (*MdAAT1*) mesmo quando tratadas com 1-MCP em comparação a ACD – FC.

O armazenamento de maçãs em ACD – QR resulta em significativo aumento de compostos do metabolismo anaeróbico, porém também incrementa a síntese de açúcares-álcoois como sorbitol e glicerol, diminuindo a permeabilidade da membrana celular mesmo em condições de estresse por baixo O_2 e acúmulo de acetaldeído e etanol.

A determinação do LMO pode ser realizada de maneira precisa e em tempo real durante o armazenamento apenas pela medição da produção de CO_2 através da aplicação de um algoritmo (FruitAtmo®) a fim de determinar o *set point* de O_2 de maneira dinâmica durante o armazenamento de maçãs de várias cultivares.

O armazenamento em ACD monitorada pela produção de CO_2 das maçãs (ACD – DC) mantém a qualidade similar àquelas armazenadas em ACD – QR e superior àquelas armazenadas em AC, AC + 1-MCP e ACD – FC.

Maçãs armazenadas em ACD – DC resultam em *set points* de O_2 similares à ACD – QR, resultando em frutas com maior emissão de compostos voláteis do que AC + 1-MCP e ACD – FC.

O armazenamento de maçãs em AC e ACD afetam de maneira diferenciada o metabolismo de açúcares e ácidos do ciclo de Krebs, sendo que o maior impacto nesses metabólitos ocorre quando as frutas são armazenadas em ACD – QR e ACD – DC.

O etileno não desempenha regulação direta na dinâmica de açúcares e ácidos do ciclo de Krebs.

Há interação na manutenção da qualidade entre AC, ACD (QR, FC e DC) e 1-MCP de maçãs após longos períodos de armazenamento.

1.2.OBJETIVOS

Verificar a interação entre o armazenamento em AC, ACD – FC e ACD – QR com a aplicação de 1-MCP sobre a manutenção da qualidade, perfil volátil e expressão de enzimas relacionadas a síntese de etileno e compostos voláteis em maçãs ‘Galaxy’ após seis e nove meses de armazenamento mais sete dias de vida de prateleira a 20 °C.

Avaliar o efeito do armazenamento em ACD – QR sobre a dinâmica do metabolismo anaeróbico e indução de açúcares-álcoois, como sorbitol e glicerol, e sua relação com a permeabilidade da membrana celular em maçãs ‘Elstar’ e ‘Nicoter’ produzidas na Europa, após a colheita, seis e nove meses de armazenamento.

Desenvolver, calibrar e validar um método de ACD monitorada pela produção de CO_2 das frutas (ACD – DC) e comparar a estimação do LMO com o método de ACD – QR para várias cultivares de maçãs.

Avaliar o efeito do armazenamento em ACD – DC de maçãs ‘Elstar’ sobre a qualidade, enzimas do metabolismo anaeróbico e perfil volátil em comparação a AC e outros métodos de ACD. Verificar também a necessidade/efeito da aplicação de 1-MCP quando as frutas são armazenadas em ACD – DC e comparar com outros métodos de ACD + 1-MCP.

Identificar o efeito do armazenamento em AC, ACD – FC, ACD – QR e ACD – DC, com ou sem aplicação de 1-MCP, sobre o metabolismo dos açúcares, ácidos do ciclo de Krebs e compostos do metabolismo anaeróbico em maçãs ‘Elstar’ e ‘Nicoter’ após a colheita, seis e nove meses de armazenamento mais sete dias de vida de prateleira a 20 °C.

2. REVISÃO DE LITERATURA

2.1. PRODUÇÃO DE MAÇÃS NO BRASIL E NA ALEMANHA

A maçã é uma das frutas mais produzidas no mundo, sendo a terceira frutífera mais produzida em nível mundial (FAOSTAT, 2017). Já no Brasil é a quinta fruta mais produzida, com produção anual de 1 a 1,1 milhões de toneladas (KIST et al., 2018). Desta produção, a maior parte é da cultivar Gala e seus mutantes, como a 'Galaxy', 'Maxi Gala', 'Royal Gala', entre outras (AGAPOMI, 2018). Em contrapartida, na Alemanha as cultivares de maçãs mais produzidas são do grupo 'Elstar', 'Jonagold' e 'Braeburn' (WAPA, 2018). Brasil e Alemanha estão colocados no ranking de produtores de maçã entre o nono e décimo maiores produtores mundiais (WAPA, 2018).

Como a colheita da fruta é sazonal/concentrada em apenas alguns meses do ano, fevereiro a abril no Brasil e agosto a outubro na Alemanha, o armazenamento de parte da produção é de fundamental importância para oferta de frutas de qualidade durante o ano todo. Atualmente, a maior parte dessa produção é armazenada em sistemas de atmosfera controlada (AC).

2.2. ATMOSFERA CONTROLADA (AC)

A AC é uma técnica de armazenamento que consiste no controle da temperatura de armazenamento, umidade relativa (UR) e composição gasosa, com redução das pressões parciais de O_2 e aumento das de CO_2 (ARGENTA; FAN; MATTHEIS, 2000; BRACKMANN et al., 2008; KITTEMANN; NEUWALD; STREIF, 2015; WEBER et al., 2011). Essa modificação das condições atmosféricas no ambiente de armazenamento resulta em melhor manutenção da qualidade das frutas em comparação ao armazenamento refrigerado (AR), onde apenas a temperatura e UR são controladas (BRACKMANN et al., 2008). De maneira geral, o armazenamento em AC resulta em frutas com melhor manutenção da firmeza de polpa, acidez e menor produção de etileno, respiração e ocorrência de distúrbios fisiológicos (ARGENTA; FAN; MATTHEIS, 2000; BRACKMANN et al., 2008; LUMPKIN et al., 2015; STEFFENS et al., 2013; WEBER et al., 2011).

A redução das pressões parciais de O_2 no armazenamento em AC é baseado em experimentos prévios, ou seja, é definida uma condição de O_2 para cada cultivar e/ou mutante, que é mantida constante durante todo o período de armazenamento. Para maçãs das cultivares Gala e Fuji e seus mutantes, a pressão parcial de O_2 em AC varia de 1,0 a 1,2 kPa (ARGENTA;

FAN; MATTHEIS, 2000; BRACKMANN et al., 2009; CORRÊA et al., 2010; WEBER et al., 2011). Já para cultivares europeias, como Elstar, Nicoter, Braeburn e Jonagold, as pressões parciais de O_2 empregadas em AC são, geralmente, mais altas, variando de 1,0 a 2,0 kPa de O_2 (HO et al., 2013; KITTEMANN; NEUWALD; STREIF, 2015; KÖPCKE, 2015; VELTMAN; VERSCHOOR; VAN DUGTEREN, 2003). O modo de ação do baixo O_2 na AC resulta de sua atuação como aceptor final de elétrons na enzima citocromo *c* oxidase na respiração aeróbica (WRIGHT et al., 2015), ou seja, a redução de sua disponibilidade resulta em redução na respiração das frutas e, consequentemente, em maior período de conservação. O baixo O_2 também tem papel fundamental na redução da síntese de etileno, sendo o O_2 necessário para a atividade da enzima ACC oxidase, que converte ACC em etileno (YANG; HOFFMAN, 1984). Nesse sentido, poder-se-ia inferir que quanto menor a concentração de O_2 na câmara de armazenamento, melhor a manutenção da qualidade durante o armazenamento. Contudo, se a pressão parcial de O_2 for reduzida abaixo do PCA ocorre a indução do metabolismo anaeróbico, resultando no acúmulo de acetaldeído e etanol, os quais em altas concentrações podem causar *off-flavours* (WRIGHT et al., 2015). Assim, no armazenamento em AC a pressão parcial de O_2 utilizada está sempre bem acima do LMO, não reduzindo ao máximo o metabolismo das frutas e, consequentemente, não mantendo a máxima qualidade.

A exemplo das baixas pressões parciais de O_2, o aumento da pressão parcial de CO_2 nas câmaras de AC também influencia o metabolismo das frutas, uma vez que o CO_2 é um produto da respiração. Aumentando a sua concentração no ambiente de armazenamento, ocorre redução na taxa respiratória das frutas (MATHOOKO, 1996). O CO_2 também influencia no metabolismo do etileno, reduzindo a sua produção e ação, especialmente em função de sua ação inibitória na expressão gênica da enzima ACC sintase (GORNY; KADER, 1997; MATHOOKO, 1996). Entretanto, as respostas das frutas a elevadas concentrações de CO_2 durante o armazenamento são variadas e complexas, havendo interação entre cultivares e concentração de CO_2 utilizada (KITTEMANN; NEUWALD; STREIF, 2015; WRIGHT et al., 2015). Nesse sentido, se a concentração de CO_2 for muito elevada podem ocorrer perdas de qualidade em função da ocorrência de distúrbios fisiológicos, como a degenerescência de polpa (ARGENTA; FAN; MATTHEIS, 2000; DE CASTRO et al., 2007, 2008; KITTEMANN; NEUWALD; STREIF, 2015) e ocorrência de cavernas internas (DE CASTRO et al., 2008; SAQUET; STREIF; BANGERTH, 2000; SAQUET; ALMEIDA, 2017). A presença de CO_2 nas câmaras de armazenamento também afeta diversas enzimas do metabolismo das frutas, como succinato desidrogenase e citocromo *c* oxidase (KUBO; INABA; NAKAMURA, 1990; LIU et al., 2004; MATHOOKO, 1996).

O armazenamento em AC traz grandes vantagens na manutenção da qualidade de maçãs após o armazenamento em comparação ao AR. Entretanto, como mencionado acima, as condições de O_2 e CO_2 utilizadas são definidas previamente e, não levam em consideração a modificação do metabolismo das frutas em função da estação de cultivo e do tempo de armazenamento, fatores que influenciam diretamente na tolerância das frutas a baixas pressões parciais de O_2 (BESSEMANS et al., 2016; BOTH et al., 2017; THEWES et al., 2017c; WEBER et al., 2015). Nesse sentido, as pressões parciais de O_2 e CO_2 nas câmaras de AC são reguladas em níveis seguros, ou seja, o O_2 bem acima do LMO e o CO_2 abaixo do limite superior. Isto não permite a máxima manutenção da qualidade, especialmente em função da pressão parcial de O_2 estar acima do LMO, resultando em maior metabolismo das frutas (THEWES et al., 2017a). Para melhorar o armazenamento em AC, estão sendo desenvolvidos e aplicados na prática, diversos métodos que permitem monitorar o metabolismo das frutas em tempo real, otimizando as pressões parciais de O_2 durante o armazenamento. Pesquisas nesse sentido estão sendo realizadas em diversas partes do mundo e com diferentes técnicas de monitoramento do LMO (WRIGHT et al., 2015). Esta nova forma de armazenamento é conhecida mundialmente como atmosfera controlada dinâmica (ACD) sendo o seu princípio explicado nos próximos tópicos.

2.3. ATMOSFERA CONTROLADA DINÂMICA (ACD)

O armazenamento de maçãs em níveis de O_2 extremamente baixos, menores que 0,5 kPa pode causar danos as frutas em função da indução do metabolismo anaeróbico, causando *off-flavours* (KE et al., 1994; KÖPCKE, 2015; WRIGHT et al., 2015). Assim, sempre que for utilizada uma pressão parcial de O_2 extremamente baixa, é de fundamental importância a adoção de um método de determinação do LMO a fim de evitar perdas durante o armazenamento. Atualmente, a detecção do LMO em tempo real durante o armazenamento em ACD pode ser realizada basicamente de três formas: [1] monitorando a produção/acúmulo de compostos do metabolismo anaeróbico, como o etanol (SCHOUTEN et al., 1998; VELTMAN; VERSCHOOR; VAN DUGTEREN, 2003); [2] monitorando a emissão da fluorescência de clorofilas em função da redução da pressão parcial de O_2 (PRANGE et al., 2003, 2015) e [3] pela determinação do quociente respiratório (WEBER et al., 2015). Essas formas de determinação do LMO no armazenamento em ACD estão atualmente disponíveis no mercado.

2.3.1. ACD monitorada pela produção de etanol (ACD – EtOH)

O monitoramento do LMO pela produção de etanol pode ser realizado de duas maneiras, uma no *headspace* da câmara, denominada DCS® (*Dynamic Control System* - DCS®) (SCHOUTEN et al., 1998; VELTMAN; VERSCHOOR; VAN DUGTEREN, 2003) e através da determinação da concentração de etanol no suco do fruto (ILOS-Plus®) (KÖPCKE, 2015). O princípio do método DCS®, desenvolvido na Holanda, baseia-se no monitoramento contínuo da concentração de etanol no *"headspace"* da câmara frigorífica. No momento em que a concentração de etanol na atmosfera da câmara aumenta significativamente, a pressão parcial O_2 é aumentada para que reduza o metabolismo anaeróbico e a concentração de etanol volte ao normal (DEUCHANDE et al., 2016; SCHOUTEN et al., 1998; VELTMAN; VERSCHOOR; VAN DUGTEREN, 2003). O monitoramento da câmara é feito durante todo período de armazenamento.

Estudos realizados utilizando a tecnologia DCS® demonstraram que maçãs 'Elstar' mantêm maior firmeza de polpa, menor incidência de escaldadura e maior coloração verde em comparação àquelas armazenadas em AC (SCHOUTEN et al., 1998; VELTMAN; VERSCHOOR; VAN DUGTEREN, 2003). Entretanto, em peras 'Rocha', o armazenamento em DCS® resultou em acumulação de acetaldeído e etanol na polpa das frutas, resultando em significativo aumento na degenerescência de polpa (DEUCHANDE et al., 2016). Assim, percebe-se que os resultados obtidos utilizando esta tecnologia são um tanto contraditórios e não conclusivos. Esses resultados podem ser explicados pelo fato do etanol ser um metabólito intermediário e precursor de vários outros compostos, sendo a determinação deste composto na atmosfera da câmara comercial complicada em função de sua alta variabilidade e necessidade de equipamentos de alto custo e difícil manejo, como o cromatógrafo. O etanol aplicado na câmara ou o etanol produzido pelas frutas é rapidamente convertido em ésteres etílicos, como o acetato de etila (BRACKMANN; STREIF; BANGERTH, 1993; JIN et al., 2013; LIU et al., 2012). Esse fato, tornou essa tecnologia de ACD pouco difundida, sendo apenas utilizada em algumas poucas câmaras comerciais em nível mundial.

Outra tecnologia de ACD baseada na produção do etanol é a sua determinação no suco extraído das frutas. Esse método é comercializado pela empresa Marvil da Itália e é denominado ILOS-Plus® (MARVIL, 2018). De acordo com as informações fornecidas pela empresa, o método possui a vantagem de uma rápida determinação da concentração de etanol nas frutas, sendo que a mão de obra não necessita ser especializada em função do fácil manuseio do

biossensor enzimático de etanol. Esse biossensor imobiliza algumas biomoléculas na superfície do sensor, possibilitando a determinação da concentração de etanol no suco (BESSEMANS et al., 2016). Entretanto, esse método possui o mesmo problema observado no método DCS®, pois o etanol produzido pelas frutas pode ser convertido em outros compostos causando erros na determinação do LMO durante o armazenamento, além de necessitar a abertura da câmara para retirada de amostras para a determinação de etanol, o que torna o método pouco prático em nível comercial.

2.3.2. ACD monitorada pela fluorescência de clorofilas (ACD – FC)

O monitoramento do LMO pela fluorescência de clorofilas é o mais amplamente utilizado em nível comercial no mundo. No início do armazenamento, a pressão parcial de O_2 é reduzida até o momento em que é detectado um incremento na fluorescência emitida pelas clorofilas presentes na epiderme das maçãs. Neste ponto, a pressão parcial de O_2 deve ser aumentada em 0,2 a 0,3 kPa acima da pressão parcial em que foi detectado o pico de fluorescência de clorofilas, porém, o O_2 nunca deve ser menor que 0,4 kPa (PRANGE, 2018; PRANGE et al., 2003, 2015; WRIGHT et al., 2012). Após a detecção da fluorescência e o aumento do O_2, continua-se o monitoramento da fluorescência para verificar se o O_2 não continua ou volta a ficar abaixo do LMO. A fluorescência das clorofilas ocorre em situações de estresse, que impossibilitam a transferência da energia luminosa captada pela clorofila para os centros de reação (TAIZ; ZEIGER, 2013). Os estresses podem ser de várias origens, como secas, excesso de luz, temperatura (TAIZ; ZEIGER, 2013), além do baixo oxigênio durante o armazenamento (PRANGE, 2018; WRIGHT et al., 2012).

O mecanismo fisiológico que conecta o metabolismo respiratório, como a glicólise, o ciclo de Krebs, e as clorofilas, que estão localizadas nos cloroplastos, ainda não está completamente elucidado. Adicionalmente, também não está claro como a indução do metabolismo anaeróbico poderia induzir a fluorescência de clorofilas. Contudo, na literatura são levantadas basicamente quatro hipóteses, descritas a seguir, para explicar essa relação: [1] a redução das pressões parciais de O_2 a níveis extremamente baixos resulta na produção e acúmulo de acetaldeído, etanol e acetato de etila, que podem causar danos nas membranas celulares e suas organelas, como os cloroplastos, danificando os fotossistemas (FS), dificultando a transferência da energia luminosa e resultando no aumento da fluorescência (MAXWELL; JOHNSON, 2000); [2] níveis extremamente baixos de O_2 provocam a acidose celular e de suas organelas (KE et al., 1994), o que também pode danificar os FS aumentando

a fluorescência de clorofilas (PRANGE; DELONG; HARRISON, 2005); [3] a adoção de pressões parciais de O_2 extremamente baixas resulta no acúmulo de compostos reduzidos no citosol e na mitocôndria que podem ser transportados para o cloroplasto, sendo utilizados para redução do *pool* de plastoquinona (PQ), o que pode frear o transporte da energia luminosa (elétrons) e, consequentemente, aumentar a fluorescência de clorofilas (WRIGHT et al., 2015), gerando um sinal indicando o LMO tolerado pelas frutas e [4] o processo de dissipação da energia lumínica na forma não fotoquímica (fluorescência) tem ligação com o ciclo das xantofilas, mais especificamente a zeaxantina (WRIGHT et al., 2011), nesse ciclo para a transformação de zeaxantina para anteraxantina, é necessária a presença de O_2 para atividade da enzima zeaxantina epoxidase. Assim, a redução da concentração de O_2 pode resultar no acúmulo de zeaxantina, aumentando a fluorescência de clorofilas.

Na prática, para a medição da fluorescência de clorofilas, as frutas são mantidas em um ambiente escuro, sendo utilizadas amostras representativas de toda câmara comercial, geralmente amostra de seis frutas por sensor. Essas frutas são acondicionadas em caixas plásticas que possuem um sensor FIRM (*Fluorescence Interactive Response Monitor*) acoplado na parte superior. Além do sensor, há também quatro lâmpadas LED (*Light Emitting Diode*) que servem com fonte luminosa para determinação do LMO. De maneira geral, durante todo período de armazenamento, a determinação do LMO pela fluorescência de clorofilas é realizada de hora em hora. Para tanto, as quatro lâmpadas LED são ligadas como fonte de energia para o processo fotoquímico das clorofilas da epiderme das frutas. Caso as frutas estejam em condições de O_2 estressantes, o processo fotoquímico é dificultado, resultando na reemissão de luz em um comprimento de onda um pouco superior ao fornecido pelo LED, a chamada fluorescência de clorofilas. Esse sinal é captado pelo sensor FIRM e transferido para um *software* específico que registra a intensidade da fluorescência enquanto a pressão parcial de O_2 é reduzida durante o início do armazenamento. A pressão parcial de O_2 é reduzida até o momento em que é detectado um pico na emissão de fluorescência. A partir desse momento, o O_2 é incrementado em 0,2 kPa, porém nunca a pressão parcial da câmara deverá ser menor que 0,4 kPa, e este nível é mantido assim até o final do armazenamento ou até que um novo pico de fluorescência seja detectado, quando o nível de O_2 deve ser novamente incrementado (PRANGE; DELONG; HARRISON, 2005; THEWES et al., 2015a; TRAN et al., 2015; WRIGHT et al., 2015; ZANELLA; CAZZANELLI; ROSSI, 2008).

Atualmente, a ACD - FC é a tecnologia mais estuda dentre os métodos de ACD (AUBERT; MATHIEU-HURTIGER; VAYSSE, 2015; GASSER et al., 2008; HENNECKE; KÖPCKE; DIEREND, 2008; KITTEMANN; NEUWALD; STREIF, 2015; KÖPCKE, 2015;

RAFFO et al., 2009; THEWES et al., 2015a; TRAN et al., 2015; WEBER et al., 2020; WRIGHT et al., 2012, 2010; ZANELLA; CAZZANELLI; ROSSI, 2008; ZANELLA; STÜRZ, 2015) e utilizada comercialmente no mundo. Atualmente, as tecnologias de ACD – FC são comercializadas por duas empresas, a Isolcell e Besseling, sob o nome comercial de HarvestWatch™ e FruitObserver®, respectivamente (PRANGE, 2018). A grande vantagem da utilização da ACD – FC em comparação à AC é a possibilidade de redução das pressões parciais de O_2 a níveis extremamente baixos, o que reduz drasticamente a respiração e a produção de etileno das frutas, mantendo melhor qualidade das frutas após o armazenamento, comparado à AC (HENNECKE; KÖPCKE; DIEREND, 2008; KÖPCKE, 2015; PRANGE; DELONG; HARRISON, 2005; THEWES et al., 2015a; WRIGHT et al., 2011; ZANELLA; CAZZANELLI; ROSSI, 2008). Entretanto, em todos os estudos citados acima, o período durante o armazenamento que ocorre maior variação da pressão parcial de O_2 em função da fluorescência de clorofilas é nos primeiros meses de armazenamento, o que aproxima essa tecnologia ao armazenamento em ultra baixo oxigênio (ULO) (THEWES et al., 2015a), pois a tecnologia não prevê redução da pressão parcial de O_2 abaixo de 0,4 kPa.

Estudos desenvolvidos com mutantes de maçãs ‘Gala’, ‘Royal Gala’ e ‘Galaxy’, cultivadas no Brasil demonstram que o armazenamento em ACD – FC não trouxe benefícios quando comparado a condições de ULO com 0,4 kPa O_2 (THEWES et al., 2015a). Em contrapartida, esses autores reportam que a redução rápida do O_2, para detectar o pico de fluorescência de clorofilas, no período inicial de armazenamento pode resultar em danos nas frutas, reduzindo a qualidade dos mesmos. O longo período com o O_2 próximo a 0,0 (em torno de 2 a 5 dias), até que ocorra o pico de fluorescência, deve ser função da fluorescência de clorofilas não ser uma resposta direta do efeito do baixo oxigênio no metabolismo da fruta, mas sim uma resposta da mudança do metabolismo e seus produtos na célula, como acidose citoplasmática, redução da PQ ou produção de etanol. Esses eventos ocorrem bem depois do momento em que o O_2 foi reduzido, podendo o O_2 ter ficado em concentrações abaixo do LMO por tempo demasiado, resultando em distúrbios fisiológicos e, consequentemente, não manutenção da máxima qualidade.

Diversos trabalhos na literatura relatam e comparam o armazenamento em ACD – FC à AC e ULO (<0.7 kPa), na manutenção da qualidade de maçãs e outras frutas. Em geral ocorre uma redução na ocorrência de distúrbios fisiológicos quando comparado à AC, especialmente da escaldadura superficial (EREN et al., 2015; MDITSHWA et al., 2017a, 2017b; MDITSHWA; FAWOLE; OPARA, 2018; PRANGE et al., 2015; ZANELLA et al., 2005), degenerescência de polpa (BOTH et al., 2017; DEUCHANDE et al., 2016; KÖPCKE, 2015;

RIZZOLO et al., 2015; THEWES et al., 2015a), manchas na epiderme (HENNECKE; KÖPCKE; DIEREND, 2008; KÖPCKE, 2015; RIZZOLO et al., 2015) e maior manutenção da firmeza de polpa (BOTH et al., 2017; GABIOUD REBEAUD; GASSER, 2015; KÖPCKE, 2015; THEWES et al., 2015a; TRAN et al., 2015; WEBER et al., 2019) e da porcentagem de frutos sadios. Entretanto, as frutas armazenadas em ACD – FC geralmente tem menor emissão/concentração de ésteres em comparação à AC (AUBERT; MATHIEU-HURTIGER; VAYSSE, 2015; BOTH et al., 2017; DONADEL et al., 2019; THEWES et al., 2017b, 2017c), o que resulta em frutas com menor qualidade sensorial, especialmente no que diz respeito a aroma e flavor (AUBERT; MATHIEU-HURTIGER; VAYSSE, 2015; TRAN et al., 2015). Essa redução na emissão de compostos voláteis pelo armazenamento em ACD – FC necessita de mais estudos para explicar o porquê disso. Outro ponto crítico para adoção da ACD – FC é a pouca representatividade do número de frutas utilizados por sensor, de 6 a 8 frutas por sensor, sendo alocados em média seis sensores por câmara comercial, o que resulta em 36 - 48 frutas para representar uma câmara de 300 toneladas, por exemplo. Adicionalmente, a detecção do estresse é realizada nas primeiras camadas de células da epiderme (WRIGHT et al., 2015), local onde tem maior disponibilidade de O_2 em comparação ao interior do fruto (HO et al., 2013), sendo assim, quando as células da epiderme acusam estresse por baixo O_2 (pico de fluorescência de clorofilas) as células no interior da polpa, provavelmente, já foram danificadas pelo metabolismo anaeróbico. De acordo com pesquisas realizadas no Núcleo de Pesquisa em Pós-Colheita da Universidade Federal de Santa Maria (NPP – UFSM), o quociente respiratório (QR) no momento da detecção da fluorescência de clorofilas é superior a 10, o que indica alta taxa de metabolismo anaeróbico nas maçãs antes da emissão de fluorescência de clorofilas (*Informação pessoal*). Nesse sentido, a determinação do LMO pelo QR poderia ser mais precisa em comparação à ACD – FC, pois mede diretamente a relação entre produção de CO_2 e o consumo de O_2.

2.3.3. ACD monitorada pelo quociente respiratório (ACD – QR)

A tecnologia de ACD – QR é baseada no princípio de que a razão entre a produção de CO_2 e o consumo de O_2 é próxima a 1,0 em condições normóxicas, ou seja, em condições de suprimento de O_2 adequado as frutas. Todavia, mesmo em condições normóxicas, o valor de QR pode variar um pouco acima ou abaixo de 1,0, em função do substrato que está sendo utilizado na respiração, sendo exatamente igual a 1,0 quando o substrato são açúcares (GOYETTE et al., 2012). Estudos realizados recentemente demostram que valores de QR

menores do que 1,0 podem ser função da solubilização do CO_2 no suco celular (DELELE et al., 2019), porém podem ser função dos substratos utilizados na respiração serem lipídios (PATEL; BHARDWAJ, 2019). Desta maneira, pode-se monitorar o LMO das frutas em função de cálculos periódicos do QR e sua comparação com um QR desejado (GASSER et al., 2008; VAN SCHAIK et al., 2015; WEBER et al., 2015). Níveis de QR acima de 1,0 indicam indução do metabolismo anaeróbico das frutas e, consequentemente, produção e acúmulo de acetaldeído, etanol e ésteres de etila, o que pode ser benéfico na manutenção da qualidade das frutas (ASODA et al., 2009; JIN et al., 2013; LIU et al., 2012; WEBER et al., 2016, 2020). Em contrapartida, se a indução do metabolismo anaeróbico for demasiada podem ocorrer a produção e acúmulo de *off-flavours* (WRIGHT et al., 2015).

A partir dessas constatações, estudos foram realizados tentando aplicar essa técnica de monitoramento do LMO em nível comercial, com a medição do QR em câmaras comerciais. As primeiras câmaras de ACD – QR se basearam na medição do QR em toda a câmara comercial, sendo a tecnologia patenteada pela empresa Van Amerongen CA Technology da Holanda, com o nome de Advanced Control Respiration (ACR) – My Fruit Dynamic. Para tanto, a recomendação da empresa é que seja realizado o desligamento de todo o sistema de ventilação, refrigeração e absorção de CO_2 durante a medição do QR, para evitar mudanças de pressão no interior da câmara e permitir a determinação do acúmulo de CO_2 e consumo do O_2 pelas frutas (VELTMANN, 2013). Além disso, a câmara é equipada com um sensor de pressão que mantém a câmara sempre sob pressão através da injeção de nitrogênio puro durante esse período. Assim, caso a câmara não esteja completamente vedada, a tendência é sair gás da câmara e não entrar ar, resultando assim numa determinação de QR mais confiável. Entretanto, mesmo com esse manejo podem ocorrer erros em função da entrada de O_2 pelas paredes da câmara, resultando em superestimação do LMO e, consequentemente, o *set point* de O_2 não é ajustado ao mínimo tolerado pelo fruto (BESSEMANS et al., 2018). Estudos realizados na Holanda encontraram resultados satisfatórios na estimação do LMO usando essa tecnologia em maçãs do cultivar Elstar (VAN SCHAIK et al., 2015).

O desligamento de todos os equipamentos e aplicação de pressão no interior da câmara não é desejável, pois ocorre a elevação da temperatura das frutas e movimentação dos painéis (paredes) da câmara em função da variação da pressão, o que diminuí consideravelmente a vida útil das câmaras. Para resolver esse problema poder-se-ia instalar no interior da câmara comercial uma outra minicâmara especialmente para determinação do QR e estimação do LMO para a câmara comercial, nesse sentido foram desenvolvidos dois métodos: [1] Safepod® e [2] RQ – StoreFresh.

A empresa norte americana Storage Control System Inc. (Sparta, MI, USA) desenvolveu um dispositivo chamado Safepod®, que permite o fechamento hermético automatizado (SCHAEFER; BISHOP, 2012). No interior desse recipiente são colocadas as frutas, amostras representativas de todas as frutas da câmara, para realizar a determinação do QR. Com o Safepod® reduz-se muito as chances de erro na determinação do QR e, consequentemente, na estimação do LMO durante o armazenamento. Entretanto, nesse sistema e naquele em que o QR é determinado em toda câmara, são necessários analisadores de O_2 muito precisos e sem muita variação temporal, pois, a relação entre produção de CO_2 e consumo de O_2 é feita através da leitura em um determinado período de tempo de fechamento do dispositivo com as frutas.

Nesse sentido, foi desenvolvido no NPP – UFSM um dispositivo para contornar os problemas dos analisadores, sendo o mesmo patenteado e comercializado pela empresa Isolcell com o nome de RQ – StoreFresh (BRACKMANN, 2015). Esse dispositivo é muito similar ao Safepod®, porém, além do dispositivo com amostra de frutas, ele possui um reservatório de gás com a concentração de gases igual ao da atmosfera da câmara no momento de fechamento do dispositivo com as frutas para determinação do QR. Esse reservatório é fechado no início do processo de medição do QR e é usado para determinação dos níveis de O_2 e CO_2 inicial, no momento da leitura da concentração gasosa no dispositivo com as frutas. Dessa maneira evita-se erros no cálculo do QR em função dos analisadores, além de possibilitar várias determinações do QR e, assim, ter um valor mais confiável de LMO (BRACKMANN, 2015).

Mais recentemente, foi desenvolvido um modelo matemático que simula qual é a entrada de O_2 em uma câmara comercial em função da sua estanqueidade e variação da pressão atmosférica, a fim de possibilitar a medição de QR na câmara comercial inteira (BESSEMANS et al., 2016, 2018), e patenteada pela Universidade de Leuven na Bélgica (DELELE et al., 2015). A dificuldade da adoção dessa tecnologia é a determinação exata de qual a área de painel que contém furos que possibilitam vazamentos na câmara comercial e também em função da variação do vazamento da câmara durante o período de armazenamento. Recentemente, estavam sendo conduzidos estudos em câmaras comerciais com essa tecnologia na Itália, no instituto de Laimburg e na empresa Isolcell (*Informação Pessoal*). Todavia, essa tecnologia de ACD – QR ainda não está sendo empregada em nível comercial até o momento.

A tecnologia da ACD – QR, independentemente de como é determinado o QR na câmara comercial, possui a vantagem, em relação a DCS e ACD – FC, da detecção do LMO das frutas diretamente (pela relação entre produção de CO_2 e consumo de O_2), enquanto que os métodos apresentados anteriormente apresentam como mecanismo a detecção do LMO a partir do monitoramento de um evento que é resultante de um período de exposição das frutas ao baixo

O_2. Outro ponto favorável da ACD – QR é a possibilidade de indução do metabolismo anaeróbico de maneira controlada, pelo uso de QRs acima de 1,0, o que resulta em um leve incremento na produção de etanol, que por sua vez reduz a produção de etileno, retardando o amadurecimento (ASODA et al., 2009; JIN et al., 2013; PESIS, 2005; WEBER et al., 2016, 2020). Assim, a ação da ACD – QR no metabolismo das frutas não é função apenas do baixo O_2, mas também do acúmulo de metabólitos do metabolismo anaeróbico. Porém, a tolerância ao metabolismo anaeróbico é dependente da cultivar, estádio de maturação, tempo de armazenamento, temperatura, entre outros fatores. Assim, é de fundamental importância a determinação do QR ideal ou a faixa de QR ideal.

Diversos estudos foram realizados comparando a tecnologia de ACD – QR com AC e ACD – FC, sendo observados resultados positivos tanto no Brasil (BOTH et al., 2017; BRACKMANN; WEBER; BOTH, 2015; THEWES et al., 2017c; WEBER et al., 2015) quanto na Europa (BESSEMANS et al., 2016, 2018; GASSER et al., 2008; VAN SCHAIK et al., 2015 WEBER et al., 2019, 2020). Esse trabalhos demostram que o armazenamento em ACD – QR proporciona maçãs com menor incidência de escaldadura superficial (BESSEMANS et al., 2016), degenerescência de polpa, maior firmeza de polpa e frutos sadios quando comparado à AC convencional (BOTH et al., 2017; THEWES et al., 2017c; WEBER et al., 2015, 2019) e maior emissão de compostos voláteis, especialmente ésteres (BOTH et al., 2017; THEWES et al., 2017b, 2017c), independentemente do estádio de maturação na colheita (THEWES et al., 2017a, 2017b).

Resultados promissores foram observados na interação entre o armazenamento em ACD – QR e temperatura de armazenamento, a fim de possibilitar economia de energia pelo aumento da temperatura. Para maçãs do grupo ‘Gala’ o armazenamento em ACD – QR possibilitou o aumento da temperatura de armazenamento de 1,0 °C para 2,0 a 2,5 °C, reduzindo consideravelmente a incidência de degenerescência de polpa (BOTH et al., 2018; DE OLIVEIRA ANESE et al., 2019). Já em maçãs ‘Nicoter’ e ‘Braeburn’ é possível aumentar a temperatura de armazenamento de 1,0 °C para 3,0 °C sem prejuízos à qualidade das frutas (KITTEMANN; NEUWALD; STREIF, 2015; WEBER et al., 2019, 2020). Esses resultados evidenciam o potencial de aplicação na prática, em câmaras comerciais, da tecnologia ACD – QR, reduzindo custos de armazenamento e mantendo frutas de alta qualidade durante longos períodos de armazenamento. Em função disso, é importante a realização de estudos avaliando o armazenamento em ACD – QR para as diversos cultivares, além de comparar o efeito da ACD - FC a outros métodos de ACD e a sua interação com a aplicação de 1-MCP, a fim de melhorar o armazenamento de maçãs.

2.3.4. ACD monitorada pela produção de CO_2 (ACD – DC)

Como mencionado acima, um dos maiores entraves da adoção da ACD é a necessidade de equipamentos adicionais nas câmaras de AC e a falta de estanqueidade das mesmas, o que resulta em entrada de O_2, dificultando o armazenamento em pressões parciais de O_2 extremamente baixas. Assim, há dificuldade na obtenção de pressões parciais de O_2 extremamente baixas para verificação do LMO pela fluorescência de clorofilas e problemas na determinação do QR, quando o mesmo é avaliado diretamente na câmara comercial. Desta maneira, é necessária a instalação destes equipamentos nas câmaras comerciais ou o desenvolvimento de outras técnicas de ACD que irão facilitar a sua adoção em nível comercial.

O metabolismo das frutas pode ser monitorado pela respiração dos mesmos (produção de CO_2), uma vez que a produção de CO_2 diminui com a redução da pressão parcial de O_2 na câmara, até um determinado nível de O_2, quando a produção de CO_2 tende a aumentar pelo metabolismo anaeróbico (Figura 1). Assim, a sua medição na câmara comercial pode ser utilizada como indicativo para determinação do LMO durante o armazenamento. A grande vantagem da medição de CO_2, em comparação à de O_2, é a pouca influência do ambiente externo sobre a sua determinação (BESSEMANS et al., 2016, 2018), uma vez que é a difusão do mesmo é menor do interior da câmara para fora, em função do gradiente de concentração e do tamanho da molécula de CO_2 (maior que a de O_2). Desta maneira, o desenvolvimento de um método de ACD baseado na produção de CO_2 das frutas poderá facilitar a adoção dessa tecnologia em nível comercial, uma vez que, a determinação da produção de CO_2 pode ser realizada em câmaras comerciais sem maiores problemas e sem a necessidade da instalação de equipamentos adicionais, como são necessários nas tecnologias disponíveis atualmente.

Nesse sentido, durante os últimos quatro anos de pesquisas no NPP, da UFSM, foi iniciado um projeto tentando desenvolver uma técnica de ACD baseada unicamente na avaliação da produção de CO_2 das frutas durante o armazenamento. Essa técnica se baseia na resposta da produção de CO_2 das frutas à redução do O_2 durante o armazenamento. A produção de CO_2 das frutas reduz de acordo com a redução da O_2, até que a pressão parcial de O_2 atinja o ponto de compensação anaeróbico (PCA) (BOERSIG; KADER; ROMANI, 1988; GASSER et al., 2003). Caso o O_2 seja reduzido abaixo do PCA, a produção de CO_2 aumenta drasticamente. Assim, poder-se-ia monitorar o LMO durante o armazenamento apenas pela produção de CO_2 das frutas e controlar dinamicamente a pO_2 durante o armazenamento. De acordo com (PRANGE, 2018), o aumento na produção de CO_2 das frutas em função da redução

da pressão parcial de O_2 durante o armazenamento ocorre um pouco antes da fluorescência de clorofilas. Em estudos preliminares realizados no NPP, utilizando essa técnica de monitoramento do LMO, os resultados foram bastante positivos e similares aos observado com ACD – QR. Assim, o estudo com diversas cultivares de maçãs e locais de origem é importante para testar essa nova tecnologia de ACD, o que pode resultar em melhorias nas técnicas de armazenamento no Brasil e no mundo, especialmente, aumentando a adoção da ACD em nível comercial.

2.4. EFEITO DO 1-METILCICLOPROPENO (1-MCP) SOBRE A FISIOLOGIA DAS FRUTAS

O 1-MCP é um composto químico que se liga de maneira irreversível nos receptores de etileno, impedindo sua ligação e, consequentemente, retardando o amadurecimento das frutas (BLANKENSHIP; DOLE, 2003; SISLER; SEREK, 1997; WATKINS, 2006; YANG et al., 2016). A ligação do 1-MCP aos receptores de etileno ocorre de maneira competitiva com o etileno, porém ele possui uma afinidade muito maior aos receptores que o próprio etileno (SISLER; SEREK, 1997). Assim, o 1-MCP tem vantagem competitiva pelos receptores, justificando sua alta habilidade no bloqueio do amadurecimento das frutas.

O 1-MCP, além de inibir a ação do etileno, também apresenta influência na expressão gênica das frutas. Os seus efeitos sobre a expressão gênica estão relacionados a genes que codificam as enzimas ACC oxidase (*MdACO1*), ACC sintase (*MdACS1*), receptores de etileno (*MdERS1* e *MdERS2*) (TATSUKI; ENDO, 2006; TATSUKI; ENDO; OHKAWA, 2007), enzimas relacionadas à lipoxigenase e síntese de compostos voláteis (YANG et al., 2016). Assim, com a aplicação de 1-MCP ocorre um bloqueio de genes relacionados a enzimas produtoras e receptores de etileno, resultando no retardamento da senescência das frutas e, consequentemente, em frutas com melhor manutenção da firmeza de polpa (BRACKMANN et al., 2013; NOCK; WATKINS, 2013; WATKINS, 2006), menor taxa respiratória (NOCK; WATKINS, 2013; PRE-AYMARD; WEKSLER; LURIE, 2003; THEWES et al., 2015b, 2018; WATKINS, 2006), menor incidência de polpa farinácea (BRACKMANN et al., 2014; STEFFENS et al., 2008; THEWES et al., 2015b) e manutenção de vários outros caracteres de qualidade de frutas, como acidez e açúcares.

Contudo, o efeito da aplicação de 1-MCP sobre a ocorrência de degenerescência de polpa é contraditório, dependente, dentre vários outros, da cultivar e estádio de maturação (JUNG; WATKINS, 2011; KITTEMANN; NEUWALD; STREIF, 2015; LEE et al., 2012;

THEWES et al., 2017a). A aplicação de 1-MCP também aumenta o acúmulo de patulina em maçãs ‘Galaxy’ e ‘Fuji Kiku’, independentemente do método de armazenamento (DOS SANTOS et al., 2018) em função de sua aplicação incrementar a susceptibilidade a incidência de podridões (BOTH et al., 2018; DOS SANTOS et al., 2018) . Entretanto, ainda não está bem clara a interação do armazenamento em ACD, especialmente ACD – DC, com a aplicação de 1-MCP sobre a qualidade geral, metabolismo aeróbico, anaeróbico e perfil volátil das frutas. Trabalhos recentes têm demonstrado que a aplicação de 1-MCP tem seu efeito reduzido quando as maçãs são armazenados em ACD – QR, com pressões parciais de O_2 extremamente baixas (< 0,3 kPa) (BOTH et al., 2018; DE OLIVEIRA ANESE et al., 2019), todavia os estudos ainda são escassos. Além do reduzido efeito sobre a qualidade físico-química, a aplicação de 1-MCP têm efeito inibitório sobre a síntese de voláteis, especialmente ésteres (DEFILIPPI; KADER; DANDEKAR, 2005; JUNG; WATKINS, 2011; KONDO et al., 2005; LURIE et al., 2002; THEWES et al., 2015b, 2017b; YANG et al., 2016), contudo, também ocorre redução na emissão de metabólitos considerados *off-flavours*, como etanol e acetato de etila (THEWES et al., 2018).

A redução na emissão de ésteres pelas frutas tem efeito negativo sobre o desenvolvimento do aroma (FAN; MATTHEIS, 1999). Esse efeito do 1-MCP deve-se à inibição de enzimas do metabolismo de lipídeos, da beta-oxidação e lipoxigenase (LOX) (YANG et al., 2016), na atividade de algumas isoformas da enzima AAT e ADH (ORTIZ et al., 2010; YANG et al., 2016) e no acúmulo de precursores de ésteres, como álcoois e ácidos voláteis. Todavia, quando as frutas são armazenadas em ACD – QR e ACD – DC, é possível induzir, de forma controlada, o metabolismo anaeróbico e a síntese de precursores de compostos voláteis através desta via metabólica. Assim, o efeito prejudicial do 1-MCP sobre a síntese de ésteres poderia ser reduzida pelo emprego dessas novas tecnologias de armazenamento, o que justifica estudos avaliando o efeito do 1-MCP quando as maçãs são armazenadas nessas novas tecnologias de ACD.

2.5. METABOLISMO DE AÇÚCARES E ÁCIDOS ORGÂNICOS

A principal forma de armazenamento de carboidratos em maçãs é o acúmulo de amido (DOERFLINGER et al., 2015), o qual é convertido em açúcares simples para poder ser metabolizado pelas frutas. No momento da colheita as frutas têm a quantidade máxima de reservas que podem ser metabolizadas. Entretanto, esse fato não significa que a fruta tenha a máxima concentração de açúcares livres na colheita, como sacarose, glicose, frutose e sorbitol,

mas sim, depois da hidrólise de todo amido (DOERFLINGER et al., 2015; LINDO-GARCÍA et al., 2019; WANG et al., 2010). Essas modificações de amido para açúcares livres na polpa das frutas são induzidas basicamente pelo metabolismo do etileno, podendo ser manejada pela aplicação de 1-MCP (DOERFLINGER et al., 2015; LINDO-GARCÍA et al., 2019). Estudos recentes também têm demostrado que a velocidade de hidrólise de amido para açúcares livres é afetada pelo tempo que a fruta permaneceu ligada à planta mãe durante a maturação (LINDO-GARCÍA et al., 2019; NEUWALD; STREIF; KITTEMANN, 2010).

A partir da hidrólise do amido é liberada basicamente glicose, que pode ser transformada em frutose e sacarose (DOERFLINGER et al., 2015), sendo que os açúcares metabolizados pelas frutas na glicólise são glicose e frutose (BEKELE et al., 2015, 2016; THEWES; BRACKMANN; NEUWALD, 2019). O acúmulo de glicose e frutose na polpa da fruta também pode ser função da degradação de açúcares-álcoois, como o sorbitol (DOERFLINGER et al., 2015; WANG et al., 2010), a partir da enzima sorbitol desidrogenase (DOERFLINGER et al., 2015; JAIN et al., 2012; TEO et al., 2006). Essa interconversão de açúcares pode influenciar o aroma das frutas e, consequentemente, a aceitabilidade pelo consumidor (APREA et al., 2017).

Nesse sentido, na literatura é descrito que cada um dos açúcares livres tem uma determinada intensidade de sabor doce, sendo a frutose o açúcar mais doce (KOEHLER; KAYS, 1991) e também o mais abundante em maçãs (APREA et al., 2017; DOERFLINGER et al., 2015; WANG et al., 2010). Assim, a modificação do metabolismo das frutas, seja pela modificação da atmosfera ou pelo manejo do etileno, afeta diretamente a glicólise e, consequentemente, o metabolismo dos açúcares, o que pode resultar em modificações no sabor das frutas após o armazenamento. O uso de ACD – QR resultou em modificação no metabolismo de açúcares em comparação à AC (THEWES; BRACKMANN; NEUWALD, 2019). Esses autores, levantaram a hipótese de que frutas armazenadas em ACD – QR poderiam ter sabor diferenciado em comparação à AC, uma vez que acumularam maior concentração de frutose e sorbitol. Sendo que o sorbitol em conjunto com os compostos voláteis possui maior impacto sobre sabor de maçãs (APREA et al., 2017).

O acúmulo/metabolismo de açúcares também tem importância na tolerância a estresses, como mencionado em leveduras (JAIN et al., 2011, 2012; SHEN et al., 1999) e maçãs ‘Granny Smith’ (BUSATTO et al., 2018), especialmente os açúcares-álcoois, como o sorbitol e glicerol. Em maçãs ‘Granny Smith’ a aplicação de 1-MCP resultou em aumento na expressão da enzima sorbitol desidrogenase (BUSATTO et al., 2018), resultando no acúmulo de sorbitol (BUSATTO et al., 2018; LEE et al., 2012). De acordo com esses estudos, a fruta tem a tendência de acumular

sorbitol em condições de estresse, como aplicação de 1-MCP combinado ao armazenamento em baixas temperaturas.

Nessa mesma linha de raciocínio, o armazenamento em ACD, com pressões de O_2 extremamente baixas, também resulta em estresses na fruta (BRACKMANN; WEBER; BOTH, 2015; DONADEL et al., 2019; WEBER et al., 2015). Adicionalmente, o armazenamento em ACD – QR permite a indução de metabolismo anaeróbico, resultando no acúmulo de acetaldeído e etanol, que podem ser estressantes para o metabolismo das frutas se em concentrações acima das toleradas (KE et al., 1994; LIU et al., 2012; PESIS, 2005; WEBER et al., 2016, 2020). Um estudo recente comprovou que no armazenamento de maçãs em condições de O_2 extremamente baixas (<0,3 kPa) há tendência de acúmulo de sorbitol, que auxilia na redução da permeabilidade da membrana celular, mesmo com acúmulo de etanol e acetaldeído (THEWES; BRACKMANN; NEUWALD, 2019). Entretanto, ainda são escassos os estudos relacionando o acúmulo de açúcares-álcoois à maior tolerância a estresses pelas frutas, como por exemplo, o armazenamento em ACD + 1-MCP, onde há dois estresses combinados: o baixo O_2 mais a inibição da ação do etileno.

De maneira geral, a concentração de sacarose, glicose e frutose sofre pouca influência da inibição da ação do etileno durante o armazenamento, como demostrado para maçãs ‘Empire’ (LEE et al., 2012), ‘Jonagold’ (BEKELE et al., 2015) e ‘Gala’ (DOERFLINGER et al., 2015). Entretanto, durante o armazenamento em AC e ACD, há um tendência da fruta consumir primeiro glicose e depois frutose (THEWES; BRACKMANN; NEUWALD, 2019), sendo que no armazenamento de maçãs em AC (2,0 kPa O_2 + 1,0 kPa CO_2) ocorreu o acúmulo de sacarose e hexoses (ZHU et al., 2013). Porém, quando a pressão parcial de O_2 é reduzida, pelo armazenamento em ACD – QR e ACD – DC, ocorre a indução do metabolismo anaeróbico, o que acelera a glicólise para produção de energia em nível de substrato (KE et al., 1994; SAQUET; STREIF, 2008). Esse fato pode resultar em modificações no metabolismo da glicose e frutose, justificando a realização de estudo sobre o metabolismo de carboidratos quando as frutas são armazenadas nessas novas tecnologias, como ACD – FC, ACD – QR, ACD- DC e 1-MCP.

O produto final da glicólise é o piruvato, composto chave no metabolismo das frutas, uma vez que este faz a ligação entre glicólise, ciclo de Krebs, síntese de aminoácidos, metabolismo anaeróbio e compostos voláteis (BEKELE et al., 2016). A modificação de uma rota metabólica para outra é, principalmente, função do pH do citosol, o qual modula as enzimas PDC e PDH (KE et al., 1994; MATHOOKO, 1996; SAQUET; STREIF, 2008). De acordo com a literatura, os ácidos do ciclo de Krebs sofrem pouca influência da condição de AC, com

exceção do succinato que acumula em condições de alto CO_2 (BEKELE et al., 2016; MATHOOKO, 1996), sendo os aminoácidos mais influenciados pela AC (BEKELE et al., 2015, 2016; MBONG VICTOR et al., 2017). Entretanto, esses trabalhos foram realizados em condições normóxicas, sendo a literatura escassa no que diz respeito ao efeito da ACD nos ácidos do ciclo de Krebs.

A inibição da ação do etileno retarda o acúmulo de alguns ácidos do ciclo de Krebs, como succinato e citrato (BEKELE et al., 2015) e incrementa a síntese de aminoácidos pelas frutas, como glutamato e alanina (BEKELE et al., 2015; LEE et al., 2012). Esse fato pode explicar o porquê da não redução na emissão de ésteres de cadeia ramificada, oriundos de aminoácidos, quando as frutas são tratadas com 1-MCP (THEWES et al., 2015b; YANG et al., 2016). Entretanto, ainda não está claro qual o efeito do etileno no metabolismo de maçãs armazenadas em condições de ACD, especialmente ACD – QR e ACD – DC. Assim, o estudo da dinâmica de açúcares e ácidos em condições de ACD poderia trazer informações valiosas sobre o modo de ação da ACD no metabolismo das frutas e quais os possíveis efeitos sobre o metabolismo de compostos voláteis e resistência a estresses por baixo O_2 em maçãs.

2.6. PRODUÇÃO/ACÚMULO DE COMPOSTOS VOLÁTEIS

O sabor de maçãs é uma interação complexa entre vários compostos, como açúcares, ácidos e compostos voláteis (APREA et al., 2017; SALAZAR; OROZCO, 2011). O perfil de compostos voláteis de maçãs é bastante complexo, com mais de 400 compostos de diferentes grupos orgânicos, como ésteres, álcoois, aldeídos, cetonas, terpenos, ácidos, entre outros. Destes 400, em torno de 40 a 50 compostos, na maioria ésteres, apresentam fundamental importância no odor de maçãs (FELLMAN et al., 1993; SALAZAR; OROZCO, 2011). De uma maneira geral, para maçãs do grupo ‘Gala’ os principais ésteres são acetato de butila, de hexila e de 2-metilbutila (SALAZAR; OROZCO, 2011; YOUNG et al., 1996), já para maçãs do grupo ‘Fuji’ os principais ésteres são butanoato de etila, 2-metilbutanoato de etila e acetato de 2-metibutila (DONADEL et al., 2019; ECHEVERRÍA et al., 2004; NIU et al., 2019).

A síntese de ésteres nas frutas é realizada pela enzima álcool acil CoA tranferase (AAT), que catalisa a combinação de um álcool com um ácido (DEFILIPPI; KADER; DANDEKAR, 2005; LUCCHETTA et al., 2007; YANG et al., 2016), sendo que os ácidos definem se os ésteres serão acetatos, propanoatos, butanoatos, pentanoatos, hexanoatos, entre outros. Os álcoois necessários para a formação de ésteres podem ser originários basicamente de três rotas, degradação de ácidos graxos, aminoácidos e açúcares. Em condições normóxicas o álcool mais

produzido por maçãs é o 1-butanol (MEHINAGIC et al., 2006), o qual tem como precursor ácidos graxos. Em contrapartida, maçãs armazenada em ACD – QR tem como álcool mais produzido e acumulado, após longos períodos de armazenamento, o etanol (BOTH et al., 2017; DONADEL et al., 2019; THEWES et al., 2017c), que é oriundo do metabolismo anaeróbico de açúcares.

Os ácidos graxos precursores de voláteis são obtidos por diversas rotas metabólicas nas frutas. Entre elas podemos citar a degradação de lipídeos pela β-oxidação e ação da lipoxigenase (LOX) (CONTRERAS; BEAUDRY, 2013; CONTRERAS; TJELLSTRÖM; BEAUDRY, 2016; SONG; BANGERTH, 2003), síntese *de novo* de ácidos graxos (CONTRERAS; TJELLSTRÖM; BEAUDRY, 2016; SONG; BANGERTH, 2003), pelo acúmulo de ácidos graxos livres na polpa das frutas, entre outras formas. Contudo, a principal rota de obtenção de precursores é a partir da β-oxidação e lipoxigenase (LOX), processos que necessitam de oxigênio para funcionar de maneira adequada.

Como o O_2 é necessário para a oxidação de lipídeos e formação de precursores de compostos voláteis, é esperado que a redução das pressões parciais de O_2 no armazenamento em AC reduza a emissão de compostos voláteis (BOTH et al., 2014; BRACKMANN; STREIF; BANGERTH, 1993; ECHEVERRÍA et al., 2004, 2008; LUMPKIN et al., 2014; RAFFO et al., 2009). Todavia, não apenas a redução da pressão parcial de O_2 influencia negativamente a formação de compostos voláteis, mas também o incremento da pressão parcial de CO_2, especialmente ésteres de cadeia linear, que são originários da degradação de lipídeos (BRACKMANN; STREIF; BANGERTH, 1993; LUMPKIN et al., 2015), porém os ésteres de cadeia ramificada são pouco influenciados pelo aumento da pressão parcial de CO_2 (BRACKMANN; STREIF; BANGERTH, 1993). Por outro lado, a redução da pressão parcial de O_2 e o aumento da de CO_2 reduzem a produção de etileno e a taxa respiratória e, consequentemente, a expressão e atividade de enzimas responsáveis pela produção de ésteres (BOTH et al., 2014; DEFILIPPI; KADER; DANDEKAR, 2005; LUMPKIN et al., 2014; SONG; BANGERTH, 1996, 2003; YANG et al., 2016).

Atualmente, há uma tendência de armazenar maçãs em pressões parciais de O_2 cada vez mais baixas, por permitir melhor conservação da firmeza de polpa, acidez titulável, açúcares e maior percentagem de frutos sadios (BOTH et al., 2016; THEWES et al., 2015a; TRAN et al., 2015). Entretanto, pouca importância é dada no efeito das baixas pressões parciais de O_2 sobre a emissão de ésteres pelas frutas. Estudos recentes têm demostrado que a redução da pressão parcial de O_2, em AC, em níveis próximos de 0,5 kPa suprime fortemente o acúmulo de ésteres de cadeia linear (BOTH et al., 2014, 2016). Resultados similares foram observados quando as

frutas são armazenadas em ACD – FC, onde os ésteres de cadeia linear são drasticamente reduzidos (AUBERT; MATHIEU-HURTIGER; VAYSSE, 2015; BOTH et al., 2017; DONADEL et al., 2019; RAFFO et al., 2009; THEWES et al., 2017c). Já quando as frutas foram armazenadas em ACD – QR, especialmente QRs entre 1,5 e 2, ocorre uma melhor manutenção dos principais ésteres de maçãs do grupo 'Gala' (BOTH et al., 2017; THEWES et al., 2017b) e 'Fuji' (DONADEL et al., 2019; THEWES et al., 2017c), evidenciando que essa tecnologia de armazenamento permite melhor manutenção da qualidade. Entretanto, na literatura não há informações sobre o efeito do armazenamento em ACD – DC sobre o perfil volátil de maçãs.

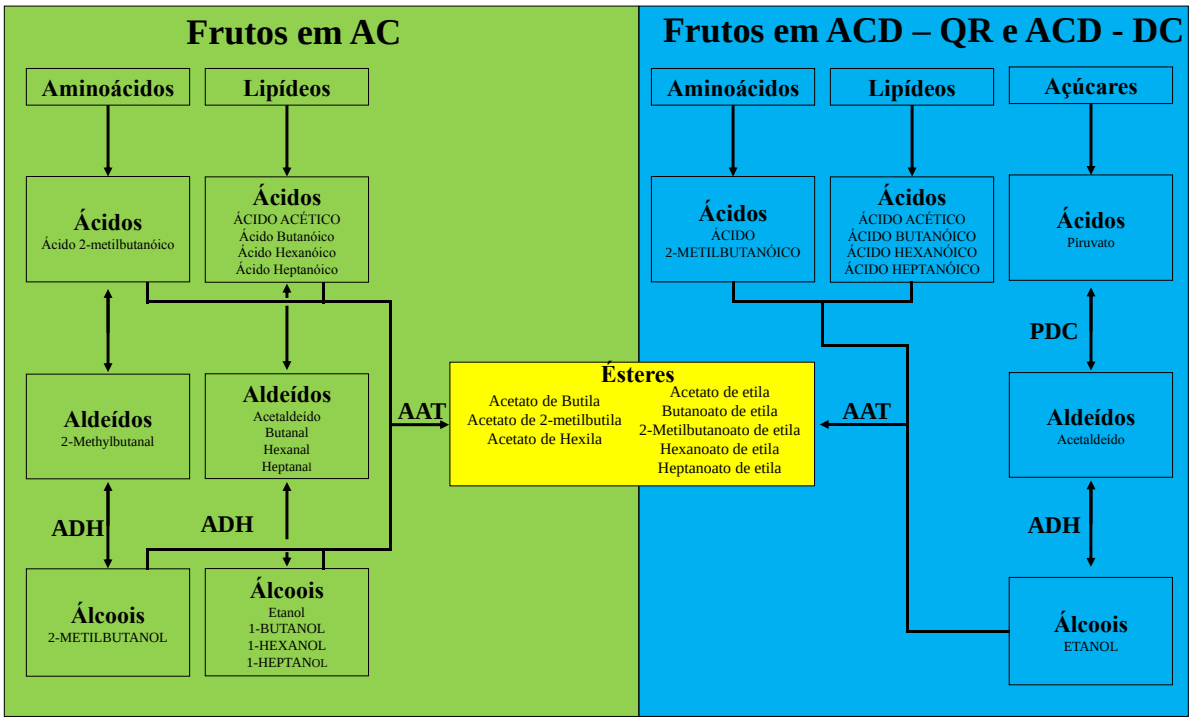
A redução das pressões parciais de O_2 durante o armazenamento em ACD – QR e ACD – DC permite a indução de maneira controlada do metabolismo anaeróbico, resultando no acúmulo de acetaldeído, etanol e acetato de etila. Esses compostos voláteis, quando em concentrações adequadas auxiliam na manutenção da qualidade das frutas, pela inibição do metabolismo do etileno (ASODA et al., 2009; JIN et al., 2013; PESIS, 2005; WEBER et al., 2016, 2020), redução na atividade de enzimas de parede celular (WEBER et al., 2020) e para conferir às frutas o aroma típico de maçã (ECHEVERRÍA et al., 2008). Entretanto, se a concentração destes compostos voláteis ficar acima do desejado pode ocorrer a formação de *off-flavours*, reduzindo a qualidade organolépticas das maçãs (FORNEY; KALT; JORDAN, 2000; WRIGHT et al., 2015). Tentando contornar o problema da formação de *off-flavours*, pesquisas recentes têm demonstrado que a aplicação de 1-MCP em maçãs armazenadas em ACD – QR 1,5 têm capacidade de suprimir o metabolismo anaeróbico em maçãs 'Galaxy', 'Fuji' e 'Pink Lady®', mesmo em pressões parciais de O_2 extremamente baixas (THEWES et al., 2018). Nesse sentido, mais estudos avaliando como e para quais cultivares o 1-MCP tem a capacidade de inibir o metabolismo anaeróbico são necessários. Também é importante estudar para qual rota metabólica o piruvato é direcionado em ACD + 1-MCP.

A produção de compostos voláteis é altamente dependente de etileno (MATTHEIS; FAN; ARGENTA, 2005). Como o uso de AC e ACD reduz a produção de etileno nas frutas (BOTH et al., 2017; BRACKMANN; STREIF; BANGERTH, 1993; BRACKMANN; WEBER; BOTH, 2015; THEWES et al., 2017b) é esperada uma redução na emissão de compostos voláteis, especialmente quando combinados ao tratamento com 1-MCP (BANGERTH; SONG; STREIF, 2012). Todavia, estudos recentes têm demonstrado que quando maçãs do grupo 'Gala' são armazenadas em ACD – QR, não foi observada uma correlação positiva entre etileno e emissão de compostos voláteis (THEWES et al., 2017b), mostrando que em condições de hipóxia a produção de compostos voláteis não é dependente do

etileno. Isso evidencia que deve haver outra rota de síntese de precursores de voláteis, como a síntese *de novo*.

Em condições de hipóxia há acúmulo de compostos reduzidos nas células (KE et al., 1994; WRIGHT et al., 2015), que geralmente são oxidados no metabolismo anaeróbico. Porém, em pressões parciais de O_2 extremamente baixas os compostos reduzidos presentes na célula podem ser transportados para os cloroplastos permitindo a síntese de precursores de voláteis através da biossíntese *de novo* de ácidos graxos ao invés da sua oxidação pelo metabolismo anaeróbico. Estudos com *Arabidopsis thaliana* demonstraram que a rota da biossíntese *de novo* de ácidos graxos pode aliviar a toxidez de acetaldeído e etanol (LI-BEISSON et al., 2013), compostos estes que são acumulados em ACD – QR. Maçãs 'Scarlett Spur Red Delicious' armazenadas em concentrações estáticas de O_2 extremamente baixa (0,3 kPa) não apresentaram redução na concentração de acetato de hexila, porém, os acetatos de butila e propila foram significativamente reduzidos (LUMPKIN et al., 2014).

Figura 2 – Esquema demonstrativo das principais rotas de síntese de ésteres em maçã armazenadas em AC, ACD – QR e ACD – DC. Compostos/precursores com letras maiúsculas são os mais utilizados, em cada sistema de armazenamento, na síntese de ésteres.



Fonte: o Autor.

Recentemente, um estudo avaliando a emissão de compostos voláteis por maçãs do grupo ‘Fuji’, levantou a hipótese de que no armazenamento em ACD – QR há tanto acúmulo de etanol que a enzima AAT apenas combina esse álcool com ácidos para formação de ésteres (THEWES et al., 2017c), como demonstrado esquematicamente (Figura 2). De acordo com esse estudo, maçãs em ACD – QR tem acúmulo de etanol em altas concentrações fazendo a enzima AAT combinar o mesmo com intermediários da β-oxidação, como o ácido 3-hidroxibutanoato e ácido 3-hidroxihexanoato. Assim, pode-se inferir que maçãs armazenadas em ACD – QR sintetizam compostos voláteis como uma forma de detoxificação das frutas do excesso de etanol. Em leveduras, a síntese de compostos voláteis, especialmente conversão de aldeídos em álcoois, é a principal forma de dissipação do potencial redutor em condições de anaerobiose (JAIN et al., 2011, 2012), podendo o mesmo ocorrer em frutas no armazenamento em ACD. Em maçãs ‘Royal Gala’ e ‘Galaxy’ o armazenamento em ACD – QR resultou em frutas com maior acúmulo de 1-butanol em comparação as frutas armazenadas em ACD – FC (BOTH et al., 2017; THEWES et al., 2017b), demonstrando que realmente a síntese de álcoois e ésteres pode ser uma forma de dissipação do potencial redutor nas células em baixo O_2. Esse fato demonstra que o estudo do efeito do armazenamento em ACD, especialmente em ACD – QR e ACD – DC, sobre a síntese de compostos voláteis é necessário para entender como ocorre a adaptação das frutas a condições de O_2 extremas. Trabalhos recentes demostraram que em ACD a pressão parcial de O_2 pode chegar a 0,08 kPa sem ocorrer danos nos tecidos internos da fruta (BOTH et al., 2017; THEWES; BRACKMANN; NEUWALD, 2019). Se no ambiente em volta da fruta a concentração de O_2 é tão baixa, então, no miolo da fruta a concentração deve ser muito menor (HO et al., 2013) e, mesmo assim, não ocorrem danos ao tecido.

3. ARTIGO 1

3.1. DYNAMIC CONTROLLED ATMOSPHERE × 1-MCP: IMPACT ON VOLATILE ESTERS SYNTHESIS AND OVERALL QUALITY OF 'GALAXY' APPLES[1]

Abstract

Previous study showed that dynamic controlled atmosphere based on respiratory quotient (DCA – RQ) contributes to extend apple storage potential, as well as aroma production. However, these studies did not explain if the reason of higher volatile compounds synthesis is due to constant/repeated low oxygen stress that increases precursors or due to expression of higher level of enzymes. Thus, we aimed to evaluate the expression of *MdACO1, MdADH1, MdLOX1, MdAAT1* at long time of storage, quantify the main volatile esters and its precursors and evaluate the overall quality of 'Galaxy' apples at 6 and 9 months of storage plus 7 d of shelf life at 20 °C. The storage conditions evaluated were: [1] Controlled atmosphere (CA) – 1.2 kPa O_2 + 2.0 kPa CO_2; [2] DCA – CF; [3] DCA – RQ 1.1; [4] DCA – RQ 1.5; [5] CA + 1-MCP; [6] DCA – CF + 1-MCP; [7] DCA – RQ 1.1 + 1-MCP and [8] DCA – RQ 1.5 + 1-MCP. All DCA – RQ conditions were submitted to two low oxygen stresses a week due to RQ calculation. The storage under DCA or CA + 1-MCP have a suppression effect on *MdACO1* expression and consequently reducing the ethylene production and fruit ripening. Lower overall metabolism in DCA – RQ stored fruit resulted in higher flesh firmness, lower mealiness and flesh breakdown. Storage under DCA – RQ 1.5 also resulted in higher decay incidence after 9 months of storage. The expression of *MdAAT1* appears to be regulated by ethylene in CA, but in DCA – RQ 1.5 its expression may be induced by another signal, because even when fruit are treated with 1-MCP the expression of *MdAAT1* is increased by the storage under DCA – RQ 1.5 as compared to CA. This could help to explain why the oxygen reduction to obtain an RQ 1.5 increases the ester production in 'Galaxy' apples. Higher volatile compounds were detected when fruit were stored under CA and DCA – RQ 1.5, without 1-MCP application. 1-MCP application suppressed the volatile compound production in almost all storage conditions, especially the esters. Hexenyl esters are increased by the storage under DCA – RQ1.5.

Keywords: *Malus domestica;* respiratory quotient; chlorophyll fluorescence; *MdAAT1; MdADH1*; *MdLOX1*.

[1] Artigo formatado de acordo com as normas da revista Food Packaging and Shelf Life.

3.1.1. Introduction

Volatile compounds are one of the main quality attributes of apples. According to López et al. (2007), their presence is criticall for consumer acceptance. These compounds are affected by several factors, such as harvest maturity, storage temperature, oxygen and carbon dioxide partial pressures (pO_2 and pCO_2), growth regulators applications, ethylene production and action, despite other factors (Bangerth, Song, & Streif, 2012; Both et al., 2017; Brackmann, Streif, & Bangerth, 1993; López et al., 2007; Lumpkin, Fellman, Rudell, & Mattheis, 2014, 2015; Thewes, Brackmann, de Oliveira Anese, et al., 2017). Nevertheless, a part of the apple production need to be stored to offer fruit in the off-season, even when is know that volatile compounds will be reduced in CA.

The volatile compounds reduction over storage is a result of reduced metabolism, which reduces β-oxidation and lipoxygenase activity (LOX) (Brackmann et al., 1993; Echeverría, Fuentes, Graell, & López, 2004; Song & Bangerth, 2003). The low pO_2 (Lara, Graell, López, & Echeverría, 2006) and 1-MCP application (Yang et al., 2016), also decrease the expression and activity of the alcohol acyltransferase (AAT), which is responsible to produce esters. During the last years, the pO_2 lowering to extremely low levels, below 0.7 kPa in apple storage, is performed in many countries, and this management suppresses more the straight-chain esters formation as compared to branched-chain one (Both, Brackmann, Thewes, Ferreira, & Wagner, 2014; Both et al., 2016; López et al., 2007; Lumpkin et al., 2014). The pO_2 reduction to extremely low levels is only possible by adoption a technique that allow to monitor the fruit metabolism, the so-called dynamic controlled atmosphere (DCA).

Throughout the storage under DCA, the pO_2 change according to a bio-response of fruit. Commercially, there are three methodologies to detect the bio-response of fruit to low pO_2 during the storage period. Schouten, Prange, Verschoor, Lammers, & Oosterhaven, (1998) proposed a method based on the ethanol production by fruit. Latter, a technology based on chlorophyll fluorescence was developed (Wright, DeLong, Gunawardena, & Prange, 2012; Wright, DeLong, Harrison, Gunawardena, & Prange, 2010). More recently, the respiratory quotient has been used in some rooms as fruit bio-response (Bessemans, Verboven, Verlinden, & Nicolaï, 2016, 2018; Brackmann, Weber, & Both, 2015; Gasser, Eppler, Naunheim, Gabioud, & Hoehn, 2008). The storage under dynamic controlled atmosphere based on chlorophyll fluorescence (DCA – CF) resulted in fruit with higher overall quality (Aubert, Mathieu-Hurtiger, & Vaysse, 2015; Eren, Çalhan, Onursal, & Güneyli, 2015; Thewes, Both, Brackmann, Weber, & de Oliveira Anese, 2015; Tran, Verlinden, Hertog, & Nicolaï, 2015; Zanella & Stürz,

2015), but with lower volatile compounds emission, when compared to conventional controlled atmosphere (CA) (Aubert et al., 2015; Both et al., 2017; Raffo, Kelderer, Paoletti, & Zanella, 2009; Thewes, Brackmann, Both, et al., 2017). Nevertheless, when apples were stored under DCA based on respiratory quotient (DCA – RQ), where the pO_2 are lower than in DCA – CF, and fruit are submitted to repeated low oxygen stress due to RQ calculation, the volatile compounds emission increased and were the same/or higher as compared to fruit stored in CA (Both et al., 2017; Thewes, Brackmann, Both, et al., 2017; Thewes, Brackmann, de Oliveira Anese, et al., 2017), and extremely higher as compared to fruit stored under CA + 1-MCP (Thewes, Brackmann, de Oliveira Anese, et al., 2017). According to Anese (2017), the repeated low oxygen stress caused by RQ calculation influences the quality maintenance, where fruit submitted to the stress had higher overall quality. Nevertheless, no results were found in the literature about the effect of DCA – RQ with repeated low oxygen stress on the precursor accumulation and volatile compounds enzymes expression.

The 1-MCP application is worldwide performed in commercial storage rooms. Its application blocks ethylene receptors and consequently delays the fruit ripening (Watkins, 2006). With the ethylene action inhibition, the expression of several genes and enzymes activity, such as ACC oxidase (*MdACO1*) (Tatsuki, Endo, & Ohkawa, 2007; Wakasa et al., 2006), ethylene receptors (*MdERS1* and *MdERS2*) (Tatsuki et al., 2007), and enzymes of the volatile compounds formation (*MdLOX*, *MdPDC2*, *MdAAT1* and *MdAAT2*) (Yang et al., 2016) are downregulated. These results clearly showed that the volatile compounds production are partially coordinated by ethylene under normoxic conditions. Nevertheless, under DCA – RQ, the ethylene production is extremely low, and the volatile compounds are increased (Both et al., 2017; Thewes, Brackmann, Both, et al., 2017; Thewes, Brackmann, de Oliveira Anese, et al., 2017). Additionally, Thewes, Brackmann, de Oliveira Anese, et al. (2017) found a negative correlation between the volatile compounds emission and ethylene production when 'Galaxy' apples were stored under DCA – RQ 1.5, where a little anaerobic metabolism of fruit is induced.

The studies that reported higher volatile compounds emission by the pO_2 reduction to extremely low levels, under DCA – RQ, did not explain the reason of the higher volatile compounds. They did not show if it is a result of the higher precursor's amount, or due to a higher level of ester forming enzymes expression. Thus, we aimed to evaluate the *MdACO1*, *MdADH1*, *MdLOX1*, *MdAAT1* expression at 6 months of storage, quantify the main esters and its precursors, and evaluate the overall quality of 'Galaxy' apples at 6 and 9 months of storage under CA, DCA – CF, DCA – RQ 1.1 and DCA – RQ 1.5 with low oxygen stresses applied twice a week, either with or without 1-MCP, plus 7 d of shelf life at 20 °C.

3.1.2. Material and Methods

3.1.2.1. Plant material and sample preparing

Apples of the cultivar Galaxy were harvested at a commercial orchard at Vacaria, RS, Brazil. The apples were harvested in the morning, and transported to the postharvest research center of the Federal University of Santa Maria in the afternoon. In the morning of the next day, the fruit were randomly sampled, to perform samples of 25 fruit each. Fruit with damage were discharged.

3.1.2.2. Treatment application

Immediately after sample preparing, they were randomly put into 20 kg boxes, four samples in each box. Each of this box was considered a treatment. The boxes were put into 230 L gas-tight experimental chambers. After, the temperature of fruit was reduced to 5 °C and held during 5 d. Then, the temperature was gradually reduced to the storage temperature (2 °C) in 5 d.

3.1.2.3. 1-MCP treatment

Fruit were put into a 450 L chamber at temperature of 2 °C. Together with the fruit was put a solution of 1-MCP 0.625 μL L^{-1} (SmartFresh, 0.14 % of active ingredient). The chamber was hermetically closed during 24 h, and the air continuously circulated with a fan.

3.1.2.4. CA and DCA condition setup

Immediately after 1-MCP treatment, the fruit were put back into 230 L experimental chamber to setup the atmosphere conditions. The atmosphere conditions were: [1] CA – 1.2 kPa O_2 + 2.0 kPa CO_2; [2] DCA – CF; [3] DCA – RQ 1.1; [4] DCA – RQ 1.5; [5] CA + 1-MCP; [6] DCA – CF + 1-MCP; [7] DCA – RQ 1.1 + 1-MCP and [8] DCA – RQ 1.5 + 1-MCP. Fruit stored under DCA – RQ were submitted to RQ calculation twice a week, which causes a repeated low oxygen stress (Anese, 2017). The frequency of RQ calculatiuon (low oxygen stresses) was defined in a preliminar experiment (Supplementary Figure 1 and 2). Therefore,

from this point onward in the present study every time we refer to DCA – RQ it means that apples were submitted to RQ calculation twice a week (Figure 1). The carbon dioxide partial pressure for all DCA conditions was 1.2 kPa.

To reduce the pO_2, the chambers were flushed with nitrogen down to 5.0 kPa. Thereafter its concentration was reduced by fruit respiration until the correct concentration for CA and 0.5 kPa for DCA conditions. The carbon dioxide partial pressure was obtained by its accumulation due to the fruit respiration. This process was undertaken in 5 d. At the day that the pO_2 reached 0.5 kPa for DCA conditions, the fruit bio-response to low O_2 was started, as described below, in order to determine the optimal pO_2 in real time during storage. The oxygen set point variation is showed in Figure 1.

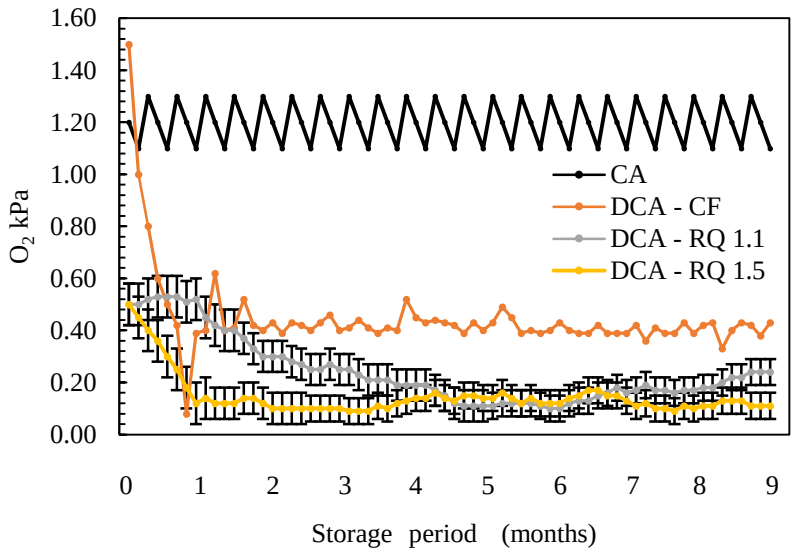


Figure 1. Oxygen set-point variation of 'Galaxy' apples over 9 months of storage under controlled atmosphere (CA), dynamic controlled atmosphere monitored by chlorophyll fluorescence (DCA - CF) and respiratory quotient (DCA – RQ). Standard deviation show the repeated low oxygen stress due to RQ calculation.

3.1.2.4.1. Fruit bio-response based on chlorophyll fluorescence

Two batches of six fruit were put into two baskets, with a chlorophyll fluorescence sensor in its upper side, and accommodate inside the DCA – CF chamber. These two baskets were connected to a software that monitored the chlorophyll fluorescence every hour. The pO_2 were changed according to the recommendation of Zanella, Cazzanelli, & Rossi, (2008).

3.1.2.4.2. Fruit bio-response based on respiratory quotient (RQ)

The bio-response of fruit based on RQ was performed according to Weber et al. (2015). In brief, the chambers were closed during 13 h, being the pO_2 and pCO_2 measured before and after this period. The ratio between CO_2 release and O_2 uptake was the RQ. At the present work were tested two RQs: 1.1 and 1.5. When the RQ was below defined, the O_2 set point was decreased, and if RQ was above defined, the set point of O_2 was increased. The rate of increase and decrease of pO_2 was between 0.01 – 0.03 kPa. This process of RQ calculation cause a repeated low oxygen stress over the 9 months of storage (Figure 1). In the present study when we write DCA – RQ it means that the LOL was monitored with RQ calculation twice a week (two low oxygen stresses a week).

3.1.2.5. Relative mRNA accumulation

3.1.2.5.1. RNA extraction

The total RNA was extracted according to the methodology proposed by Zeng & Yang, (2002), with minor modifications. Samples of the equatorial region of fruit were frozen in liquid nitrogen. These samples were ground to a fine powder with a ball mill. The powder was transferred into 2 mL eppendorf with 750 µL of a buffer (CTAB 2%, PVP40 2%, 300 mM Tris pH 8, 25 mM EDTA, 2M NaCl, H_2O 0.05 %, 2 % β-mercaptoetanol). Immediately, the samples were put into a water bath at 65 °C during 10 min. At the moment that the samples were removed from the water bath was added 750 µL of CIA (a solution containing chloroform and isoamylic alcohol in a proportion 24:1), and centrifuged for 15 min at 7000 g (4 °C). The supernatant were the nucleic acids. The supernatant was transferred into another Eppendorf and CIA added again at same volume as supernatant recovered. These extract was centrifuged again, as described previously. Afterward, the supernatant was taken, put into another Eppendorf and centrifuged at 20000 g for 15 min (4 °C). The supernatant was taken and mixed with 0.6 mL of cold isopropanol and 0.1 mL of sodium acetate (3M, pH 5.5), these tubes were put in ultra-freezer (-80 °C) for 25 min, and then centrifuged at 20000 g for 15 min (4 °C). The pellet formed on the bottom was the total RNA. The RNA quality was evaluated in 1 % agarose gel after electrophoresis and by spectrometry, using A260/A280 and A260/A230 ratios. RNA concentration was measured in the spectrophotometer (Spectra Max, Molecular Devices). The DNA absence was assured by PCR.

3.1.2.5.2. cDNA synthesis

Total RNA (3 µg) was treated with 1U RNase-Free DNAse and RNase-Free DNAse 10 × reaction buffer (Promega) before cDNA synthesis, to eliminate genomic DNA. All treated RNA was reverse transcribed using the High-capacity cDNA Reverse Transcription Kit, according to manufacturer's instructions (Applied Biosystems™).

3.1.2.5.3. RT-qPCR analysis

Two reference genes that showed high expression stability in previous postharvest experiments were used in the present study, the gene *UBIQUITIN- CONJUGATING ENZYME E2* (*MdUBC)* and the gene *HISTONE 1* (*MdH1)* (Storch et al., 2015). For *alcohol desidrogenase (MdADH), lipoxygenase (MdLOX), alcohol acyl transferase (MdAAT) and ACC Oxidase (MdACO1)* genes, primers were designed based on the sequences of *Malus* extracted from GenBank, designed for previous experiments. All primers were again validated by the analyses of the amplification curve employing a pool of cDNAs from all tested conditions of our experiments, at four distinct concentrations.

Quantitative PCR (qPCR) was carried out using the equipment CFX96 Real time PCR detection system (Bio-Rad) and the GoTaq® qPCR Master Mix (Promega). The reactions were performed in a total volume of 12.5 µL, consisting of 6.25 µL of qPCR Master Mix 2X, 2.5 µL cDNA and 200 nM each, primer pair, and started with a denaturation step at 95 °C for 10 min, followed by 40 cycles consisting of 30 s at 95 °C, and 1 min at 58 °C and 1 min at 72 °C, finalized by the dissociation curve with denaturation at 95 °C for 15 s, cooling at 65 °C for 0.05 minute and gradual heating, at 0.3 °C steps, up to 95 °C for 0.5 min. A negative, no template control (NTC), was used to check the absence of DNA contamination.

3.1.2.6. ACC oxidase enzyme activity

The ACC oxidase enzyme activity was evaluated according to the methodology proposed by Bufler, (1986). The results were expressed in ng kg^{-1} s^{-1}.

3.1.2.7. Ethylene production and respiration rate

Samples of fruit (±1.5 kg) were put into a glass of 5 L and hermetically closed during 2 hours. After this period, two samples of 1mL were taken of the glass and injected into a Varian® gas chromatograph model Star CX 3400 (Varian, Palo Alto, CA, USA), equipped with a flame ionization detector (FID) and a Porapak N80/100 column to determine the ethylene concentration in the container headspace. The temperature of the injector, column and detector were: 140, 90 and 200 °C, respectively. The results are showed in ng kg^{-1} s^{-1}. The same glass was connected to a gas analyzer (Isolcell®, Oxycarb 6, Italy), to quantify the CO_2 concentration inside the glass. The respiration rate was determined by the difference between CO_2 concentration inside the glass and the ambient. Results were expressed as µg kg^{-1} s^{-1}.

3.1.2.8. Internal ethylene concentration (IEC)

Quantified according to Mannapperuma, Singh, & Montero (1991), in 10 fruit per replicate. The internal fruit air was injected in the same gas chromatograph to ethylene production. The IEC was expressed in ug L^{-1}.

3.1.2.9. Decay, mealiness, flesh breakdown and healthy fruit

These quality attributes were determined according to Thewes et al. (2015). In brief, fruit with any decay, mealiness and flesh breakdown incidence were accounted and expressed in percentage of the total fruit of each treatment. Healthy fruit were considered those without any incidence of decay and internal or external disorder. The results were expressed in percentage.

3.1.2.10. Flesh firmness

The pulp firmness was measured in two opposite sides of each fruit, were previously the skin was removed. An 11 mm in diameter tip was inserted and measured the force necessary to penetrate into the pulp. Results expressed as Newton (N).

3.1.2.11. Soluble solids and titratable acidity

Slices of the equatorial region of fruit were taken and make a juice with a juicer (Philips Walita®). Drops of this juice were used to measure the soluble solids by refractometry, results

expressed in %. From the same juice were taken 10 mL and diluted in 100 mL of distillated water. This solution was titrated with a 0.1 N NaOH solution up to pH 8.1. Results expressed as % of malic acid.

3.1.2.12. Volatile compounds analysis

3.1.2.12.1. Sample preparation

Ten fruit of each sample were cooled to 0 °C (pulp temperature). These fruit were centrifuged under low temperature (<5 °C). The juice was immediately put into amber flasks and frozen to -30 °C until the analysis. The process was undertaken under low temperature to avoid enzymatic oxidation. At the day of the volatile compounds analysis, the juice was thawed in water up to the juice reached 0 °C. Immediately, were taken 10 mL of the juice put into a 20 mL vial, with 3 g of NaCl and 10 µL of 3-octanol standard solution (82.2 µg mL^{-1}). The vial was sealed with PTFE-coated silicon lid seals.

3.1.2.12.2. Volatile compounds quantification

The solution of juice, NaCl and 3-octanol was warmed up to 35 °C in a water bath during 5 min. After, the volatile compounds were extracted via solid phase microextration (HS-SPME) during a period of 60 min and constant juice stirring (400 rpm). A Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/Car/PDMS) fiber (Supelco, 50/30 µm × 20 mm) was preconditioned following the manufacturer protocol. After 60 min of volatile compounds adsorption on the fiber, they were thermally desorbed into the injection port of a DANI® (Dani Intruments Spa., Viale Brianza, Cologno Monzese, Italygas) gas chromatograph at 250 °C for 10 min in a split less mode. The volatile compounds were separated in a DN-WAX (60 m × 0.25 mm; 0.25 µm of thickness film) capillary column. The carrier gas (Nitrogen) was seated in a constant flow of 1.0 mL min^{-1}. For the volatile compounds separation was adopted a temperature ramp: initial temperature 35 °C held for 3 min, then a ramp of temperature of 2 °C min^{-1} up to 80 °C, thereafter, another ramp of 5 °C min^{-1} up to 230 °C and held at this temperature for 5 min. The FID detector temperature was 230 °C. A series of n-alkanes were analyzed at the same conditions described above, to calculate the linear retention index (LRI). The quantification of the volatile compounds was performed in relation to the area of the internal standard (Both et al., 2014), and the results expressed in µg L^{-1}.

3.1.2.12.3. Volatile compounds identification

To identify the volatile compounds, the same method described above was followed in a Shimadzu QP2010 Plus gas chromatography coupled to mass spectrometry (GC/MS; Shimadzu Corporation, Kyoto, Japan) or by standard solutions as showed in Supplementary table 1. The detector was operated in the electron impact ionization, ionization energy of +70 eV and a scan mass from 35 up to 350 m/z. The mass spectra of each compound was compared with mass spectra available in the National Institute of Standards and Technology (NIST) library and by comparing the linear retention index (LRI) with those available in the scientific literature.

Table 1. Initial analysis carried out before storage (at day of harvest).

Compounds	LRI[1]	Average (μg L^{-1} ± SD*)
Acetaldehyde**	716	0.40 ± 0.20
Methyl acetate	839	1.25 ± 1.04
Ethyl acetate**	902	0.52 ± 0.50
2-Methyl-1-Butanal	923	2.59 ± 1.44
2-Propanol	942	2.65 ± 2.18
Ethanol**	950	4.92 ± 3.58
Ethyl propanoate	968	0.25 ± 0.12
Methyl 2-Methyl butanoate	1017	3.94 ± 0.41
2-Methylpropyl acetate	1018	6.06 ± 1.06
Ethyl butanoate	1046	2.33 ± 0.86
Butyl acetate	1082	1,315.79 ± 190.75
Hexanal	1092	119.63 ± 22.73
2-Methylbutyl acetate	1130	162.45 ± 12.19
Butyl propanoate	1147	5.86 ± 1.53
1-Butanol	1158	368.57 ± 187.10
Pentyl acetate	1178	73.56 ± 9.26
3-Hexanol	1201	4.52 ± 0.92
2-Methyl-1-Butanol	1212	27.13 ± 12.52
E-2-Hexenal	1227	70.17 ± 13.88
Z-2-Pentenyl acetate	1199	0.75 ± 0.47
1-Pentanol	1256	8.07 ± 2.54
Hexyl acetate	1273	972.12 ± 100.11
E-5-Hexenyl acetate	1316	1.66 ± 0.58
E-2-Hexenyl acetate	1338	9.21 ± 1.63
6-Methyl-5-hepten-1-one	1345	6.31 ± 0.36
1-Hexanol**	1361	350.00 ± 59.76

Heptyl acetate	1364	4.20 ± 0.82
Z-3-Hexen-1-ol	1380	2.27 ± 0.40
E-2-Hexen-1-ol	1413	12.55 ± 4.72
E-5-Hexen-1-ol	1407	2.97 ± 0.35
E-3-Hepten-1-ol	1361	5.16 ± 0.54
Ethanoic acid	1468	21.43 ± 4.30
6-Methyl-5-hepten-2-ol	1473	1.59 ± 0.39

*SD = Standard deviation. ** Identified by standards. [1] Linear retention index.

3.1.2.13. Statistical analyses

The data were submitted to a principal component analysis (PCA) using The Unscrambler® X software (version 9.7, CAMO A/S, Trondheim, Norway). Before the PCA the data matrix was auto scaled for each variable in order to obtain the same weight for all variables (mean = 0 and variance = 1). Additionally, a variance analysis (ANOVA) was carried out (Perecin & Cargnelutti Filho, 2008). Data that showed significant difference by ANOVA were subjected to the Tukey's test at 5 % error probability.

3.1.3. Results

3.1.3.1. MdACO1, MdLOX1, MdADH1 and MdAAT1 relative RNA accumulation

The ACC oxidase is a key enzyme of ethylene metabolism, and its expression was suppressed by the storage under DCA, regardless the DCA method, and 1-MCP application (Figure 1). When fruit were treated with 1-MCP there was no difference between fruit stored under CA and DCA for *MdACO1*. Contrarily to *MdACO1*, *MdADH1* expression was increased in all fruit treated with 1-MCP (Figure 1). The storage under DCA resulted in higher *MdADH1* in fruit without 1-MCP. On the other hand, when fruit were treated with 1-MCP the storage under DCA – RQ 1.1 resulted in lower *MdADH1* expression.

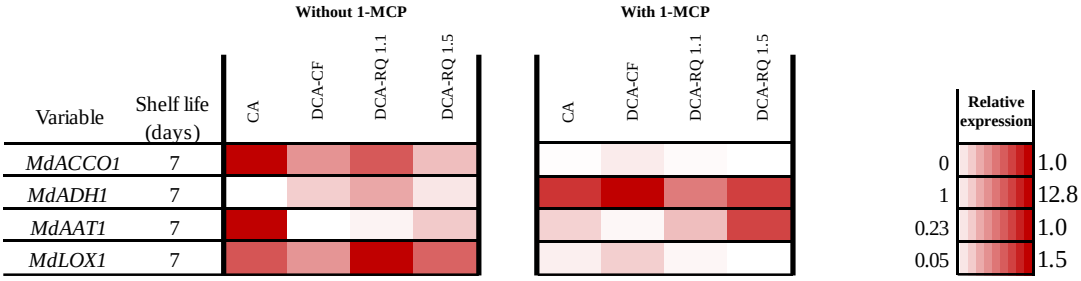


Figure 1: Heat map showing the expressions of *MdACCO1*, *MdADH1*, *MdAAT1* and *MdLOX1* of 'Galaxy' apples at 6 months of storage in controlled atmosphere (CA), dynamic controlled atmosphere monitored by chlorophyll fluorescence (DCA - CF) and respiratory quotient (DCA – RQ), either without or with 1-methylcyclopropene (1-MCP) treatment, plus 7 d of shelf life at 20 °C. Treatments with the same color are not significantly different by Tukey's test, at 5 % probability of error.

The AAT enzyme is responsible to ester formation. For fruit without 1-MCP, the storage under CA resulted in the highest *MdAAT1* expression (Figure 1). Fruit stored under DCA – CF and DCA – RQ 1.1 had the lowest *MdAAT1* expression. A noteworthy fact is that when the fruit were stored under DCA – RQ 1.5 the expression of *MdAAT1* increased as compared to DCA – CF and DCA – RQ 1.1. When the fruit were treated with 1-MCP, the *MdAAT1* expression was higher in fruit stored under DCA – RQ 1.5. Again, fruit stored under DCA – CF had the lowest *MdAAT1* expression. CA and DCA – RQ 1.1 had an intermediary *MdAAT1* expression. These results showed that the RQ 1.5 induced the expression of *MdAAT1*, either in fruit without or with 1-MCP, showing that its induction in DCA – RQ 1.5 is not ethylene dependent (Figure 2).

Volatile acids are important ester precursors, and they are synthetized by the lipoxygenase (LOX) from fatty acids. *MdLOX1* expression was significantly reduced by 1-MCP application, regardless the storage condition (Figure 1). For fruit without 1-MCP, the storage under DCA – RQ 1.1 resulted in the highest *MdLOX1* expression. Fruit stored under DCA – CF had the lowest *MdLOX1* expression. CA and DCA – RQ 1.5 had an intermediary *MdLOX1* expression, higher than DCA – CF and lower than DCA – RQ 1.1. When the fruit were treated with 1-MCP, the storage under DCA – CF resulted in higher *MdLOX1* expression as compared to the other storage conditions, but far bellow all conditions without 1-MCP.

3.1.3.2. Ethylene metabolism and respiration rate

ACC oxidase enzyme activity was higher in fruit stored under CA after 6 months of storage, irrespective of with or without 1-MCP, and after 9 months in fruit without 1-MCP (Figure 2). 1-MCP application reduced ACC oxidase enzyme activity when fruit were stored under CA. After 6 months of storage plus 7 d of shelf life, the storage under DCA resulted in lower ACC oxidase, even when fruit were treated with 1-MCP. The lower ACC oxidase enzyme activity by storage under DCA and 1-MCP treatment is probably due to the lower *MdACO1* RNA accumulation (Figure 1).

Higher ACC oxidase enzyme activity in fruit stored under CA resulted in higher IEC and ethylene production after both 6 and 9 months of storage, in fruit without 1-MCP (Figure 2). Ethylene production increased during the shelf life period in a greater rate when fruit were stored under CA as compared to fruit in DCA. When fruit were treated with 1-MCP, the storage under DCA had little effect on IEC and ethylene production. Fruit stored under CA also had higher respiration rate during shelf life period, when compared to DCA stored one (Figure 2). Similarly to ethylene production, the respiration rate increased during shelf life until end shelf life after 6 months of storage and until 4 days shelf life after 9 months storage, regardless the storage condition.

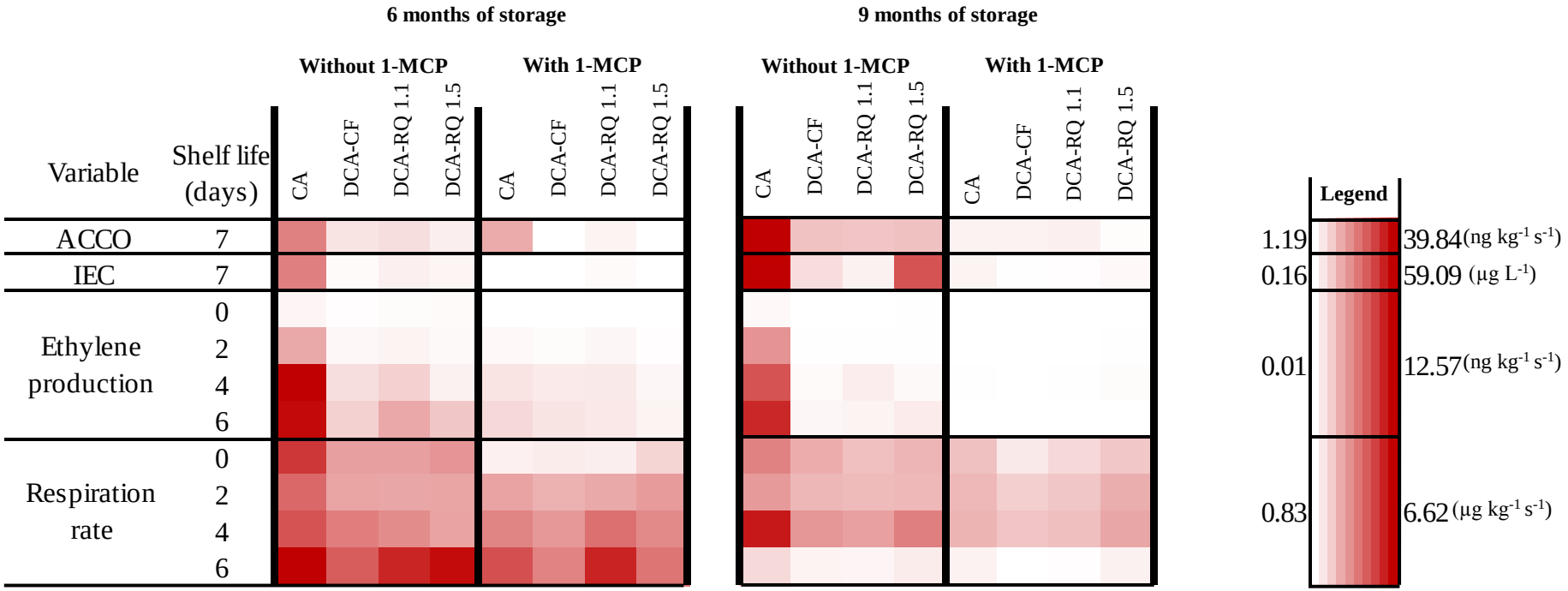


Figure 2: Heat map of the ACC oxidase enzyme activity (ACCO), internal ethylene concentration (IEC), ethylene production and respiration rate of 'Galaxy' apples after 6 and 9 months of storage in controlled atmosphere (CA), dynamic controlled atmosphere monitored by chlorophyll fluorescence (DCA - CF) and respiratory quotient (DCA – RQ), either without or with 1-methylcyclopropene (1-MCP) treatment, plus 7 d of shelf life at 20 °C. Treatments with the same color are not significantly different by Tukey's test, at 5 % probability of error.

3.1.3.3. Overall quality

Decay is one of the main reason of fruit losses over the storage period. There was no decay incidence after 6 months of storage plus 7 d of shelf life at 20 °C (Figure 3). Nevertheless, after 9 months of storage, there was decay incidence and some differences among treatments. At chamber opening, fruit stored under DCA – CF had higher decay incidence for fruit without 1-MCP. However, when fruit were treated with 1-MCP, higher decay was observed under DCA – RQ 1.1. After 7 d of shelf life at 20 °C, fruit stored under DCA – RQ 1.5 had the highest decay incidence, for fruit without 1-MCP. When fruit were treated with 1-MCP, the storage under DCA – RQ 1.1 and DCA – RQ 1.5 resulted in higher decay as compared to the other storage conditions.

Mealiness incidence was higher in fruit stored under CA as compared to the other storage conditions, for fruit without 1-MCP after 6 months of storage. The 1-MCP application to fruit stored under CA was able to inhibit mealiness. After 9 months of storage, fruit stored under CA had higher mealiness again. Fruit stored under DCA – RQ 1.5 had the lowest mealiness, when fruit were stored without 1-MCP. The 1-MCP application inhibit mealiness, especially in CA after 9 months (Figure 3). Flesh breakdown occurrence, characterized as flesh browning, was higher in fruit stored under CA after 6 months of storage without 1-MCP. 1-MCP application reduced flesh breakdown incidence in CA stored fruit after 6 and 9 months (Figure 3). Fruit stored under DCA – RQ 1.1 and 1.5 had the lowest flesh breakdown after 9 months of storage without 1-MCP. When the fruit were treated with 1-MCP, the storage under DCA resulted in lower flesh breakdown incidence, differing from CA. The 1-MCP application was able to reduce flesh breakdown in all atmosphere conditions after 9 months of storage plus 7 d at 20 °C.

Taken in account the number of fruit affected by decay and internal disorders, it is possible to obtain the amount of healthy fruit, which means the one without any damage. After 6 months of storage plus 7 d of shelf life, fruit stored under DCA had higher healthy fruit amount as compared to CA, when fruit were stored without 1-MCP (Figure 3). If 1-MCP was applied, there was no difference among treatments for healthy fruit after 6 months. 1-MCP resulted in higher healthy fruit amount in CA only, but for DCA was not observed this positive effect after 6 months. After 9 months of storage, DCA had higher healthy fruit amount as compared to CA, for fruit without 1-MCP (Figure 3). On the other hand, when fruit were treated with 1-MCP, there was no significant difference between treatments for healthy fruit.

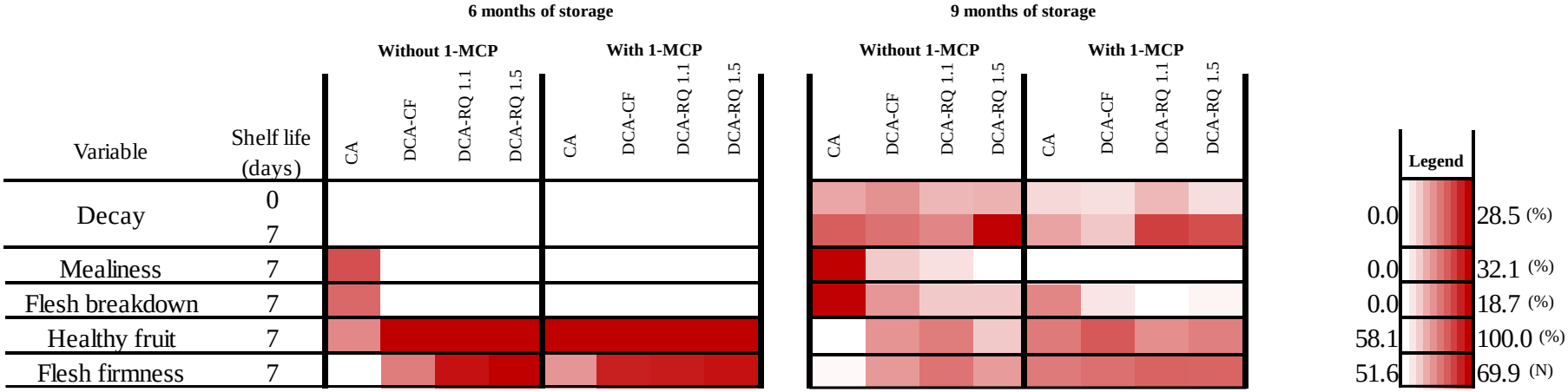


Figure 3: Heat map of the decay incidence, mealiness, flesh breakdown, healthy fruit and flesh firmness of 'Galaxy' apples after 6 and 9 months of storage in controlled atmosphere (CA), dynamic controlled atmosphere monitored by chlorophyll fluorescence (DCA - CF) and respiratory quotient (DCA – RQ), either without or with 1-methylcyclopropene (1-MCP) treatment, plus 7 d of shelf life at 20 °C. Treatments with the same color are not significantly different by Tukey's test, at 5 % probability of error.

After 6 months of storage plus 7 d of shelf life, the storage under DCA – RQ 1.1. and 1.5 resulted in higher flesh firmness maintenance, when the fruit were stored without 1-MCP (Figure 3). For fruit treated with 1-MCP, all DCA conditions resulted in higher firmness as compared to CA. The 1-MCP application resulted in higher flesh firmness maintenance for CA and DCA – CF only. Fruit firmness reduced after 9 months of storage plus 7 days shelf life in all storage conditions as compared to 6 months. When fruit were stored without 1-MCP, DCA resulted in higher flesh firmness as compared to CA after 9 months. Olny in CA storage the 1-MCP application resulted in significantly higher flesh firmness (Figure 3). CA and DCA storage had the same firmness after 9 months of storage when treated with 1-MCP.

The storage conditions and 1-MCP application had no effect on the titratable acidity and soluble solids (data not showed).

3.1.3.4. Volatile compounds concentration

Volatile compounds were evaluated after harvest (Table 1), 6 and 9 months of storage plus 7 d of shelf life at 20 °C (Figure 4). At the three evaluations were detected 35 volatile compounds in all samples. Five groups of volatile compounds were found: 4 aldehydes, 13 alcohols, 1 ketone, 1 acid and 16 esters.

3.1.3.5. Anaerobic metabolism volatiles

Apple storage under DCA – RQ, with a RQ higher than 1, start the anaerobic metabolism of fruit. The first volatile produced during ethanolic anaerobic metabolism is acetaldehyde, and its concentration was higher in fruit after 9 months of storage in DCA – RQ 1.5 (Figure 4). After 6 months of storage, fruit in CA had higher acetaldehyde, when they were stored without 1-MCP. 1-MCP application reduced anaerobic metabolism when fruit were stored under DCA – RQ 1.5 over 9 months of storage plus 7 d of shelf life. Higher acetaldehyde in DCA – RQ 1.5 resulted in higher ethanol accumulation (Figure 4). Analyzing the *MdADH1* expression (Figure 1) and alcohol production, is noteworthy that the precursor concentration had higher importance on ethanol production by fruit as compared to new *MdADH1* expression (Figure 5 and 6).

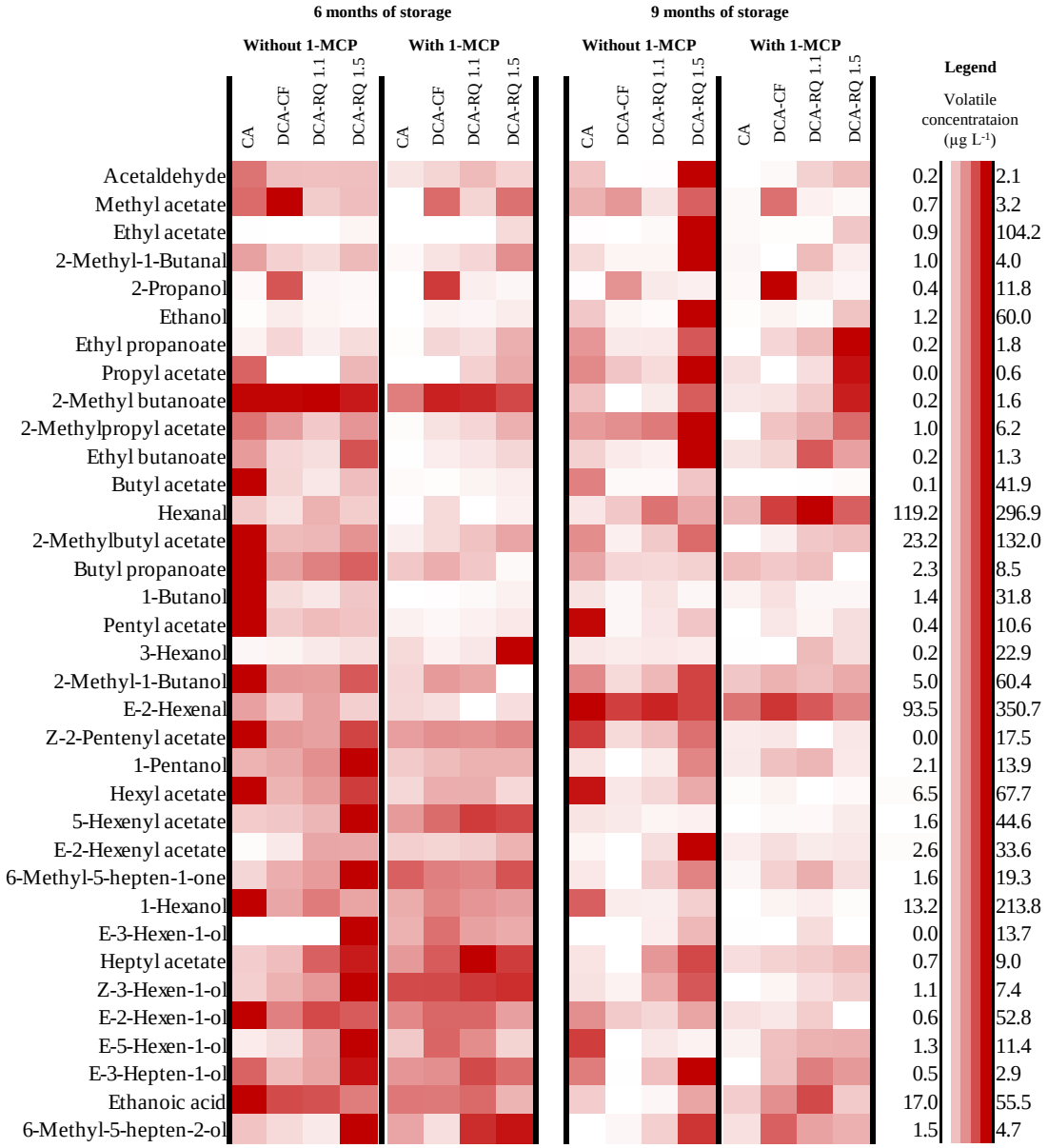


Figure 4. Heat map of the volatile compounds concentration (µg L^{-1}) of 'Galaxy' apples after 6 and 9 months of storage in controlled atmosphere (CA), dynamic controlled atmosphere monitored by chlorophyll fluorescence (DCA - CF) and respiratory quotient (DCA – RQ), either without or with 1-methylcyclopropene (1-MCP) treatment, plus 7 d of shelf life at 20 °C. Treatments with the same color are not significantly different by Tukey's test, at 5 % probability of error.

The AAT enzyme combines ethanol with ethanoic acid forming ethyl acetate. Fruit stored over 9 months under DCA – RQ 1.5 plus 7 d of shelf life had the highest amount of ethyl acetate (Figure 4). This is a result of higher ethanol concentration in fruit stored under DCA – RQ 1.5 and also due to the high *MdAAT1* expression in these fruit (Figure 1). 1-MCP application suppressed the ethyl acetate formation in fruit stored under DCA – RQ 1.5. Storage under CA,

DCA – CF and DCA – RQ 1.1 had the same ethyl acetate concentration, regardless the storage period and 1-MCP application. These result clearly showed that the anaerobic metabolism is started significantly by RQ 1.5 only.

The storage under DCA – RQ 1.5 also increased the concentrations of other ethyl esters, like ethyl propanoate and ethyl butanoate, which are esters that contribute positively to fruit flavor (Figure 4). Ethyl propanoate and ethyl butanoate were in higher concentration after 9 months of storage under DCA – RQ 1.5 with and without 1-MCP, respectively. A noteworthy fact is that 1-MCP application increased the ethyl propanoate in juice when fruit were stored under DCA – RQ 1.5, and reduced it concentration when stored under CA, especially after 9 months plus 7 d of shelf life. On the other hand, ethyl butanoate was reduced by 1-MCP when fruit were stored under DCA – RQ 1.5.

3.1.3.6. Main volatile esters of 'Galaxy' apples

Butyl acetate concentration was higher in fruit stored under CA after 6 months plus 7 d of shelf life (Figure 4). The lowest butyl acetate was observed under DCA – RQ 1.1, after 6 months of storage. A noteworthy fact is that the pO_2 reduction to have an RQ 1.5 increased the butyl acetate as compared to DCA – RQ 1.1. 1-MCP application reduced it accumulation in fruit juice when stored under CA, DCA – CF and DCA – RQ 1.5. The higher butyl acetate in CA is a result of higher 1-Butanol and ethanoic acid accumulation in these fruit (Figure 4), which are substrates for AAT, which was also expressed in a higher level in CA stored fruit (Figure 1). After 9 months of storage, fruit under CA and DCA – RQ 1.5 had the highest butyl acetate, when stored without 1-MCP. When treated with 1-MCP, concentration was low and there was no difference among storage conditions.

2-Methylbutyl acetate is one of the most important ester of 'Gala' apple group, and its concentration in juice was higher when fruit were stored under CA after 6 months, but after 9 months of storage, there were no difference between CA and DCA – RQ 1.5 (Figure 4). A noteworthy fact is that when fruit were treated with 1-MCP, the storage under DCA - RQ 1.5 resulted in higher 2-methylbutyl acetate as compared to CA. DCA – CF had the lowest 2-methylbutyl acetate after 9 months of storage without 1-MCP. The higher 2-methylbutyl acetate in CA and DCA – RQ 1.5 is a result of higher 2-methyl-1-butanol and ethanoic acid (Figure 4), which are the precursors of 2-methylbutyl acetate, and due to higher *MdAAT1* expression in these treatments (Figure 1). Additionally, the higher 2-methyl-1-butanol is a result of higher 2-methyl-1-butanal when fruit were stored under CA and DCA – RQ 1.5 (Figure 4).

Another important ester for ‘Galaxy’ apples is hexyl acetate, and fruit stored under CA and DCA – RQ 1.5, without 1-MCP, had higher hexyl acetate as compared to DCA – CF and DCA – RQ1.1 after 6 and 9 months of storage plus 7 d of shelf life (Figure 4). When fruit were treated with 1-MCP, ethyl acetate was low and there were no differences between atmosphere conditions, after either 6 or 9 months of storage plus 7 d of shelf life at 20 °C, and its application reduced hexyl acetate production when fruit were stored under CA and DCA – RQ 1.5 (Figure 4). The hexyl acetate accumulation in juice is closely correlated to the 1-hexanol concentration and *MdAAT1* expression (Figure 5 and 6).

3.1.3.7. Pentenyl and hexenyl volatiles

The storage under CA and DCA – RQ 1.5 without 1-MCP resulted in higher concentration of Z-2-pentenyl acetate either after 6 or 9 months of storage plus 7 d of shelf life at 20 °C (Figure 4). On the other hand, when fruit were treated with 1-MCP, the concentrations of Z-2-pentenyl acetate were not affected by the storage conditions after 6 months. After 9 months the storage, DCA – RQ 1.1 + 1-MCP resulted in the lowest Z-2-pentenyl acetate concentration (Figure 4).

Lipoxygenase action on the unsaturated fatty acids results in the formation of precursor of hexenyl esters. 5-Hexenyl acetate was accumulated in higher concentration by the storage under DCA – RQ 1.5 without 1-MCP, after 6 months of storage plus 7 d of shelf life (Figure 4). A noteworthy fact is that the concentration of 5-hexenyl acetate decrease after 9 months of storage. When the fruit were treated with 1-MCP, the storage under DCA – RQ, regardless the RQ, resulted in higher 5-hexenyl acetate after 6 months, but after 9 months of storage, concentration was lower and there were no differences between atmosphere conditions. The higher 5-hexenyl acetate in fruit stored under DCA – RQ 1.5 is a result of higher E-5-hexen-1-ol (Figure 4) and due to high *MdAAT1* expression in fruit stored under DCA – RQ1.5 (Figure 1).

Similarly to 5-hexenyl acetate, the concentration of E-2-hexenyl acetate was higher when fruit were stored under DCA – RQ, regardless the RQ level, after 6 months of storage without 1-MCP (Figure 4). After 9 months of storage, DCA – RQ 1.5 resulted in higher concentration of E-2-Hexenyl acetate in fruit without 1-MCP. When fruit were treated with 1-MCP, there were no differences between atmosphere conditions for E-2-Hexenyl acetate, after either 6 or 9 months of storage.

Other compounds originated from unsaturated fatty acids, like E-2-hexenal, E-3-hexen-1-ol, Z-3-hexen-1-ol, E-2-hexen-1-ol, E-5-hexen-1-ol and E-3-hepten-1-ol, were detected in the juice of all treatments after 6 and 9 months of storage (Figure 4). The storage under DCA – RQ 1.5 resulted in higher E-3-hexen-1-ol, Z-3-hexen-1-ol, E-5-hexen-1-ol and E-3-hepten-1-ol when fruit were stored without 1-MCP over 6 months. The results of hexen aldehydes, alcohols and esters, clearly show that the storage under DCA – RQ 1.5 probably result in higher amount of free linolenic acid and/or polar lipids as subtract for LOX for the formation of precursors of these volatiles.

3.1.3.8. 6-Methyl-5-hepten-2-ol and 6-methyl-5-hepten-1-one accumulation

The metabolism of carotenoids and alfa-farnesene could result in the production of 6-methyl-5-hepten-2-ol and 6-methyl-5-hepten-1-one, compounds that are closely correlated with the superficial scald incidence in apples. Higher levels of these two compounds were accumulated in fruit stored under DCA – RQ 1.5 after 6 and 9 months of storage without 1-MCP. When fruit were treated with 1-MCP, DCA – RQ 1.5 resulted in higher 6-methyl-5-hepten-2-ol after 6 months only (Figure 4), after 9 months, DCA – CF had higher 6-methyl-5-hepten-2-ol when treated with 1-MCP.

3.1.3.9. Principal component analysis (PCA)

To show an overview of the results and the correlations between treatments and variables were undertaken two PCA analyses, one after 6 months (Figure 5) and another after 9 months (Figure 6). According to these analyses, the treatments were separated into three groups: CA, DCA – RQ 1.5 and the other treatments applied (Figure 5 and 6). This different response of fruit stored under CA and DCA – RQ 1.5 is closely correlated to a higher amount of volatile compounds in these fruit. Mealiness and flesh breakdown were also correlated to fruit stored under CA. On the other hand, all fruit treated with 1-MCP and the ones stored under DCA – CF and DCA – RQ 1.1 are correlated to higher flesh firmness and healthy fruit amount (Figure 5 and 6).

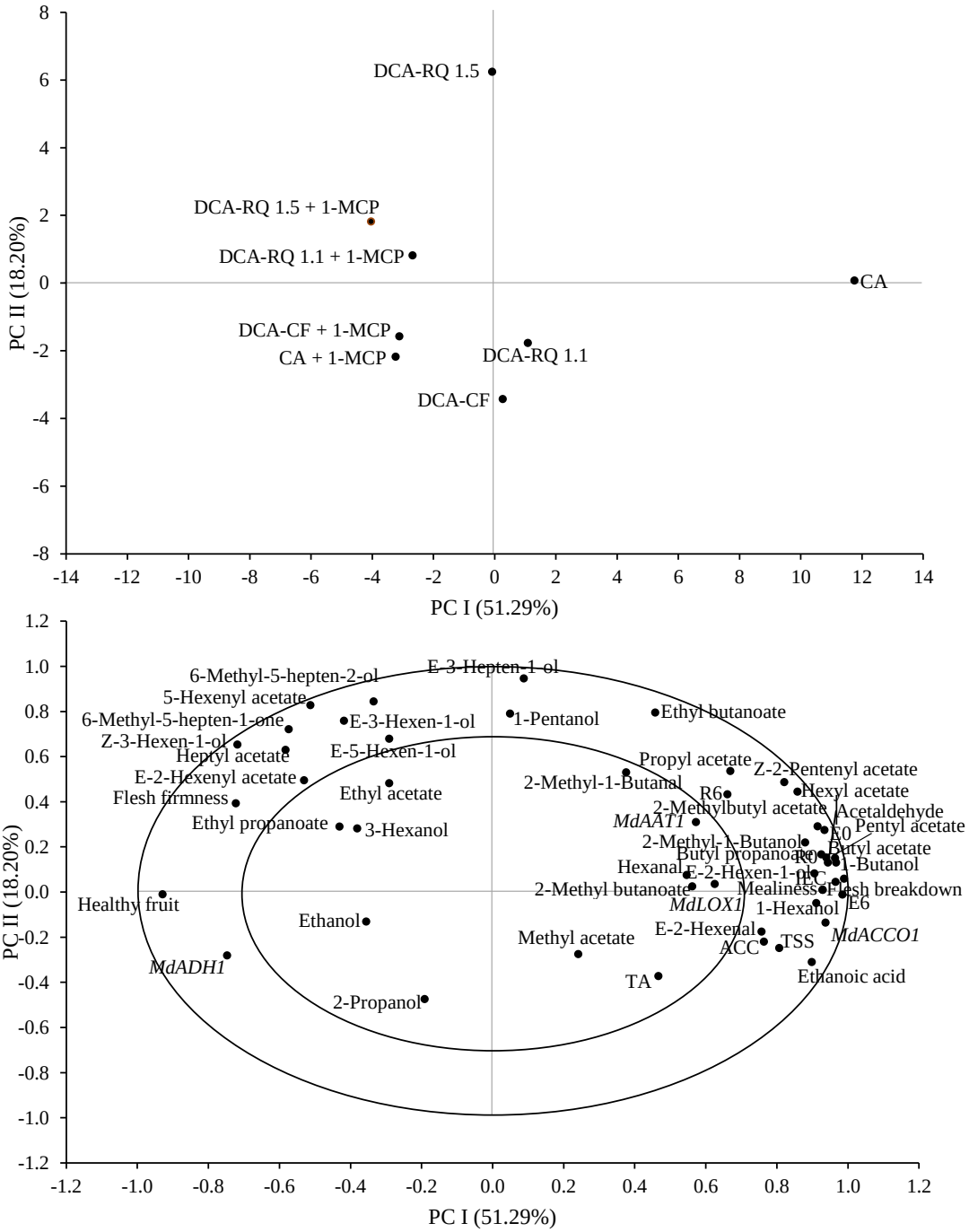


Figure 5: Principal component analysis of the metabolism, quality and volatile compounds analyses of ‘Galaxy’ apples after 6 months of storage in controlled atmosphere (CA), dynamic controlled atmosphere monitored by chlorophyll fluorescence (DCA - CF) and respiratory quotient (DCA – RQ), either without or with 1-methylcyclopropene (1-MCP) treatment, plus 7 d of shelf life at 20 °C.

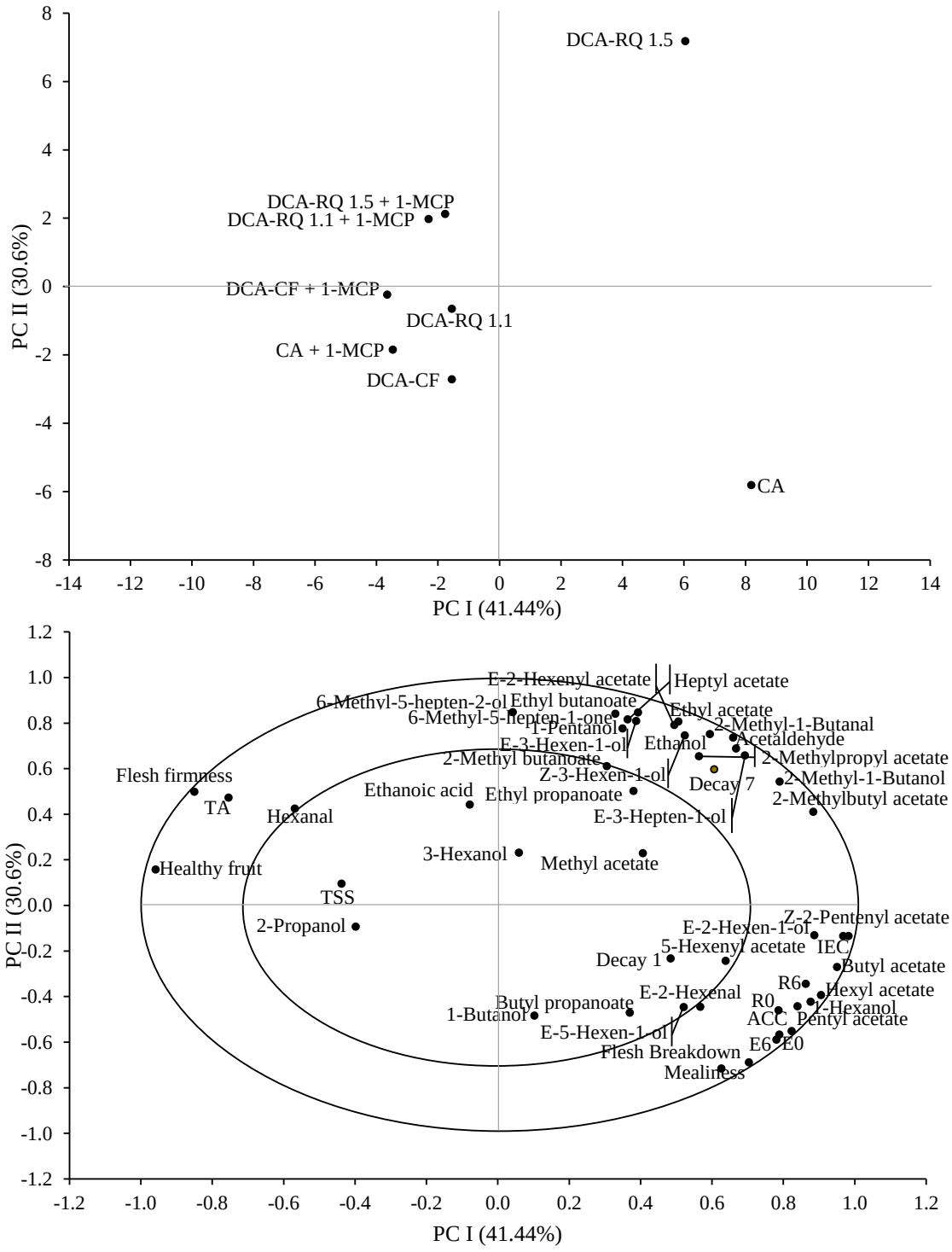


Figure 6: Principal component analysis of the metabolism, quality and volatile compounds analyses of 'Galaxy' apples after 9 months of storage in controlled atmosphere (CA), dynamic controlled atmosphere monitored by chlorophyll fluorescence (DCA - CF) and respiratory quotient (DCA – RQ), either without or with 1-methylcyclopropene (1-MCP) treatment, plus 7 d of shelf life at 20 °C.

Analyzing the correlation between gene expression analyses and the volatile compounds concentration is noteworthy that the *MdAAT1* expression is correlated with the treatments that had the highest amount of ester accumulation (CA and DCA – RQ 1.5) (Figure 5). The expression of *MdLOX1* is more associated to fruit that were stored under CA, without 1-MCP application. ACC oxidase enzyme activity had also a similar response to *MdACCO1* expression. An interesting fact is that the expression of *MdADH1* is inversely correlated to fruit that had the highest amount of alcohols, showing that its expression had little influence on the alcohol production, demonstrating that the alcohol production is more dependent of the precursors concentration as *MdADH1* expression (Figure 5).

3.1.4. Discussion

At the present study was demonstrated the effect of CA and DCA, combined or not with 1-MCP, on the expression of enzymes of the ethylene metabolism, anaerobic metabolism, LOX, volatile compounds precursor production, overall quality and the volatile compounds accumulation in the fruit juice after 6 and 9 months of storage plus 7 d of shelf life at 20 °C. We evaluated these variables because one of the main disadvantage of the storage under low pO_2 is the volatile compounds reduction, resulting in fruit with reduced quality for the consumer. We also wanted better understand what happens on the metabolism of fruit stored under DCA – RQ and the differences from the ones stored under CA.

3.1.4.1. Metabolism and overall quality

The storage under DCA – CF and DCA – RQ have a strong effect on the ethylene metabolism of apples after long-term storage (Aubert et al., 2015; Both et al., 2017; Brackmann et al., 2015; Köpcke, 2015; Thewes et al., 2015, 2018; Thewes, Brackmann, de Oliveira Anese, et al., 2017). A similar result was obtained at the present research, where the storage in DCA resulted in reduced expression of *MdACCO1* and consequently lower ACC oxidase enzyme activity and ethylene production (Figures 1 and 2). The lower ethylene production and respiration rate in fruit stored under DCA – RQ 1.5 could be attributed in part to the higher ethanol production (Figure 4), because the ethanol have an inhibitory effect of ethylene synthesis (Asoda, Terai, Kato, & Suzuki, 2009; Jin, Lv, Liu, Qi, & Bai, 2013; Liu et al., 2012; Pesis, 2005; Weber et al., 2016). Fruit metabolism (respiration rate) was inhibit at the same level by DCA or 1-MCP, results that are in line with the ones obtained by Both et al. (2018).

This shows that the storers would have the same response of fruit by storage under DCA or 1-MCP application in CA. In practical term, this is an important finding because the employment of DCA is allowed in either conventional or organic produced apples. On the other hand, 1-MCP is not allowed for organic apples produced in Europe (Gabioud Rebeaud & Gasser, 2015).

Reduced metabolism in fruit stored under DCA or treated with 1-MCP resulted in lower mealiness and flesh breakdown, and higher healthy fruit amount (Figure 3). Higher physiological disorders by fruit stored under CA led to lower flesh firmness of these fruit after either 6 or 9 months of storage plus 7 d of shelf life at 20 °C. The higher physiological disorders and lower firmness in these fruit could be attributed to the higher IEC and ethylene production, as soon as the cell-wall degrading enzymes are started by ethylene (Gwanpua et al., 2016, 2014; Payasi, Mishra, Chaves, & Singh, 2009; Prasanna, Prabha, & Tharanathan, 2007). Despite the high healthy fruit amount by storage under DCA – RQ 1.5, these fruit had higher decay incidence after 9 months of storage plus 7 d of shelf life, showing that the extremely low pO_2 could decrease the fruit resistance to fungi infection. These finding agree with Both et al. (2018), which also found higher decay incidence in fruit stored under DCA – RQ 1.5 as compared to the one in CA. This result could be explained because the effect of ethanol on decay is dose dependent (Pesis, 2005; Weber et al., 2016), showing that the ethanol production in DCA – RQ 1.5 could be too high.

3.1.4.2. Anaerobic metabolism compounds accumulation is coordinated mainly by the precursors concentrations

Analyzing the accumulation of acetaldehyde, ethanol and ethyl acetate together, is noteworthy that the production of ethanol and ethyl acetate are more influenced by the acetaldehyde accumulation than the *MdADH1* and *MdAAT1* expression (Figures 4 and 5). These results showed that the acetaldehyde production by storage under DCA – RQ 1.5 is not high enough to saturate the ADH enzyme, consequently the ethanol production is only acetaldehyde dependent. Thewes et al. (2018) also verified that the anaerobic metabolism compounds accumulation is coordinated by the accumulation of acetaldehyde, and its production is significantly suppressed by the 1-MCP treatment of fruit. Probably, the pool of anaerobic metabolism enzymes in the apple flesh was enough to detoxify the cells from acetaldehyde, and its activation was mainly based on cofactor regulation rather than on the expression/production of new enzymes (Boeckx, Hertog, Geeraerd, & Nicolaï, 2018). According to Lee, Rudell, Davies, & Watkins (2012) the accumulation of compounds derived from methanol (methyl

acetate, methyl 2-methylbutanoate) and ethanol (ethyl acetate, ethyl butanoate, ethyl propanoate) are closely correlated to the flesh breakdown occurrence in 'Empire' apples after 40 weeks of storage, disagreeing with the present study. These same authors also found that the ethanol and ethyl acetate accumulation are high correlated to the acetaldehyde concentration and its accumulation is significantly suppressed by 1-MCP treatment, corroborating the results of the present study.

3.1.4.3. MdADH1 and MdAAT1 expression are not induced by ethylene only

The major part of fruit ripening enzymes are induced by ethylene. One of these enzymes is the AAT, which is significantly induced by the ethylene in apple fruit (Defilippi, Kader, & Dandekar, 2005; Souleyre, Greenwood, Friel, Karunairetnam, & Newcomb, 2005; Yang et al., 2016). The induction of *MdAAT1* in fruit of CA is likely due to the higher ethylene production (Figures 1, 2 and 5), corroborating the results of these early works. Nevertheless, when fruit were stored under DCA – RQ 1.5, the *MdAAT1* appears to be induced by another signal, because fruit stored under DCA – RQ 1.5 had low ethylene biosynthesis and the *MdAAT1* expression is increased, even when the fruit were treated with 1-MCP (Figure 1). Ban, Oyama-Okubo, Honda, Nakayama, & Moriguchi (2010) did also not find a complete inhibition of *MdAAT1* by 1-MCP application, corroborating with our findings. These findings give evidences that the *MdATT1* expression could be induced by another compound or could be a feedback effect of the higher accumulation of precursors (ethanol) in fruit stored in DCA – RQ 1.5 (Figure 4). The increase of *MdAAT1* expression by fruit treated with 1-MCP is in accordance with some early studies (Harb, Lara, Saleh, Streif, & Khraiwesh, 2011; Ortiz, Echeverría, Graell, & Lara, 2010), but all these works were conducted in aerobic conditions. The expression of *MdADH1* was induced by 1-MCP application in all treatments, fact that did not agree with some studies (Harb et al., 2011; Yang et al., 2016). Nevertheless, according to Schaffer et al. (2007), the activity of ADH1 is reduced in the presence of ethylene, fact that can explain the higher *MdADH1* expression in fruit treated with 1-MCP, because even there is ethylene in the storage ambient the fruit would not perceive its presence.

3.1.4.4. Volatile compounds originated from linolenic acid, unsaturated fatty acids and carotenoids

Any damage on the cell membranes will result in free polar lipids in fruit flesh, and these polar lipids could be substrate for the LOX enzyme, resulting in precursors of hexenyl esters (Contreras, Tjellström, & Beaudry, 2016). On the present study appears to be that the higher ethanol production by fruit stored under DCA – RQ 1.5 (Figure 4), resulted in some type of damage on the cell membrane, resulting in higher precursors of hexenyl esters (Figure 4). In the literature, several studies had demonstrated that high ethanol concentration in the pulp, which varies according to the cultivar, harvest maturity, storage time, could increase the membrane permeability due to its dangerous effect on the lipids (Saquet, Streif, & Bangerth, 2000; Thewes, Brackmann, Anese, et al., 2017). The same effect of ethanol could occur in organelles membranes, like microsomal and chloroplast membranes, which are the richest sources of lipids. The membranes of these organelles are rich monogalactosyldiacylglycerol and digalactosyldiacylglycerol, where the predominant fatty acid is the linolenic (Wang & Faust, 1992). This could result in free linolenic acid, and consequently in higher formation of volatile compounds derived from this fatty acid, such as hexenyl esters, explaining its higher accumulation in fruit stored at the lowest oxygen partial pressure (DCA-RQ 1.5).

Other volatile compounds could been originated from pigments present into the chloroplast, like carotenoids. The main compounds originated from the oxidation of carotenoids and/or alfa-farnesene are 6-methyl-5-hepten-2-ol and 6-methyl-5-hepten-1-one (Hui, Niu, Xu, & Guan, 2016; Kobori, Wagner, Padula, & Rodriguez-Amaya, 2014; Mditshwa et al., 2017; Sabban-Amin, Feygenberg, Belausov, & Pesis, 2011). On the present study the concentration of these compounds are increased in fruit stored under DCA – RQ 1.5 (Figure 4), result that disagree with the one of the literature, which showed lower 6-methyl-5-hepten-2-ol and 6-methyl-5-hepten-1-one accumulation by low oxygen storage (Mditshwa et al., 2017; Sabban-Amin et al., 2011). These same authors also found a close relationship between the accumulation of these compounds and the occurrence of superficial scald, physiological disorder that was not observed at the present study. Additionally, the storage under DCA – RQ suppresses the superficial scald occurrence in apples after long-term storage (Bessemans et al., 2016). Thus, the occurrence of higher 6-methyl-5-hepten-2-ol and 6-methyl-5-hepten-1-one in apples stored under DCA – RQ may be related to another synthesis pathway, and not due to alfa-farnesene oxidation, as is proposed in the major part of the literature. We hypothesize that the accumulation of these compound in fruit stored under DCA – RQ 1.5 could be correlated to a damage of the ethanol on the chloroplast, resulting in free carotenoids that could be oxidized to 6-methyl-5-hepten-2-ol and 6-methyl-5-hepten-1-one (Kobori et al., 2014).

3.1.5. Conclusions

The storage under DCA associated with two low oxygen stresses a week or CA + 1-MCP have a suppression effect on the expression of *MdACO1* and consequently blocking the ethylene production and delays fruit ripening. Lower overall metabolism in DCA – RQ stored fruit resulted in higher flesh firmness, lower mealiness and flesh breakdown. DCA – RQ 1.5 resulted in higher decay at 9 months of storage, but the healthy fruit in this storage condition remained far above the ones stored in CA due to lower physiological disorders.

The expression of *MdAAT1* appears to be regulated by ethylene in CA, but not in DCA – RQ 1.5. In this case, the expression should be induced by another signal, perhaps the accumulation of precursors, because even when fruit are treated with 1-MCP the expression of *MdAAT1* is increased by the storage under DCA – RQ 1.5. This explains in part why the oxygen reduction to obtain an RQ 1.5 increased the volatile ester production in 'Galaxy' apples. The lower volatile accumulation by storage under DCA – CF is a result of lower *MdAAT1* expression, and lower precursor's accumulation, like alcohols and acids.

Higher volatile compounds were observed when fruit are stored under CA and DCA – RQ 1.5, without 1-MCP application. 1-MCP application suppressed the volatile compound accumulation in several storage conditions, especially the esters formation. This effect of 1-MCP on volatile compounds suppression is via *MdLOX1* expression, which probably resulted in lower precursor amount. The conversion of aldehydes to alcohols is more correlated to the precursor concentration as to the expression of new *MdADH1* enzyme.

Volatile compounds originated from linolenic acid and/or polar lipids, it means hexenyl esters, are increased by the storage under DCA – RQ1.5. We hypnotized that this happens because the effect of ethanol on the lipids of cell membrane and its organelles, such as microsomal and chloroplast. Fruit stored under DCA – RQ 1.5 also accumulate higher 6-methyl-5-hepten-2-ol and 6-methyl-5-hepten-1-one after 6 and 9 months of storage, especially without 1-MCP.

3.1.6. Acknowledgements

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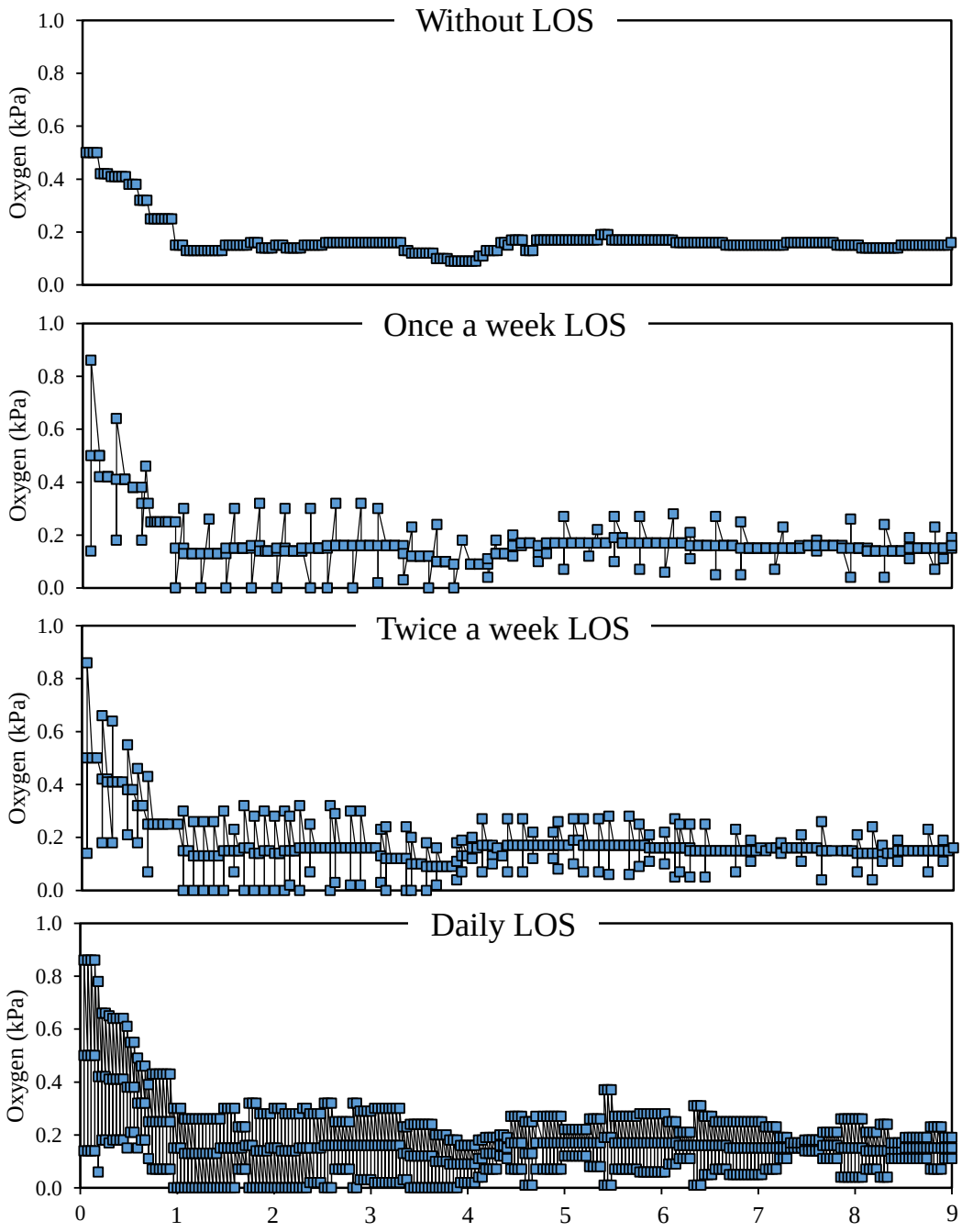
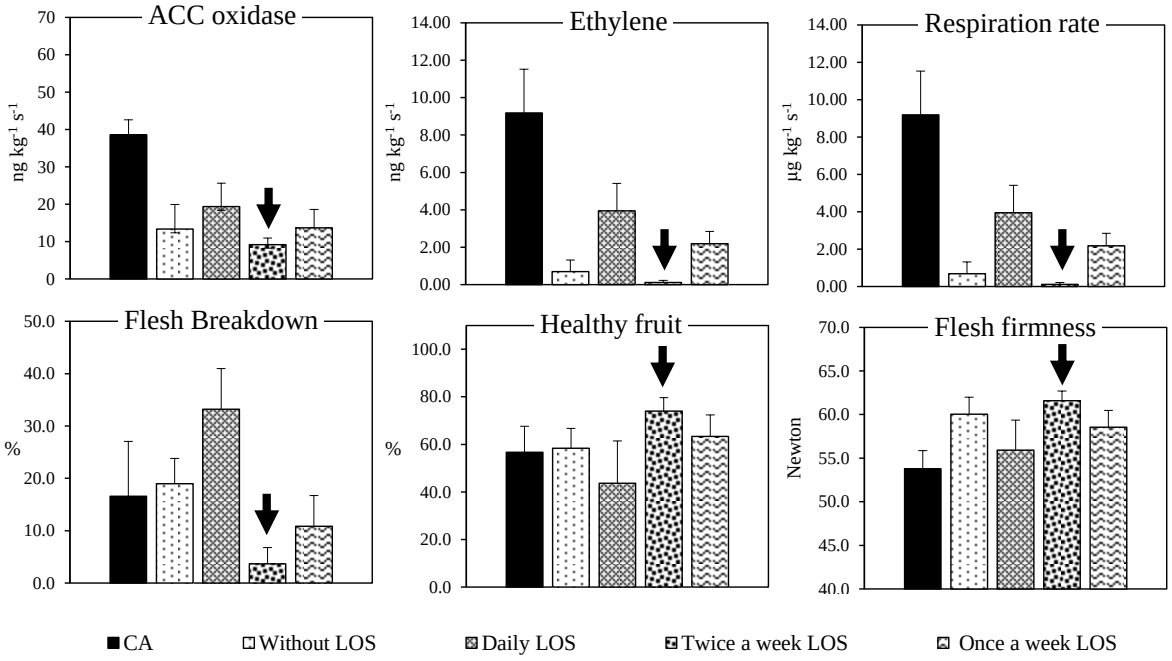
SUPLEMENTARY MATERIAL



Supplementary Figure 1. Oxygen setpoint variation of 'Galaxy' apple stored in dynamic controlled atmosphere based on respiratory quotient 1.5 (DCA – RQ 1.5), without, once, twice a week and daily low oxygen stress (LOS) during 9 months at temperature 2.0 °C.



Supplementary Figure 2. ACC oxidase enzyme activity, ethylene production, respiration rate, flesh breakdown incidence, healthy fruit amount and flesh firmness of 'Galaxy' apples stored in controlled atmosphere (CA – 1.2 kPa O_2 + 2.0 kPa CO_2) and dynamic controlled atmophere monitored by respiratory quotient 1.5 (DCA – RQ 1.5) without, once, twice and daily low oxygen stress (LOS) during 9 months plus 7 days of shelf life at 20 °C. Error bars mean standard deviation. The arrow in each graph show the best LOS frequency for 'Galaxy' apples.

4. ARTIGO 2

4.1. DYNAMICS OF SUGARS, ANAEROBIC METABOLISM ENZYMES AND METABOLITES IN APPLES STORED UNDER DYNAMIC CONTROLLED ATMOSPHERE[2]

Abstract

Apple storage under extremely low oxygen concentrations, as in dynamic controlled atmosphere, changes both aerobic and anaerobic metabolism. In this work we evaluated the effects of controlled atmosphere (CA) and dynamic controlled atmosphere based on respiratory quotient (DCA – RQ), with RQ 1.3 and RQ 1.5, on the dynamics of pyruvic acid, sugars, anaerobic metabolites and enzymes involved in anaerobic metabolism of 'Elstar' and 'Nicoter' apples after harvest, 6 and 9 months of storage plus 7 days of shelf life. We also investigated the induction of sorbitol and glycerol biosynthesis, as a response to low oxygen stress in apples stored under DCA – RQ, protecting the cell membrane from leakage. Storage under CA and DCA – RQ had different dynamics of sugars accumulation from harvest up to 9 months of storage, especially for sorbitol, which accumulated more over the storage period when fruit are stored under DCA - RQ. Glycerol was not detected in any of the cultivars or storage conditions. Storage under DCA reduces the membrane permeability even with the accumulation of anaerobic metabolism compounds, like acetaldehyde and ethanol. Perhaps, this is a result of the higher sorbitol accumulation, which acts as osmolyte. For both cultivars, the storage under DCA resulted in an increase of PDC enzyme activity from harvest to 9 months of storage. The dynamics of anaerobic metabolism compounds were different for bot cultivars: 'Elstar' apples showed an increase from harvest to 9 months storage, but 'Nicoter' had an increase until 6 months of storage and a sharply reduction until 9 months of storage. The regulation of anaerobic metabolism is performed by PDC enzyme activity, with little influence of ADH enzyme activity, when apples are stored under DCA – RQ. 'Nicoter' apples are much more sensitive to low oxygen stress conditions compared to 'Elstar'.

Keywords: *Malus domestica;* 'Elstar'; 'Nicoter'; anaerobic metabolism; sugar metabolism; membrane permeability.

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4.1.1. Introduction

The storage of apples is necessary to provide fruit during all the year. To maintain fruit quality during long-term storage, it is necessary to reduce both ethylene metabolism and the fruit respiration rate (Saquet and Streif, 2002; Steffens et al., 2007). Thus, fruit are stored under low temperatures and controlled atmosphere (CA), where the oxygen partial pressures are lowered to reduce fruit metabolism (Brackmann et al., 2010; Weber et al., 2012; Lumpkin et al., 2014; 2015). Nevertheless, the oxygen partial pressures should not be reduced below the anaerobic compensation point (ACP), the point where the CO_2 release is minimal (Boersig et al., 1988; Saquet and Streif, 2002). If the oxygen partial pressure is reduced below the ACP, anaerobic metabolism will be strongly induced, resulting in a respiratory quotient (RQ) higher than one (Gasser et al., 2008; Weber et al., 2015; Bessemans et al., 2016; Thewes et al., 2017).

Increasingly, there is a trend to store apples under dynamic controlled atmosphere (DCA), where the oxygen partial pressures are reduced to the lowest limit tolerated by the fruit (LOL), and changed continuously over all storage period (Veltmann et al., 2003; Prange et al., 2007; Gasser et al., 2008; Weber et al., 2015). To monitor the LOL in real time during storage, there are three main methods monitoring: anaerobic metabolism products (Veltmann et al., 2003), chlorophyll fluorescence (Prange et al., 2007) and the RQ (Gasser et al., 2008; Brackmann et al., 2015; Weber et al., 2015; Bessemans et al., 2016). DCA storage based on the respiratory quotient (DCA – RQ) allows the induction of anaerobic metabolism at safe levels (Weber et al., 2017), and ethanol production by the fruit (Bessemans et al., 2016; Both et al., 2017; Weber et al., 2017; Thewes et al., 2018). The ethanol produced by the storage under an RQ higher than one, has several effects on fruit metabolism, especially in suppressing ethylene production (Asoda et al., 2009; Liu et al., 2012; Jin et al., 2013; Weber et al., 2016).

Anaerobic metabolism in fruit cells starts at low oxygen partial pressures because of the accumulation of reduced compounds, like nicotinamide adenine dinucleotide (NADH), thus there are lower amounts of available electron acceptors, and ATP synthesis at the substrate level is blocked (Ke et al., 1994; Saquet and Streif, 2008; Jain et al., 2011; 2012; Mbong Victor et al., 2017). Now there is some evidence that the storage under extremely low oxygen, i.e. DCA, can result in lactic and ethanolic anaerobic metabolism being used to oxidize NADH and run glycolysis, generating ATP (Ke et al., 1994; Saquet and Streif, 2008; Taiz and Zeiger, 2013). The study of the dynamics of sugars consumption and anaerobic metabolism compounds accumulation over long-term storage under DCA – RQ could provide important information to understand the metabolism of apples under extremely low oxygen concentrations (<0.4 kPa).

Additionally, it is still unclear if the anaerobic metabolism compounds accumulation are dependent on precursor concentration only or if the enzyme activity also plays fundamental role.

The storage under DCA could also affect the consumption/accumulation of some types of sugars, especially sugar-alcohols, like sorbitol and glycerol. As showed previously for yeast, the growth under salt stress resulted in accumulation of sorbitol and glycerol (Shen et al., 1999; Jain et al., 2011; 2012). These compounds protected the cell membrane of yeast from leakage, allowing growing under salt stress conditions. Our hypothesis is that apples stored under extremely low oxygen conditions, a stress atmosphere, accumulate these compounds in order to survive under those conditions, protecting the cell membranes from damage due to high concentration of anaerobic metabolites, like acetaldehyde and ethanol. This hypothesis is supported by the fact that DCA can result in higher concentration of ethanol and lower levels of membrane permeability as compared to fruit stored under CA (Thewes et al., 2017), resulting in lower physiological disorders, like flesh breakdown (Brackmann et al., 2015; Weber et al., 2015; Both et al., 2018).

In view of the above exposed, this work evaluated the effects of CA and DCA – RQ, with RQ 1.3 and RQ 1.5, on the dynamics of pyruvic acid, sugars, anaerobic metabolites and enzymes involved in anaerobic metabolism of ‘Elstar’ and ‘Nicoter’ apples after harvest, 6 and 9 months of storage plus 7 days of shelf life. We also investigated the induction of sorbitol and glycerol biosynthesis, as a response to low oxygen stress in apples stored under DCA – RQ, protecting the cell membrane from leakage.

4.1.2. Material and menthods

4.1.2.1. Plant material

In 2017, ‘Elstar’ and ‘Nicoter’ apples were harvested at the optimal maturity for long-term storage from commercial orchards in the Constance Lake region of Southwest Germany. At-harvest, three replicates of 8 fruit each, were taken to determine the starch pattern index (SPI, scale: 1 maximal starch to 10 fully hydrolyzed starch) that resulted in values of 2.8 and 3.8 for ‘Elstar’ and ‘Nicoter’, respectively.

Immediately after harvest, fruit were transported to the Competence Centre for Fruit Growing at Lake Constance (KOB), Germany and randomly allocated into replicates of 20 fruit with each treatment having 4 replicates, for each evaluation after storage. Samples were placed

in 250 L experimental gas-tight chambers, with 3 gas-tight chambers used for each cultivar. Each chamber was connected to an automatic CA system (Isollcel®, Bolzano Italy), to continuously control the gas partial pressure setpoints.

4.1.2.2. Storage temperature and relative humidity control

Following harvest, both cultivars were step cooled, on the first storage day, the temperature was reduced to 5°C and maintained for one week, then the temperature was gradually reduced over the following 2 weeks to 1°C for 'Elstar' and 3°C for 'Nicoter'. Over this initial cooling period normal oxygen and carbon dioxide partial pressures (20.9 kPa O_2 + 0.04 kPa CO_2) were maintained. After the fruit reached their respective storage temperature, a container with calcium chloride (7.5 g kg^{-1} of fruit), was added to each chamber to remove excess humidity and help maintain an average RH of 94±2%.

4.1.2.3. Atmosphere conditions

When the fruit reached the required storage temperature, the atmosphere conditions were established. The chambers were first flushed with N_2; and the oxygen partial pressure reduced to 1.2 and 1.6 kPa for 'Elstar' and 'Nicoter', respectively. For the DCA treatments, the O_2 partial pressure was additionally reduced to 0.5 kPa for both cultivars, and then monitoring of the LOL was started by RQ. The CO_2 partial pressures of CA were 2.0 and 1.0 kPa for 'Elstar' and 'Nicoter', respectively. For DCA, the CO_2 partial pressures were 1.2 and 1.0 kPa for 'Elstar' and 'Nicoter', respectively. The CO_2 partial pressures were obtained by fruit respiration and its accumulation inside the chambers.

4.1.2.4. LOL monitoring during storage

Monitoring the LOL was based on the RQ method, according to Weber et al. (2015). In brief, the experimental chambers were closed for 13 to 14h, and the pO_2 and pCO_2 measured before and after this period. The ratio between CO_2 release and O_2 uptake was calculated to give the RQ. Two RQ treatments: 1.3 and 1.5 were tested in this experiment, and the RQ was calculated two times a week. When the RQ was below the setpoint, the pO_2 was decreased, and if the RQ was above the setpoint, the pO_2 was increased. The variation in the oxygen setpoints for both cultivars are shown in Fig. 1.

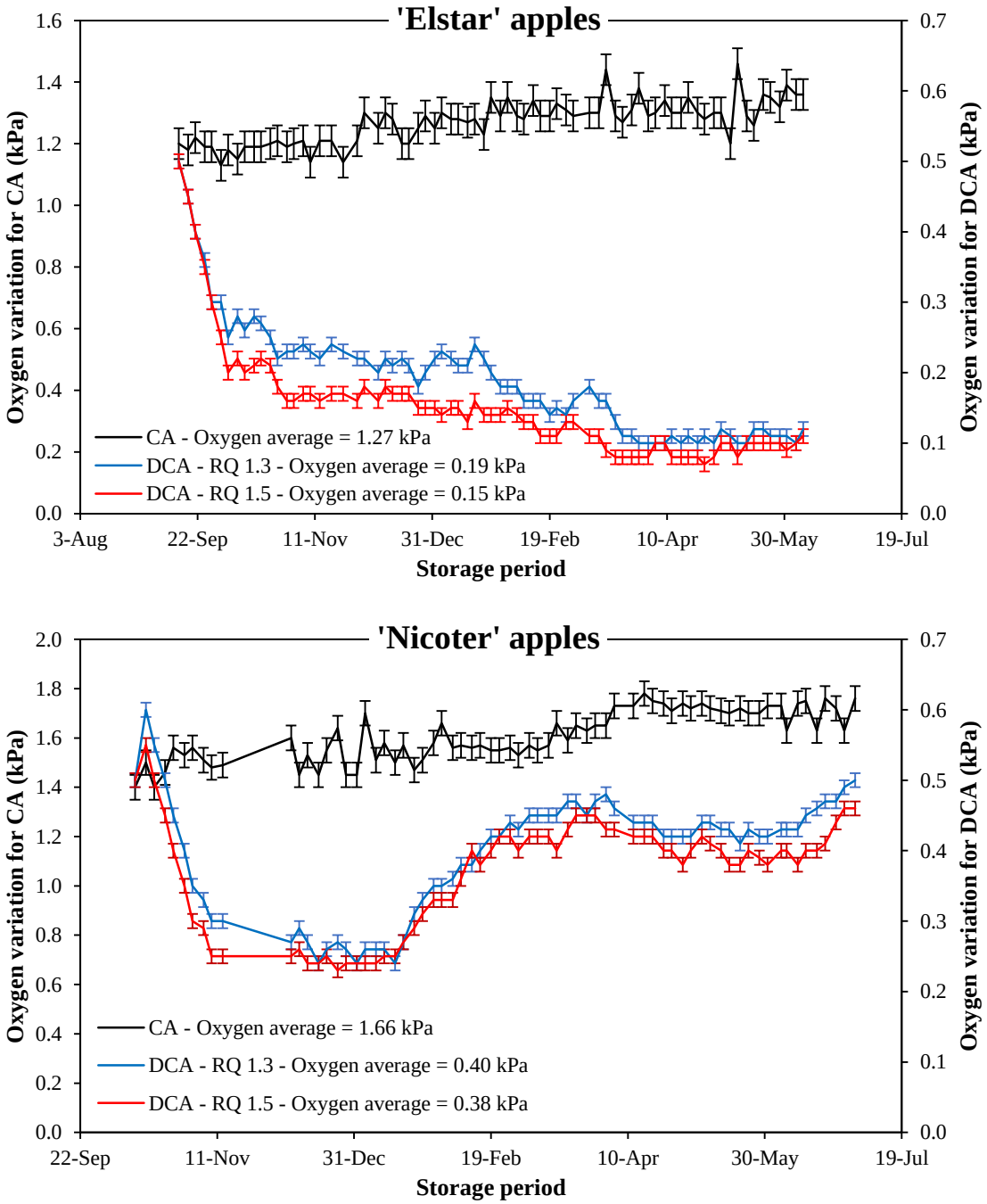

Fig. 1. Oxygen setpoint variation for fruit stored under controlled atmosphere (CA) and dynamic controlled atmosphere based on respiratory quotient (DCA – RQ) over 9 months at 1 °C for 'Elstar' and 3 °C for 'Nicoter.

4.1.2.5. Sample preparation for metabolite evaluation

4.1.2.5.1. Sugars and pyruvic acid

The fruit samples (20 fruit per replicate) were cooled to 0°C (pulp temperature) and the juice extracted under low temperature (<5°C) and 500 µL of juice, separated into lots of 2 mL, and mixed with 1500 µL of cold bi-distillated water. This solution was filtered with a 45-µm nylon filter and the samples were frozen in liquid nitrogen and stored under -30 °C until the sugars and pyruvic acid analyses.

4.1.2.5.2. Samples for anaerobic metabolism products evaluation

From the same juice as prepared for the sugar analyses, 10 mL samples were taken and immediately put into 20 mL vials and frozen to -30 °C until analysis. The juice was processed under low temperature to minimize enzymatic oxidation. For the volatile compound analysis, 3g of NaCl and 10 µL of 3-octanol standard solution (81.8 µg mL^{-1} were added to the juice samples. The vials were immediately, closed with PTFE-coated silicon lid seals and the anaerobic metabolism products evaluated as explained below.

4.1.2.5.3. Sugar and sugar alcohol determinations

The individual sugars / sugar alcohols were evaluated with high performance liquid chromatography (HPLC, Bischoff, Germany), adapted method from Wang et al. (2010). In brief, sugars were analyzed isocratically on the 305 × 7.8 mm HC-75 Ca^{2+} form cation exchange column (Hamilton, USA). Bi-distillated water at constant flow of 0.4 ml min^{-1} was used as the mobile phase. The temperature of the column isothermal was 80°C. A reflectance index detector (Bischoff, Germany) was used to determine the concentration of each sugar/sugar alcohol: sucrose, glucose, fructose, sorbitol and glycerol. The time for each sample run was 32 min. The individual sugars / sugar alcohols were identified and quantified by the comparison of retention times and the area of standards for each compound.

4.1.2.6. Pyruvic acid determination

Pyruvic acid was determined with high performance liquid chromatography (HPLC, Bischoff, Germany), with a method adapted from Wang et al. (2010). In brief, pyruvic acid was determined on a Rezex RDA-organic acid H^+ column (Phenomenex, USA), 7.8 mm × 300 mm. 50 mmol L^{-1} of H_2SO_4 at constant flow of 0.5 ml min^{-1} was used as the mobile phase. The temperature of the column isothermal was 70°C. A UV detector at 210 nm (Bischoff, Germany)

was used to determine the concentration of pyruvic acid. Pyruvic acid was identified and quantified by comparison of the retention time and area of a standard. The time for each sample run was 15 min.

4.1.2.7. Acetaldehyde, ethanol and ethyl acetate determinations

The anaerobic metabolism products were extracted from the juice via solid phase microextraction (HS-SPME). A divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fiber (Supelco, 50/30 μm × 20 mm) was preconditioned following the manufacturer's protocol. The vials containing the samples were submerged in a water bath at 35 °C for 5 min. Afterwards the fiber was exposed to the headspace of each sample for 60 min under constant stirring (400 rpm).

To quantify and identify the anaerobic metabolism compounds, a Shimadzu QP2010 Plus gas chromatography coupled to mass spectrometry (GC/MS; Shimadzu Corporation, Kyoto, Japan) was used. The fiber was thermally desorbed into the injection port at a temperature of 250 °C for 10 min, in a split less mode. The compounds were separated in a polar fused silica Zebron capillary column; ZB-WAX, 30 m × 0.25 mm i.d., 0.25 μm film thickness (Phenomenex, Aschaffenburg, Germany). Helium was used as a carrier gas at a constant flow rate 1.2 mL min^{-1}. The initial column temperature was set at 35 °C and held for 3 min. Then, a temperature gradient of 2 °C min^{-1} was started until 80 °C, followed by a 5 °C min^{-1} increase until 230 °C, and maintained at isothermal conditions for 5 min. The detector was operated in the electron impact ionization mode with an ionization energy of +70 eV and a scan mass range from 35 to 350 m/z, at a temperature of 230 °C. A series of homologous saturated n-alkanes (C7 – C30) was analyzed under the same conditions to calculate a linear retention index (LRI). The concentration of all compounds was determined by internal standardization, according to the method proposed by Both et al. (2014). The analytes were identified based on comparison with standards, mass spectra available in the National Institute of Standards and Technology (NIST) library and by comparing the calculated LRI with those available in the scientific literature.

4.1.2.8. Pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH)

The crude enzymes extracts were obtained in 100 mg of lyophilized powdered tissue taken from the equatorial region of 10 apples. This powdered was homogenized in 500 μl of

extraction solution containing 85 mM 2-(N-morpholino) ethane-sulfonic acid (MES) buffer, pH 7.5, 5 mM dithiothreitol (DTT), 1 % (w/v) polyvinyl polypyrrolidone (PVPP) and 20 µL triton X-100. The homogenate was kept below 4 °C for 30 min and stirred continuously. The samples were then centrifuged at 14,000 g for 30 min at 4 °C. These steps were repeated once, according to the method proposed by Saquet and Streif (2008). To assess alcohol dehydrogenase (ADH), a 150 µL aliquot of the crude extract was added to a reaction mixture consisting of 2.65 mL of a 0.15 mM NADH solution in MES buffer (pH 6.5) and 200 µL of 80 mM acetaldehyde. Pyruvate decarboxylase (PDC) activity was measured in a reaction mixture with 2.25 mL containing 5 mM thiamine pyrophosphate with 50 mM $MgCl_2$ in MES buffer (pH 6.5); 300 µL of 0.85 mM NADH and 180 units mg^{-1} ADH; and 300 µL of 50 mM Na-pyruvate. 150 µl of crude extract was added to this solution. For both PDC and ADH, activity was measured by following the spectrophotometric decrease in absorbance at 340 ηm over 160 s, due to NADH oxidation. Results were expressed as U kg^{-1} s^{-1}.

4.1.2.9. Membrane permeability measured as electrolyte leakage

Fifteen discs of fruit flesh were taken from 15 apples, and put into 25 mL bi-distillated water for 1 h, afterwards an electrical conductivity measurement was performed. The disc suspension was then placed in a water bath for 30 min at 120 °C and subsequently frozen at -30 °C. Thereafter, the samples were thawed (20 °C) and again the electrical conductivity measured and taken as total conductivity. Membrane permeability was expressed as a percentage of electrolyte leakage.

4.1.2.10. Statistical analysis

The experiments were conducted in a completely randomly design with three storage conditions and two cultivars. A variance analysis (ANOVA) at 5% of error probability was carried out ($p < 0.05$). Treatment means that showed a significant difference by ANOVA were subjected to a Tukey test at 5% error probability. Additionally, to show trends in the results, data were submitted to a Principal Component Analysis (PCA) using The Unscrambler® X software (version 9.7, CAMO A/S, Trondheim, Norway), before the PCA data were standardized (mean = 0 and variance = 1 for each variable).

4.1.3. Results and discussion

4.1.3.1. Influence of storage time and cultivar on the oxygen set-point variation

During the 9 months of storage, the oxygen partial pressure for both cultivars was varied according to fruit metabolism (Fig. 1). The average O_2 concentration to obtain a RQ of 1.3 for 'Elstar' was 0.19 kPa and 0.40 kPa for 'Nicoter'. To obtain a RQ of 1.5, the average O_2 concentration was reduced to 0.15 kPa for 'Elstar' and 0.38 kPa for 'Nicoter'. Analyzing the response of the two cultivars to low oxygen, is noteworthy that 'Elstar' tolerated lower oxygen partial pressures to obtain the determined RQ level, when compared to 'Nicoter'. Furthermore, the reduction of oxygen partial pressures, from an RQ of 1.3 to obtain an RQ of 1.5, was different for both cultivars, on average it was 0.04 kPa for 'Elstar' and 0.02 kPa for 'Nicoter' (Fig. 1). These results suggest that the switch between aerobic and anaerobic metabolism is more difficult to control in 'Nicoter', and this cultivar needs more precise control of O_2 concentrations as compared to 'Elstar'.

Observing how the oxygen setpoint changed over the storage time (Fig. 1), for 'Elstar' apples it reduced fast to a certain level (±0.25 kPa), and decreased slowly until the end of storage, while, for 'Nicoter', it reduced during the first storage month, remained almost constant during second month and thereafter tended to increase again until 6 months of storage, remaining constant thereafter. These results show that 'Elstar' has a mechanism to continuously adapt to extremely low oxygen concentrations, while 'Nicoter' did not demonstrate a similar adaptation, and consequently the requirement for oxygen increased again. Weber et al. (2017) observed that 'Fuji Suprema' apples adapt to low oxygen during storage, obtaining the lowest oxygen set point at end of storage period. On the other hand, Thewes et al. (2017) observed that the rate of oxygen lowering influences the adaptation of fruit to low oxygen conditions, the best adaptation to low oxygen was obtained by a slow oxygen reduction (RQ 1.3) in 'Galaxy' apples. The adaptation of fruit to extremely low oxygen over the storage period depends mainly on the expression/synthesis of specific proteins that promote fruit adaptation to the new environment (Loulakakis et al., 2006). Probably, 'Elstar' was able to adapt its metabolism to the new environment and 'Nicoter' apples were not, explaining the increase of the oxygen setpoint in this cultivar.

4.1.3.2. Effect of extremely low oxygen storage on sugar metabolism

The oxygen reduction could affect the sugar metabolism of ‘Elstar’ and ‘Nicoter’ apples, so we evaluated the sucrose, glucose, fructose, glycerol and sorbitol concentrations in both cultivars and storage methods after harvest, 6 and 9 months of storage, to better understand these processes (Fig. 2). Glycerol was not detected in any of the storage conditions or cultivars studied. For ‘Elstar’, DCA storage resulted in lower sucrose content as compared to fruit stored under CA after 6 months (Fig. 2), these differences can be explained by a higher rate of glycolysis. On the other hand, storage condition did not affect the sucrose content in ‘Nicoter’ apples, but its concentration reduced markedly after 9 months of storage (Fig. 2). Lower sucrose content in ‘Elstar’ apples stored under DCA as compared to CA could be a result of higher glycolysis to generate ATP at the substrate level (Taiz and Zeiger, 2013; Mbong Victor et al., 2017).

To be metabolized by glycolysis, sucrose is first broken into glucose and fructose. In the present study, glucose content in both cultivars reduced from harvest until 6 months of storage, without differences between storage conditions after 6 months (Fig. 2). However, after 9 months of storage, ‘Elstar’ fruit stored under DCA had lower glucose content as compared to CA. For ‘Nicoter’ apples after 9 months, the storage under DCA – RQ 1.3 resulted in higher glucose content, DCA – RQ 1.5 had intermediary concentration and fruit under CA the lowest (Fig. 2). Fructose concentration was increased from harvest to 6 months of storage and thereafter decreased until 9 months for both cultivars, with the exception of ‘Nicoter’ apples stored under DCA – RQ 1.3, that had an increase from harvest until 9 months of storage (Fig. 2). For ‘Elstar’ after 6 months, the storage under DCA resulted in a lower fructose (Fig. 2). Nevertheless, for ‘Nicoter’ apples after 6 months, only storage under DCA – RQ 1.5 resulted in lower fructose compared to CA, but DCA – RQ 1.3 did not differ from CA or DCA – RQ 1.5. After 9 months of storage, fructose concentration was not affected by the storage conditions in ‘Elstar’ apples. Nevertheless, for ‘Nicoter’ apples, the storage under DCA – RQ 1.3 resulted in higher accumulation of fructose (Fig. 2). Glucose and fructose enter glycolysis and are converted to pyruvic acid, which was higher in ‘Elstar’ stored under DCA after 6 months, and ‘Nicoter’ apples after 9 months of storage.

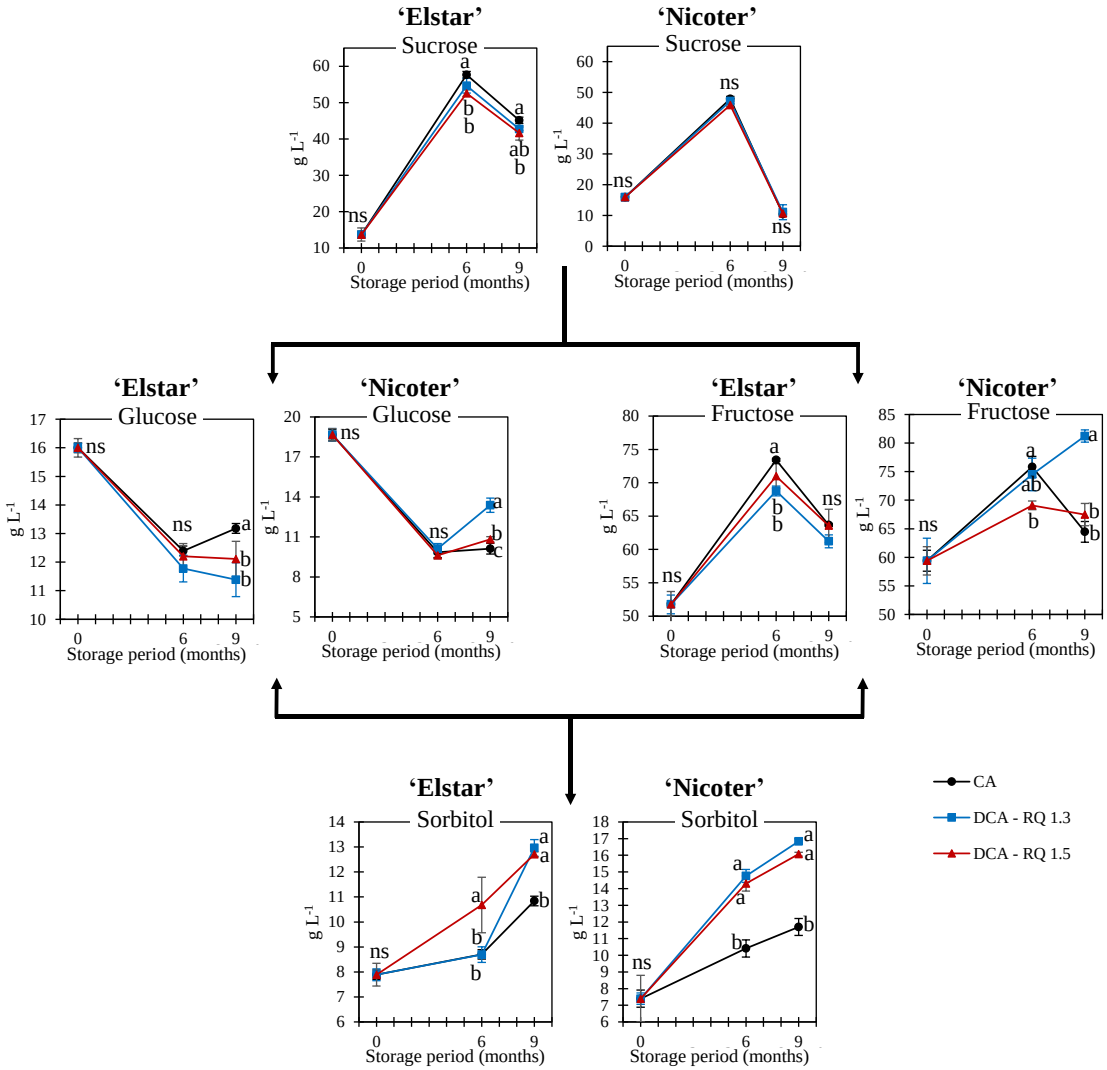

Fig. 2. Sugars metabolism of 'Elstar' and 'Nicoter' apples after harvest, 6 and 9 months of storage under controlled atmosphere (CA) and dynamic controlled atmosphere based on respiratory quotient (DCA - RQ 1.3 and 1.5) at 1 °C for 'Elstar' and 3 °C for 'Nicoter' apples. Bars with the same lower case letter, for the same cultivar, are not significantly different by Tukey's test, at 5 % probability of error. Error bars show the standard deviation.

Fructose is the sugar present in the highest concentration in most apple cultivars (Wang et al., 2010; Aprea et al., 2017). At the present study was observed a higher consumption of glucose from harvest to 6 months of storage (Fig. 2). Nevertheless, from 6 to 9 months of storage glucose concentration remains constant and fructose was consumed in a higher proportion in 'Estar' apples and 'Nicoter' apples stored under CA and DCA – RQ 1.5. These results suggest that there is a dynamic preference for glucose or fructose consumption when apples are stored under CA and DCA, being first consumed glucose, when fructose is accumulated, thereafter fructose is consumed in a higher rate (from 6 to 9 months storage). Variation in sugar

concentration can affect fruit flavor and the perception of sweetness (Zhu et al., 2013; Aprea et al., 2017), so apples stored under DCA can have a different taste as compared to fruit stored under CA. These storage effects should be tested in future sensory studies. Another key change induced by the DCA storage involves sorbitol metabolism. Sorbitol, is generally converted to fructose by sorbitol dehydrogenase and the concentration of sorbitol reduces over the storage period (Zhu et al., 2013; Doerflinger et al., 2015; Farcuh et al., 2018). This is a common response when apples are stored under normal atmospheres, but under extremely low oxygen, the response could be different (as explained further below).

4.1.3.3. Sorbitol accumulated in apples stored under DCA

As observed for both 'Elstar' and 'Nicoter', the increase in sorbitol biosynthesis correlates significantly with low oxygen storage (Fig 5 and 6). Additionally, the increase in sorbitol accumulation occurs at different RQ levels for each cultivar, with 'Nicoter' showing a higher increase as compared to 'Elstar', after 6 months. This further confirms that 'Nicoter' is much more sensitive to low oxygen storage when compared to 'Elstar', and that sorbitol synthesis could be a way to alleviate low oxygen stress. Nevertheless, after 9 months, both cultivars stored under DCA, regardless the RQ level, had higher sorbitol accumulation (Fig. 2). The sorbitol biosynthesis could be an additional way to regenerate NAD+, as previously showed in yeast, where the growth under stress conditions resulted in the accumulation of sorbitol and glycerol, as an additional way to oxidase NADH (Shen et al., 1999; Jain et al., 2011; 2012). These same authors found that in yeast, glycerol accumulated at a higher level as compared to sorbitol, however, this was not observed in our apples, because glycerol was not detected, and sorbitol was increased by oxygen lowering. Future studies should evaluate the possible induction of sorbitol synthesis in apples stored under DCA as an additional way to oxidase NADH.

Sorbitol accumulation could also protect the cell membranes and decrease membrane permeability (Shen et al., 1999; Jain et al., 2011) as well as increase the sweet flavor of apples (Aprea et al., 2017). In yeast cells, the accumulation of sorbitol and glycerol improves survival in salt stress environments (Shen et al., 1999; Jain et al., 2011). Probably, extremely low oxygen storage (Fig. 1) also causes a stress, and sorbitol accumulation could play an important protective role, especially with membrane permeability. To better understand the relationship between sorbitol and membrane permeability, we evaluated membrane permeability by determining electrolyte leakage of tissue discs taken from apples stored under CA and DCA

(Fig. 3). Apples stored under DCA had lower membrane permeability as compared to fruit stored under CA, in agreement with Shen et al. (1999) and Jain et al. (2011). The accumulation of sorbitol could partly explain why the higher ethanol accumulation did not result in higher membrane permeability, because sorbitol was active as an osmolyte, and helps to suppress membrane leakage. In an earlier study Thewes et al. (2017), evaluated the effects of the RQ on electrolyte leakage and found lower membrane permeability in fruit under DCA storage. At the present work we show that this lower electrolyte leakage could be due to sorbitol accumulation and not only a direct effect of low oxygen storage on membrane integrity, because sorbitol and electrolyte leakage are located in opposite sides in PCA analyses (inverse relationship) (Fig. 5 and 6).

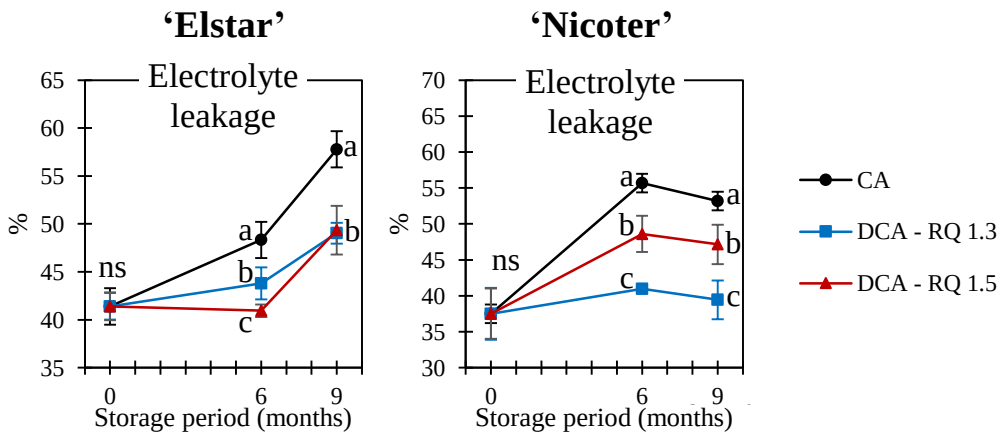

Fig. 3. Membrane permeability measured as electrolyte leakage in 'Elstar' and 'Nicoter' apples after harvest, 6 and 9 months of storage under controlled atmosphere (CA) and dynamic controlled atmosphere based on respiratory quotient (DCA - RQ 1.3 and 1.5) at 1 °C for 'Elstar' and 3 °C for 'Nicoter' apples. Bars with the same lower case letter, for the same cultivar, are not significantly different by Tukey's test, at 5 % probability of error. Error bars show the standard deviation

4.1.3.4. Anaerobic metabolism metabolites accumulation

The storage under extremely low oxygen partial pressures suppresses the electron transport chain, resulting in lower ATP synthesis and the accumulation of NADH (Ke et al., 1994; Ho et al., 2013; Taiz and Zeiger, 2013; Mbong Victor et al., 2017). Additionally, the low oxygen partial pressures (DCA) resulted in higher accumulation of pyruvic acid in 'Elstar' apples after 6 months, and in 'Nicoter' apples after 9 months of storage (Fig. 4). These results show that fruit need to produce ATP at the substrate level, in order to maintain the cell energy

under extremely low oxygen conditions (Fig. 1). A noteworthy fact is that the pyruvic acid concentration reduced in ‘Elstar’ apples after 9 months of storage under DCA – RQ 1.5 (Fig. 4), probably because the lower sucrose and glucose content (Fig. 2).

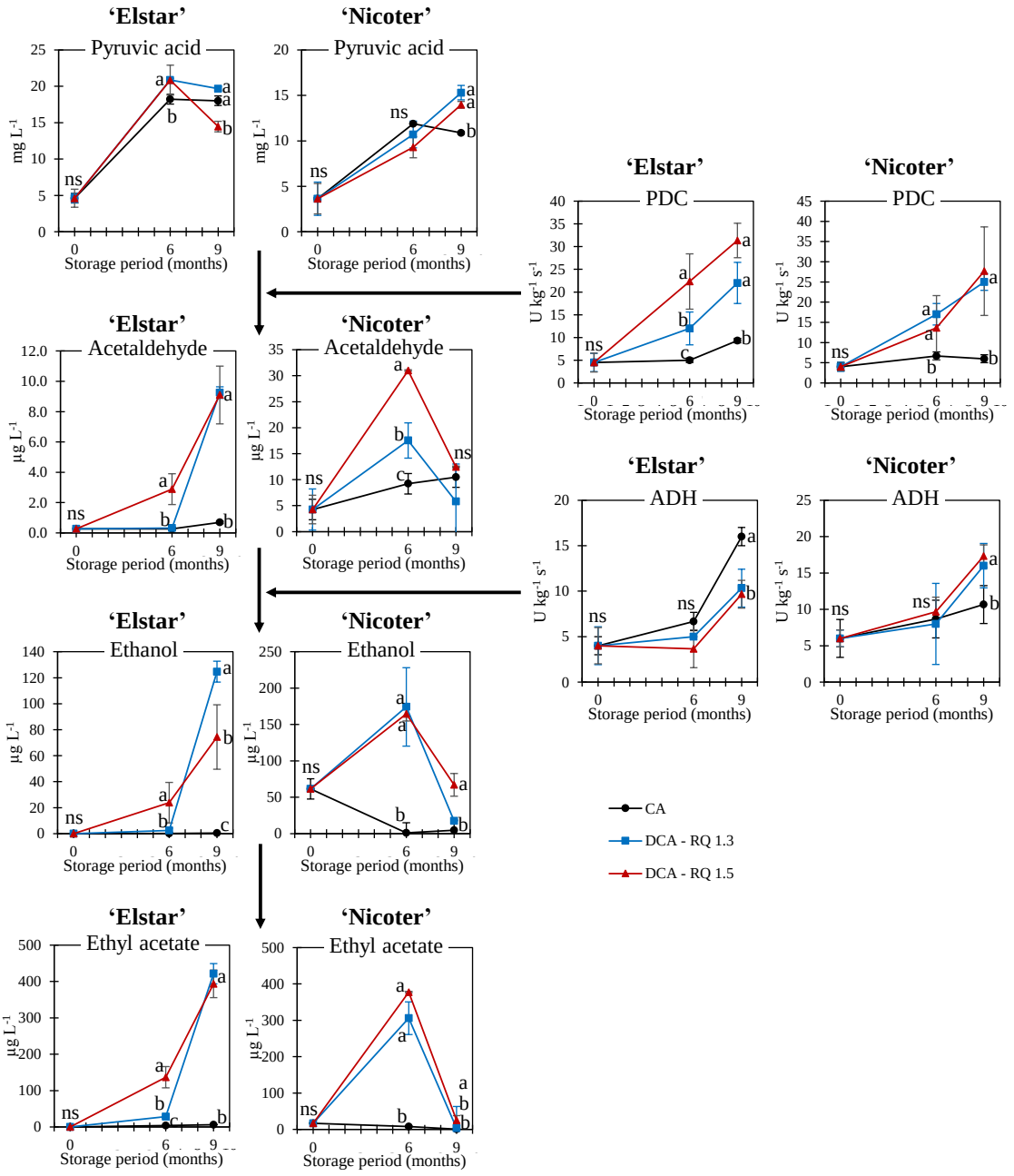

Fig. 4. Anaerobic metabolism of ‘Elstar’ and ‘Nicoter’ apples showing the metabolites concentration and pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) enzyme activity after harvest, 6 and 9 months of storage under controlled atmosphere (CA) and dynamic controlled atmosphere based on respiratory quotient (DCA - RQ 1.3 and 1.5) at 1 °C for ‘Elstar’ and 3 °C for ‘Nicoter’ apples. Bars with the same lower case letter, for the same cultivar, are not significantly different by Tukey’s test, at 5 % probability of error. Error bars show the standard deviation.

For ‘Elstar’ apples, the highest pyruvic acid concentration was observed when fruit were stored under DCA – RQ over 6 months. Probably, the oxygen partial pressure was lowered to a level that did not completely block the electron transport chain, allowing some ATP production by this pathway, while anaerobic metabolism was not yet fully active, as shown by both CA and DCA – RQ 1.3 having the same concentrations of acetaldehyde and ethanol after 6 months (Fig. 4). On the other hand, when the oxygen partial pressure was reduced to obtain an RQ of 1.5 for ‘Elstar’ (Fig. 1), the pyruvic acid concentrations were reduced after 9 months of storage, and the acetaldehyde concentration increased showing that pyruvic acid was being converted to acetaldehyde (Fig. 4).

Analyzing the PDC, acetaldehyde, ADH, ethanol and ethyl acetate data together for both cultivars, it is noteworthy that the production and accumulation of anaerobic metabolites is dependent mainly on PDC enzyme activity and the precursor concentrations (Fig. 4). These finding agree with Thewes et al. (2018), who found that ethyl acetate accumulation under DCA – RQ 1.5 is only dependent on ethanol concentration in ‘Galaxy’, ‘Fuji’ and ‘Pink Lady’ apples and that anaerobic metabolism is regulated at first step (conversion of pyruvic acid to acetaldehyde). Perhaps, the pool of anaerobic metabolism enzymes (ADH) in the apple tissue was enough to detoxify the cells from acetaldehyde, and its activation was mainly based on cofactor regulation (Boeckx et al., 2018), as soon as ADH activity was not affected by the storage condition after 6 months. However, after 9 months, ‘Elstar’ apples under DCA had lower ADH activity and ‘Nicoter’ higher (Fig. 4).

A noteworthy fact is that the same RQ level resulted in different levels of anaerobic metabolism in both cultivars, but PDC and ADH had a similar activity in both cultivars (Fig. 4), with the exception of ADH after 9 months of storage. ‘Nicoter’ showed extremely high accumulation of anaerobic metabolism products after 6 months of storage, and this was only observed for ‘Elstar’ under DCA after 9 months of storage (Fig. 4). Analyzing the oxygen setpoint variation (Fig. 1) and the accumulation of anaerobic metabolism compounds (Fig. 4) together, it is clearly observed that ‘Nicoter’ is much more sensitive to low oxygen storage conditions, and also that the same RQ did not result in the same anaerobic metabolism among

cultivars. Probably, for ‘Nicoter’ apples, anaerobic metabolism starts at lower RQs, even at a RQ of below 1, because acetaldehyde accumulation was higher in ‘Nicoter’ stored under CA (average oxygen 1.66 kPa) when compared to ‘Elstar’ stored under DCA – RQ 1.5 (average oxygen 0.15 kPa) after 6 months of storage (Fig. 4).

4.1.3.5. Multivariate analysis (PCA)

To understand the relationships among and between the storage conditions and the variables determined in this experiment a principal component analysis (PCA) for both cultivars was undertaken (Fig. 5 and 6). For ‘Elstar’ apples, the principal component one (PCI) and two (PCII) explained 87.2 % of the overall variable variation. According to Fig. 5, the storage periods are separated along PCI, and there are much more differences between CA and DCA after 9 months of storage. Sugars, like sucrose and fructose, correlated positively to storage treatments after 6 months (Fig. 5). Nevertheless, the sugar-alcohol, sorbitol, correlated with storage under DCA – RQ, as a trend of sorbitol accumulation as the average oxygen was lowered during storage. These results support the hypothesis that sorbitol biosynthesis could be an additional pathway to regenerate NAD^+ when apples are stored under extremely low oxygen, as under DCA storage. Anaerobic metabolism products also are related to the storage under DCA.

For ‘Nicoter’ apples, the principal component one (PCI) and two (PCII) explained 81.9 % of the overall variable variation. When fruit were stored under CA, there are little differences between 6 and 9 months of storage (Fig. 6), and this storage condition was correlated to electrolyte leakage (membrane permeability). On the other hand, when fruit were stored under DCA, regardless the RQ level, the storage time was separated along PCI (Fig. 6). Fruit stored over 6 months under DCA had higher acetaldehyde, ethanol, ethyl acetate and sucrose content, but after 9 months, fruit under DCA had higher concentration of pyruvic acid, glucose, fructose, sorbitol, PDC and ADH enzyme activity (Fig. 6). It could be observed for both cultivars that sorbitol and electrolyte leakage are located in opposite sides along PCII (Fig. 5 and 6) suggesting that sorbitol accumulation could be protective for cell membrane permeability. Additional, it could be observed that sorbitol is located in the opposite side of CA, showing that its concentration increase under low oxygen concentration.

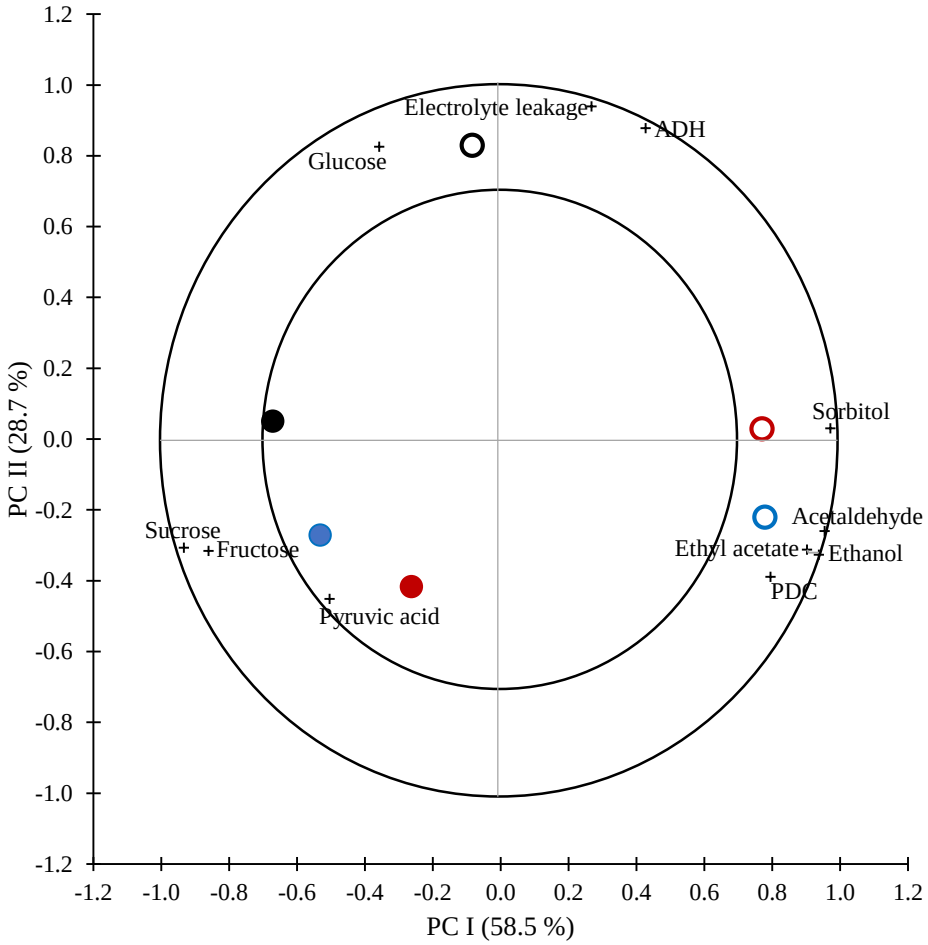

Fig. 5. Principal component analysis (PCA) showing the principal component one (PCI) and two (PCII) for 'Elstar' apples after 6 (filled symbols) and 9 (open symbols) months of storage under controlled atmosphere (CA: ●) and dynamic controlled atmosphere based on respiratory quotient (DCA - RQ 1.3: ● and DCA - RQ 1.5: ●) at 1 °C. Symbols with names are the variables evaluated.

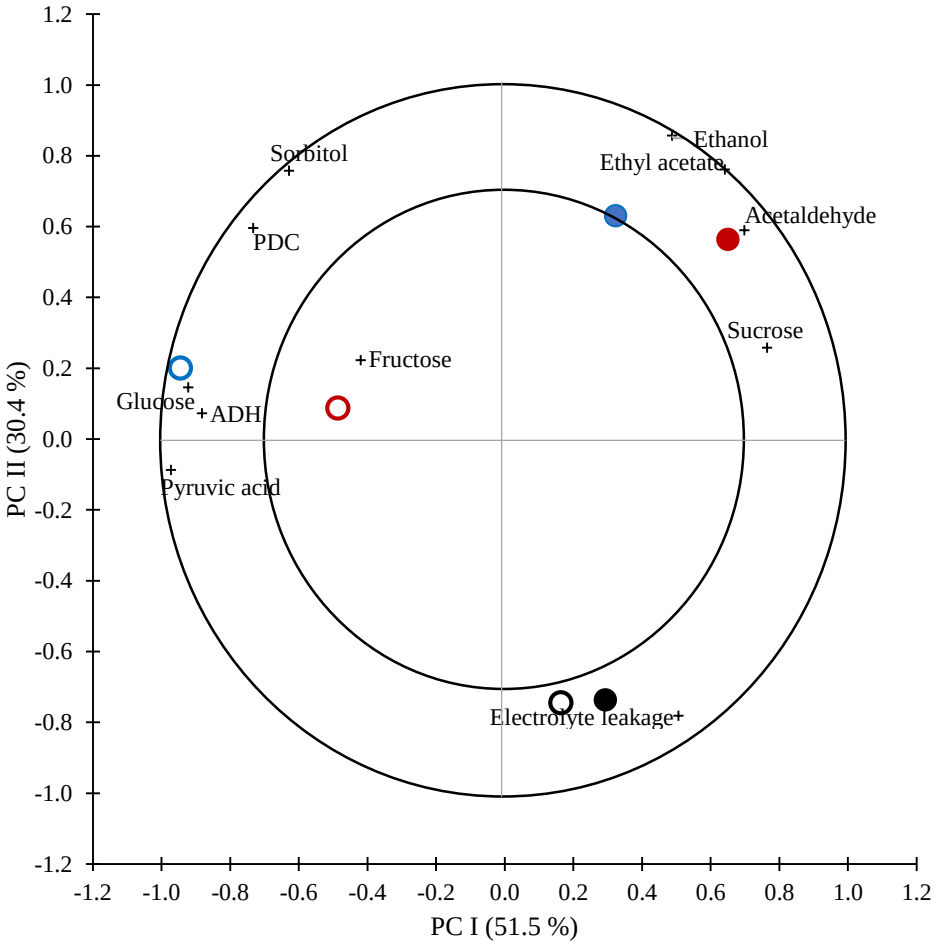

Fig. 6. Principal component analysis (PCA) showing the principal component one (PCI) and two (PCII) for 'Nicoter' apples after 6 (filled symbols) and 9 (open symbols) months of storage under controlled atmosphere (CA: ●) and dynamic controlled atmosphere based on respiratory quotient (DCA - RQ 1.3: ● and DCA - RQ 1.5: ●) at 3 °C. Symbols with names are the variables evaluated.

4.1.4. Conclusions

In our study, we demonstrated that storage under CA and DCA – RQ had different dynamics of sugars accumulation from harvest up to 9 months of storage, especially for sorbitol, which accumulated in a higher rate over the storage period when fruit are stored under DCA - RQ. Sucrose and fructose concentrations have a trend to increase from harvest to 6 months of storage and thereafter decreased until 9 months of storage, but glucose concentration reduced from harvest to 6 months of storage, remaining constant or increasing until end of storage.

Storage under DCA reduces the membrane permeability even with the accumulation of anaerobic metabolism compounds, like acetaldehyde and ethanol. Perhaps, this is a result of the higher sorbitol accumulation, which acts as osmolyte.

For both cultivars, the storage under DCA resulted in an increase of PDC enzyme activity from harvest to 9 months of storage. The dynamics of anaerobic metabolism compounds were different for bot cultivars: 'Elstar' apples showed an increase from harvest to 9 months storage, but 'Nicoter' had an increase from harvest to 6 months of storage and a sharply reduction until end of storage period. The regulation of anaerobic metabolism is performed by PDC enzyme activity, with little influence of ADH enzyme activity, when apples are stored under DCA – RQ.

'Elstar' adapts to extremely low oxygen storage while 'Nicoter' does not. Moreover, the same RQ level does not result in the same anaerobic metabolism for both apple cultivars. Overall, 'Nicoter' apples are much more sensitive to low oxygen stress conditions compared to 'Elstar'.

4.1.5. Acknowledgements

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5. ARTIGO 3

5.1. LOWER OXYGEN LIMIT ESTABLISHMENT FOR DYNAMIC CONTROLLED ATMOSPHERE STORAGE OF APPLES BASED ON CARBON DIOXIDE RELEASE (DCA – CD)[3]

Abstract

The present study aimed at developing a novel method to establish and monitor the lower oxygen limit (LOL) during dynamic controlled atmosphere storage of 'Galaxy', 'Fuji', 'Pink Lady', 'Granny Smith' and 'Golden Delicious' apples. The method was developed to monitor dynamically the oxygen partial pressure (pO_2) based only on the CO_2 release (DCA – CD) by fruit during 9 months storage period. It were carried out 16 trials during three seasons. This wide range of apple cultivars and years allowed calibrating the new DCA method in a large number of climate and fruit maturity conditions. Several respiratory quotient (RQ) were evaluated to obtain the anaerobic metabolism factor (AMF) that corresponds to each RQ in the DCA – CD method. The DCA – CD method had showed good performance for all cultivars, RQ and growth regulators applied on the field. Root mean square error ranged from 0.01 until 0.10 kPa O_2 with an average error of 0.05 kPa O_2. In DCA – CD method, the AMF varied according to the cultivar and RQ, but it remained constant during the years. In addition, the relationship between the RQ and AMF was not linear, therefore it should be calibrated for each cultivar that the DCA – CD method will be used. It was showed that the RQ variation during the storage period were more correlated to the CO_2 release than to the O_2 uptake by fruit, which was the reason of the reliable performance of DCA – CD method in LOL estimation for all cultivars used in the experiments.

Keywords: CO_2 release; *Malus domestica*; new DCA method; oxygen set point; oxygen uptake; respiratory quotient.

5.1.1. Introduction

Apples are grown and consumed worldwide, where several cultivars are produced such as Galaxy, Fuji, Cripps Pink (Pink Lady®), Golden Delicious, Granny Smith, and others. Despite the wide range of cultivars produced, a fraction of this production need to be stored, to

[3] Artigo formatado de acordo com as normas da revista Postharvest Biology and Technology.

offer fruit outside the harvest window (off-season). Thus, the major part of fruit are stored in controlled atmosphere (CA), where the oxygen partial pressure (pO_2) are lowered down to 1.2 to 2.5 kPa and the carbon dioxide partial pressure (pCO_2) are increased from 0.5 to 3.0 kPa, to reduce the fruit metabolism and prolong the storage life (Ke et al., 1991; Brackmann et al., 2009). Nevertheless, the pO_2 remains far above the lower oxygen limit (LOL) tolerated by the fruit during storage (Schouten, 1995), therefore its metabolism is not reduced at the lowest level possible and, consequently, the maximum fruit quality is not maintained. Therefore, there is a trend to store apple under extremely low pO_2 to maintain the highest fruit quality after storage.

To reduce the pO_2 to extremely low levels is necessary to monitor the LOL during the entirely storage period (Schouten, 1995; Prange et al., 2007; Weber et al., 2015). The need of LOL monitoring during storage is necessary because it varies according to the cultivar, the fruit maturity at harvest, growth regulators applied on the orchard, storage temperature, pCO_2 and other factors (Wright et al., 2015). Nowadays, there are three methodologies to monitor the metabolism in real time throughout the storage: 1) based on ethanol production by apple fruit (DCS) (Schouten, 1995; Veltman et al., 2003) and pear (Deuchande et al., 2016); 2) monitoring the chlorophyll fluorescence emission (DCA – CF) (Prange et al., 2007); and 3) monitoring changes in the respiratory quotient (DCA – RQ) (Gasser et al., 2008; Brackmann, 2015; Van Schaik et al., 2015; Weber et al., 2015). Among these technologies to detect the LOL, the DCA – CF is the most widely investigated and used worldwide for apples and pears (Wright et al., 2010; Wright et al., 2012; Aubert et al., 2015; Rizzolo et al., 2014; Eren et al., 2015; Thewes et al., 2015; Tran et al., 2015; Zanella and Stürz, 2015; Mditshwa et al., 2017; Both et al., 2017). Nevertheless, important results on apple fruit quality maintenance were obtained by the LOL monitoring with DCA – RQ (Gasser et al., 2008; Brackmann, 2015; Brackmann et al., 2015; Weber et al., 2015; Van Schaik et al., 2015; Bessemans et al., 2016; Both et al., 2017; Thewes et al., 2017a; Thewes et al., 2017b).

Almost all methodologies cited above need the addition of a device to the storage room for LOL monitoring, which increases the costs of DCA facilities. For DCS, there is necessary an ethanol sensor inside the storage room to detect the LOL by its accumulation inside the storage room (Schouten, 1995; Veltman et al., 2003). Moreover, to monitor the LOL by DCA – CF is necessary to add to the storage room several chlorophyll sensors, which demand costs. The DCA – RQ can be performed directly on the storage room, but the chamber must be tightly closed, because if it has leakage, errors occur in RQ calculation, especially due to the O_2 uptake measurements (Brackmann, 2015; Delele et al., 2015; Bessemans et al., 2016; Bessemans et al., 2018). However, for commercial storage purpose, the LOL monitoring by DCA – RQ is mainly

performed in a small room connected to each storage room, where the RQ is calculated (Brackmann, 2015). Thus, it is extremely important to develop a method to detect the LOL during storage without the need of an additional equipment, i.e., detect the LOL only with the equipment's available at the storage room.

One of the most important sign of fruit metabolism during storage is the CO_2 release rate (Saquet and Streif, 2002; Steffens et al., 2007). The CO_2 release rate decrease as the pO_2 is lowered, until the anaerobic compensation point (ACP) is achieved, where, the CO_2 release increases sharply if the pO_2 are lowered below this point (Boersig et al., 1988; Saquet and Streif, 2002; Saquet and Streif, 2017). Taken in account this response of CO_2 release to the pO_2 lowering, it would be possible to monitor the LOL in real time during storage by the CO_2 evolution in the storage room. The CO_2 release measurement in the storage room can be performed with the same gas analyzer used to control the atmosphere in a regular static CA storage rooms. Furthermore, the quantification of the CO_2 release in commercial rooms have little influence of the tightness of CA room (oxygen influx), as happen in DCA – RQ (Brackmann, 2015; Bessemans et al., 2016; Bessemans et al., 2018).

In this context, the present study aimed at developing a method to monitor the LOL during storage, for control dynamically the pO_2 in apple storage rooms, taking in account only the CO_2 release of 'Galaxy', 'Fuji', 'Pink Lady®', 'Granny Smith' and 'Golden Delicious' apple during 9 months storage period.

5.1.2. Material and methods

5.1.2.1. Plant material

The plant material was composed by several apple cultivars harvested at the commercial harvest time (Table 1). The apples were harvested in the morning and in the same day transported to Postharvest Research Center of the Federal University of Santa Maria, Rio Grande do Sul, Brazil. In the morning of the next day, the fruit were sorted to eliminate those with any type of physical damage due to the transport.

Table 1. Experiments used to monitor the LOL by carbon dioxide (DCA - CD) and compare to the LOL monitoring by respiratory quotient (DCA – RQ).

Year	Location	Cultivar	Harvest date	Growth regulator	Storage temperature	RQ	CO_2
2014	Vacaria-RS[1]	Galaxy	02/15/2014	-	2.0	1.3 and 1.5	1.2
	Vacaria-RS[1]	Fuji	04/15/2014	-	0.5	1.3 and 1.5	0.8
	Vacaria-RS[1]	Pink Lady	04/14/2014	-	2.0	1.3, 1.5 and 1.7	1.0
	Vacaria-RS[1]	Granny Smith	04/15/2014	-	1.5	1.3, 1.5 and 1.7	1.0
2015	Vacaria-RS[1]	Galaxy	02/14/2015	-	2.0	1.3 and 1.5	1.2
	Vacaria-RS[1]	Fuji	04/08/2015	-	0.5	1.3 and 1.5	0.8
	Vacaria-RS[1]	Pink Lady	05/04/2015	-	2.0	1.3 and 1.5	1.0
	Vacaria-RS[1]	Granny Smith	04/08/2015	-	1.5	1.3 and 1.5	1.0
2016	Vacaria-RS[1]	Galaxy	02/13/2016	-	2.0	1.1, 1.3, 1.5 and 2.0	1.2
	Vacaria-RS[1]	Galaxy	02/13/2016	NAA[2]	1.5	1.5	1.2
	Vacaria-RS[1]	Galaxy	02/13/2016	Ethephon[2]	1.5	1.5	1.2
	Vacaria-RS[1]	Galaxy	02/13/2016	NAA + Ethephon[2]	1.5	1.5	1.2
	Vacaria-RS[1]	Galaxy	02/13/2016	AVG[2]	1.5	1.5	1.2
	Bom Jesus-RS	Fuji	03/28/2016	-	0.5	1.3 and 1.5	0.8
	Vacaria-RS[1]	Pink Lady	05/13/2016	-	2.0	1.3 and 1.5	1.0
	São Francisco de Paula-RS	Golden Delicious	03/12/2016	-	2.0	1.3, 1.5 and 1.7	1.2

[1] All these apple cultivars were harvested in Vacaria-RS Brazil, but in several orchards.

[2] AVG: aminoethoxyvinylglycine applied 30 days before harvest, by commercial product called Retain®. Ethephon: Ethrel application 10 days before harvest. NAA: Auxins application 10 days before harvest.

5.1.2.2. Fruit maturity determination at harvest

After sample preparation, 3 samples of each cultivar were used to determine the fruit maturity at harvest. The following parameters were analyzed: a) starch-iodine index (Streif, 1984); b) ethylene production: by using about 1.5 kg of fruit inside a 5-L glass container, which was hermetically closed. After about 1 h, 2 samples of 1 mL of the headspace were taken from the container and injected into a gas chromatograph Star CX 3400 (Varian, Palo Alto, CA, USA), equipped with a flame ionization detector to determine the ethylene concentration in the container headspace. The temperature of the injector, column and detector were: 140, 90 and 200 °C, respectively. Ethylene concentration was quantified by a standard gas injection at the same chromatographic condition described above. c) respiration rate: by CO_2 concentration measured in the same container to ethylene production measurement with a gas analyzer (Isolcell®, Italy). d) flesh firmness: by the insertion of 11 mm tip in two opposite sides of fruit, where previously the skin was removed. Results expressed in N. e) titratable acidity: by the titration of 10 mL juice diluted in 100 mL of distillated water with a 0.1 N NaOH solution until pH 8.1. f) total soluble solids: determined by refractometry of juice from 10 apples.

5.1.2.3. Sample preparation and storage conditions

Apples of each cultivar and each experiment were randomly sampled to perform at least 4 replications, each with 25 fruit. Thereafter, about 60 kg of fruit were put into each experimental CA rooms of 230-L to obtain mass/volume ratio of 260 kg m^3. This mass/volume ratio was used because is a mean of the one that is used in commercial storage rooms in Brazil.

After the conditioning of samples into the CA rooms, the temperature was reduced down to 5 °C, and held for one day. From this day, the temperature was gradually reduced to the storage temperature (Table 1), in 5 d. At the day that the temperature reached the set point, the CA rooms were closed and the DCA conditions established. For this procedure, the CA rooms were flushed with nitrogen to reduce the pO_2 to 5 kPa for all experiments. From this day onwards, the pO_2 were also gradually reduced down to 0.5 kPa, in further 5 d by fruit respiration. At the moment that the pO_2 reached 0.5 kPa, the LOL monitoring begun, two times a week, by the DCA – RQ method and by the novel DCA – CD method, as will be describe below. The pCO_2 inside CA rooms was obtained by fruit respiration. During the 9 months of storage the pO_2 and pCO_2 were determined and adjusted at least 4 times every day with an automatic DCA control system (Valis®, Lajeado, RS, Brazil). Thereby, the automatic DCA control system compared the pO_2 and pCO_2 of the chamber to a set point. If the pO_2 was lower than the set point, cold air was supplied to the desired concentration and the excess of pCO_2 was absorbed with a lime scrubber. The temperature was monitored with a precision thermometer inserted in fruit flesh. The relative humidity was maintained at 94 ± 2% by the addition of calcium chloride inside experimental CA rooms.

5.1.2.4. Treatment with growth regulators

The growth regulators were applied in the field before harvest in 4 experiments at the year 2016 (Table 1). The treatments performed were: [1] AVG application (0.83 kg ha^{-1} of ReTain® (ValentBioScience, USA) 15% of active ingredient) 30 d before harvest; [2] Ethephon (0.67 L ha^{-1} of Ethrel® (Bayer Crop Science, Germany) 24% of active ingredient) applied 10 d before harvest; [3] Naphthaleneacetic acid (NAA) (40 g ha^{-1} NAA (AMVAC Chemical Corporation, USA) applied 10 d before harvest; [4] NAA + Ethephon applied 10 d before harvest. An output of 1000 L ha^{-1} of water was sprayed to the plants of the treatments in the field, according to Steffens et al. (2006).

5.1.2.5. Monitoring the LOL by DCA – RQ

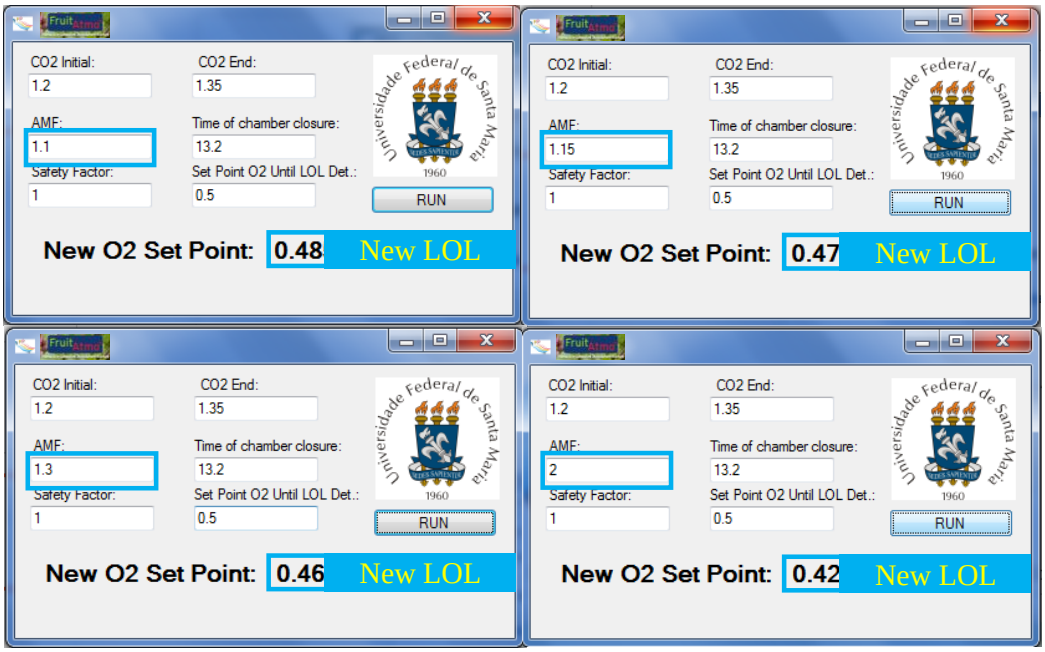
The monitoring of LOL by this method was carried out according to Brackmann (2015). The respiratory quotient (RQ) is the ratio between the CO_2 release and the O_2 uptake as showed by the following equation:

$$DCA - RQ = \frac{CO_2\ final - CO_2\ initial}{O_2\ initial - O_2\ final} \quad (1)$$

The CO_2 release and the O_2 uptake were measured after 14 h CA room closed. The pO_2 was changed to maintain the RQs showed in the table 1.

5.1.2.6. Monitoring the LOL by the CO_2 release (DCA – CD - FruitAtmo®)

To monitor the optimal oxygen partial pressure based only on CO_2 production, the software FruitAtmo® was used (Supplementary Figure 1), according to the applied patent described by Thewes et al. (2019). In brief, the CO_2 production of fruit decrease as the pO_2 is lowered, until the pO_2 reaches the anaerobic compensation point (ACP). When the pO_2 is lowered below the ACP, the CO_2 production of fruit increase drastically (Boersig et al., 1988). Therefore, it is possible to monitor the LOL during storage only by CO_2 production of fruit, and control dynamically the pO_2 throughout storage.



Supplementary Figure 1. Windows of the software (version 1.0) used to stimate LOL by CO_2 release. In blue are showed the anaerobic metabolism factors (AMF) which alow the induction of anaerobic metabolism at safe levels.

5.1.2.7. Evaluation of DCA – CD method

To evaluate the DCA – CD reliability, RMSE, BIAS and agreement index (dw) statistics were used. The RMSE statistic was calculated in several AMF, to obtain the one that resulted in the lowest DCA – CD error as compared to DCA-RQ. If the correct AMF was obtained, the RMSE for the total series and for extremely low pO_2 (bellow 0.2 or 0.3 kPa) were calculated by the following equation:

$$RMSE\ in\ kPa = \sqrt{\frac{\sum(Si-Oi)^2}{n}} \quad (2)$$

where Si is the LOL estimated by the DCA-CD, Oi is the LOL by the DCA – RQ, and n is the number of pairs estimated by DCA – CD and DCA – RQ.

To evaluate if the DCA – CD over or under estimate the LOL was used the BIAS index (Wallach, 2006):

$$BIAS\ in\ kPa = \frac{(\sum Si-\sum Oi)}{\sum Oi} \quad (3)$$

The agreement index (dw) was calculated according to Willmott (1981):

$$dw = 1 - \frac{\sum(Si-Oi)^2}{[\sum(|Si-\ddot{O}i|)+(|Oi-\ddot{O}i|)]^2} \quad (4)$$

where Si is the LOL estimated by the DCA – CD, Oi is the LOL by the DCA – RQ, Öi is the mean of LOL values by DCA – RQ.

5.1.3. Results

5.1.3.1. Metabolism and quality at harvest

Immediately after harvest, there was undertaken an analysis to gain insight about fruit maturity and variability between years, cultivars and growth regulators (Table 2). According to this analysis, the experiments performed given a wide range of variability to validate the DCA – CD method. The starch pattern index ranged from 5.2, most unripe apples, until 9.4, for overripe apples (Table 2). The ethylene production, respiration rate, acidity and soluble solids (Table 2) also confirmed the high variability of fruit maturity at harvest. This wide range of fruit variability is extremely important to test the robustness of DCA – CD method in LOL estimation, and its resilience between years, cultivars and growth regulators applied in filed.

Table 2. Metabolism and quality of apples at harvest plus one day of shelf life at 20°C.

Year	Cultivar	Growth regulator	SPI* (1-10)	Ethylene (uL kg^{-1} h^{-1})	Respiration (mL kg^{-1} h^{-1})	Firmness (Newton)	TA (meq 100mL)	TSS °Brix
2014	Galaxy	-	6.5	1.72	14.4	76.9	5.13	13.4
	Fuji	-	8.8	0.13	5.02	71.2	5.15	13.2
	Pink Lady	-	5.9	0.05	5.27	93.2	10.3	13.2
	Granny Smith	-	nd*	0.02	2.57	75.7	10.3	12.4
2015	Galaxy	-	9.4	3.33	9.97	67.1	4.23	11.8
	Fuji	-	7.8	3.99	6.36	84.4	7.94	14.1
	Pink Lady	-	8.1	0.45	4.57	72.4	10.3	14.9
	Granny Smith	-	6.7	0.03	7.21	68.4	9.67	12.0
2016	Galaxy	-	7.8	1.74	11.7	68.9	4.76	11.6
	Galaxy	NAA[1]	nd	1.46	11.4	57.1	3.87	11.0
	Galaxy	Ethephon[1]	nd	1.98	11.7	56.4	4.19	11.6
	Galaxy	NAA + Ethephon[1]	nd	3.59	12.1	51.4	3.99	11.6
	Galaxy	AVG[1]	nd	0.46	9.74	67.1	3.95	11.1
	Fuji	-	5.8	nd	nd	74.1	5.09	14.1
	Pink Lady	-	6.9	9.61	50.1	77.3	6.24	12.8
	Golden Delicious	-	5.2	0.22	10.4	72.6	7.13	13.5

[1]AVG: aminoethoxyvinylglycine applied 30 days before harvest, by commercial product called Retain®. Ethephon: Ethrel application 10 days before harvest. NAA: Auxins application 10 days before harvest. * SPI: Starch pattern index; nd: variable not determined.

5.1.3.2. Monitoring the LOL by DCA – CD in 'Galaxy' apple

The bi-plot of LOL monitoring by DCA – CD *versus* DCA – RQ for 'Galaxy' apple during three years (2014, 2015 and 2016) are showed in the figures 1 and 2. For the years 2014 and 2015 were evaluated two RQ (1.3 and 1.5), but in the year 2016 four RQ were studied (1.1, 1.3, 1.5 and 2.0) to calibrate the AMF of DCA – CD method for several RQ.

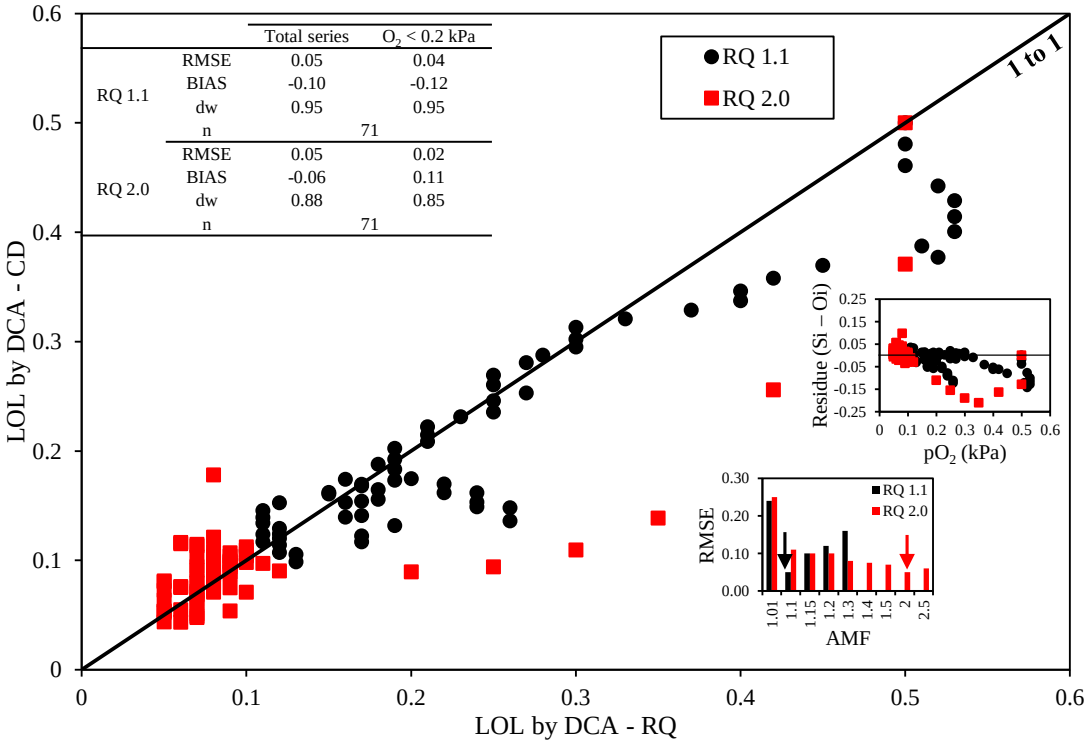


Figure 1. The estimated lower oxygen limit in kPa (LOL) by the new dynamic controlled atmosphere method based on carbon dioxide (DCA – CD) versus the LOL by respiratory quotient (DCA – RQ 1.1 and 2.0 - 2016) for 'Galaxy' apple during 9 months of storage at 2°C, relative humidity 94%. The solid line is the 1 to 1 line. Inserts are the residues, and root mean square error (RMSE) in several anaerobic metabolism factors (AMF) used to estimate the LOL by the DCA – CD method. RMSE: root mean square error, BIAS: BIAS index, dw: index of agreement, n: number of LOL determinations during the 9 months. The arrows in the inserts represent the AMF that proportionated the lowest RMSE.

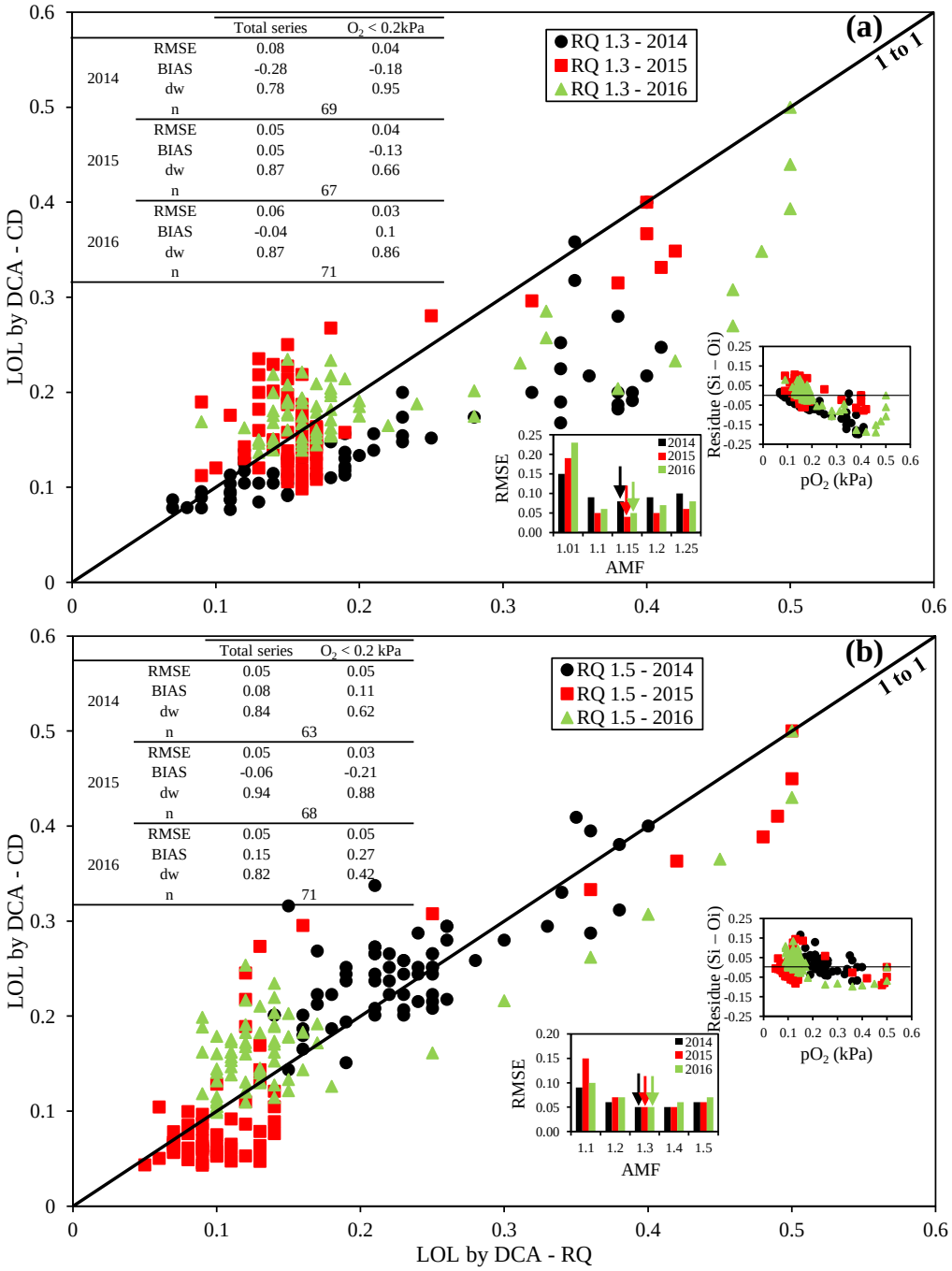


Figure 2. The estimated lower oxygen limit in kPa (LOL) by the new dynamic controlled atmosphere method based on carbon dioxide (DCA - CD) versus the LOL by respiratory quotient (DCA - RQ 1.3 and 1.5) for 'Galaxy' apple during 9 months of storage at 2°C, relative humidity 94%. The solid line is the 1 to1 line. Inserts are the residues, and root mean square error (RMSE) in several anaerobic metabolism factors (AMF) used to estimate the LOL by the DCA - CD method. RMSE: root mean square error, BIAS: BIAS index, dw: index of agreement, n: number of LOL determinations during the 9 months. The arrows in the inserts represent the AMF that proportionated the lowest RMSE.

The AMF that resulted in the lowest RMSE by the DCA – CD method for each RQ are showed in each graphic with an arrow (Figure 1 and 2). The AMF to obtain a determined RQ are constant during the 3 years evaluated, which showed that the same AMF gives similar results throughout several growth conditions and maturity of fruit. For 'Galaxy' apples, to obtain an RQ of 1.1 should be used an AMF of 1.1, for RQ 1.3 an AMF of 1.15, for RQ 1.5 an AMF of 1.3, and for RQ 2.0 an AMF of 2.0 (Figure 1 and 2). These results showed that there is not a linear relationship between the RQ level and the AMF that should be employed in DCA – CD (Figure 9).

Evaluating the RMSE of total series and below 0.2 kPa O_2, is noteworthy a higher error of DCA – CD for the total series as compared to extremely low pO_2 (Figure 1 and 2). This showed that the DCA – CD had better performance as pO_2 are lower. In practical terms, this DCA – CD performance is very important, because the risk of anaerobic metabolism is higher by lowest pO_2, so the LOL should be accurately measured below 0.2 kPa. For example, at the most extremely O_2 condition (RQ 2.0), if the pO_2 is below 0.2 kPa, the BIAS index showed an over estimation (positive value) by the DCA – CD method (Figure 1), i.e., the DCA – CD overestimate the LOL.

5.1.3.3. Monitoring the LOL by DCA – CD in 'Galaxy' apple treated with growth regulators

Fruit treated with preharvest NAA, ethephon, NAA + ethephon and AVG were stored in DCA and the LOL monitored by DCA – CD and DCA – RQ (Figure 3). Regardless the growth regulator used in field, the AMF used in DCA – CD was 1.3 to obtain an RQ of 1.5. A noticeable fact is that the AMF for 'Galaxy' apple without growth regulators (Figure 2b) was the same as 'Galaxy' apple with its application (Figure 3). The RMSE, regardless the growth regulator, ranged from 0.02 up to 0.06 kPa, which is a very low error. Contrarily to 'Galaxy' apple without growth regulators, the RMSE for total series was similar to extremely low pO_2 (<0.2 kPa), which showed that the DCA – CD method had a readable performance at high and extremely low pO_2 when the apples were treated with growth regulators at preharvest.

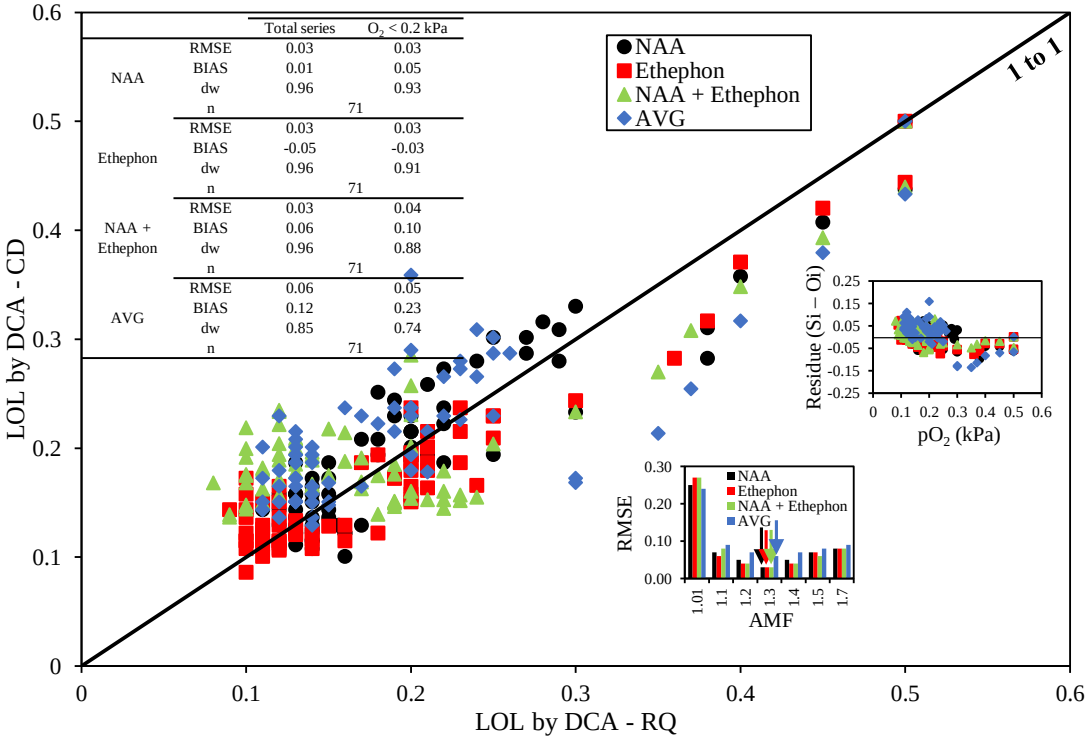


Figure 3. The estimated lower oxygen limit in kPa (LOL) by the new dynamic controlled atmosphere method based on carbon dioxide (DCA - CD) versus the LOL by respiratory quotient (DCA – RQ 1.5 - 2016) for 'Galaxy' apple treated with naphthalene acetic acid (NAA), ethephon, NAA + ethephon (C) and aminoethoxyvinylglycine (AVG) in preharvest and stored during 9 months at 1.5 °C, relative humidity 94%. The solid line is the 1 to 1 line. Inserts are the residues, and root mean square error (RMSE) in several anaerobic metabolism factors (AMF) used to estimate the LOL by the DCA - CD method. RMSE: root mean square error, BIAS: BIAS index, dw: index of agreement, n: number of LOL determinations during the 9 months. The arrows in the inserts represent the AMF that proportionated the lowest RMSE.

5.1.3.4. Monitoring the LOL by DCA – CD in 'Fuji' apple

During the three years, were evaluated two RQs, 1.3 and 1.5, and the AMF that corresponded to these RQs in the DCA – CD method were 1.05 and 1.3, respectively (Figure 4a and b). The RMSE ranged from 0.01 up to 0.09, showing that the error of the novel method to monitor the LOL is very low. According to the RMSE, BIAS and dw index, the DCA – CD had a better performance under the lowest RQ tested for 'Fuji' apple (Figure 4a). Similarly to the other cultivars, the DCA – CD method had a better performance under extremely low pO_2,

which is a very important result because at extremely low oxygen the LOL must be accurately measured due to the high risk of anaerobic metabolism and fruit damage.

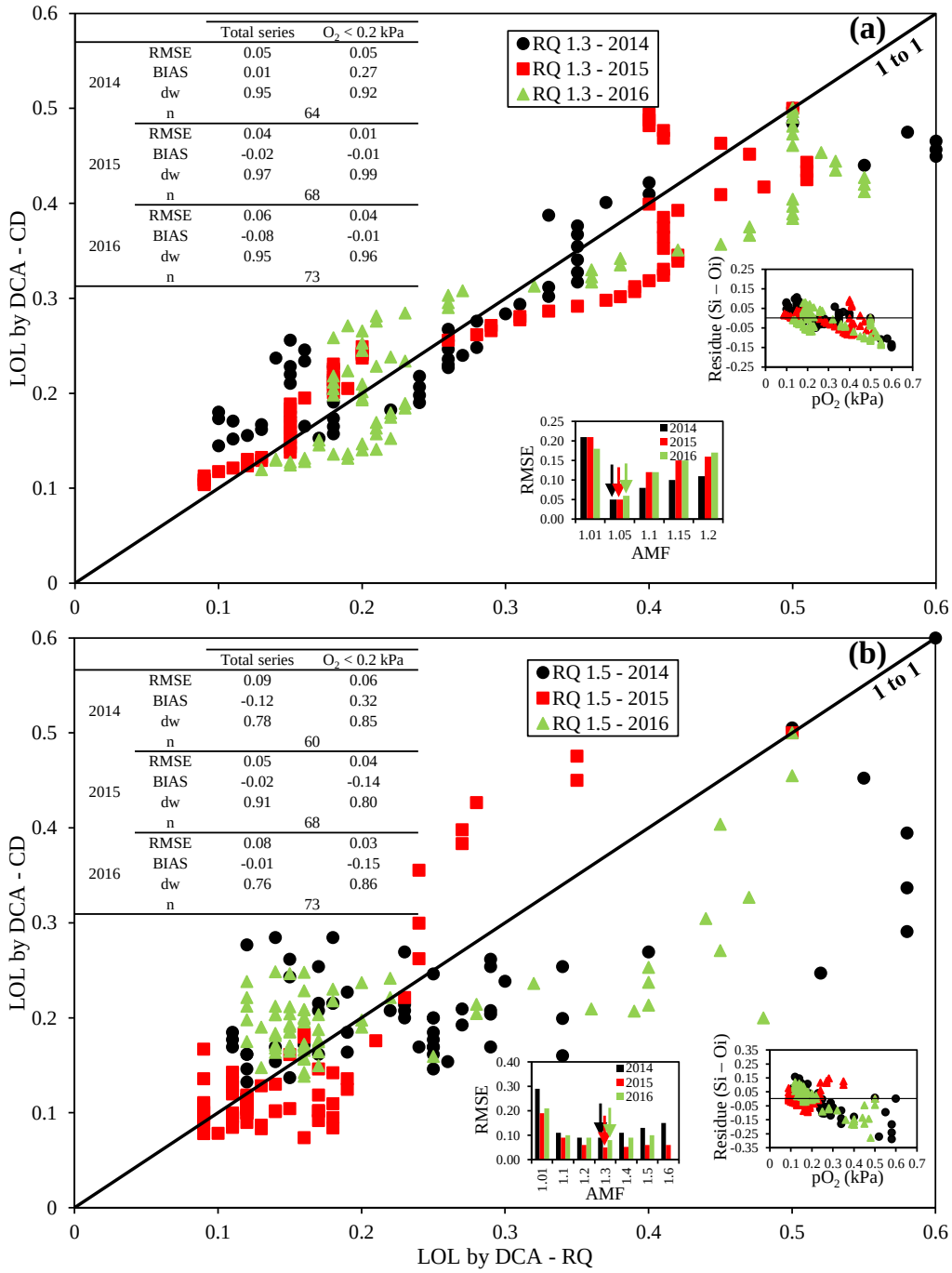


Figure 4. The estimated lower oxygen limit in kPa (LOL) by the new dynamic controlled atmosphere method based on carbon dioxide (DCA - CD) versus the LOL by respiratory quotient (DCA – RQ 1.3 and 1.5) for 'Fuji' apple during 9 months of storage at 0.5°C, relative humidity 94%. The solid line is the 1 to 1 line. Inserts are the residues, and root mean square error (RMSE) in several anaerobic metabolism factors (AMF) used to estimate the LOL by the DCA - CD method. RMSE: root mean square error, BIAS: BIAS index, dw:

index of agreement, n: number of LOL determinations during the 9 months. The arrows in the inserts represent the AMF that proportionated the lowest RMSE.

5.1.3.5. Monitoring the LOL by DCA – CD in 'Pink Lady®' apple

The bi-plots showing the LOL monitored by DCA – CD *versus* DCA – RQ of 'Pink Lady®' apple are showed in Figure 5. In 2014, the pO_2 ranged from 0.14 to 0.40 kPa, in 2015 from 0.09 to 0.60 kPa, and in 2016 from 0.13 until 0.58 kPa, to obtain RQs between 1.3 and 1.7 in the DCA – RQ method (Figure 5a and b). This high variability of LOL between the years are very important to test the DCA – CD reliability to estimate the LOL in apples of different growing seasons.

In the three years evaluated, the AMF to obtain a determined RQ was constant (Figure 5). To obtain a RQ of 1.3 the AMF employed should be 1.1, to obtain a RQ of 1.5 the AMF should be 1.2, and for RQ 1.7 the AMF should be 1.5, as showed by the arrows in the inserted graphs. The RMSE ranged from 0.02 to 0.06, and it was higher in the highest pO_2, it means that the DCA – CD method again estimated the LOL more accurately at extremely low pO_2. According to the dw index, the lowest agreement of the data with the 1:1 line was observed in the year of 2014, but in the other two years the agreement of the data was very high, ranging from 0.85 until 0.99.

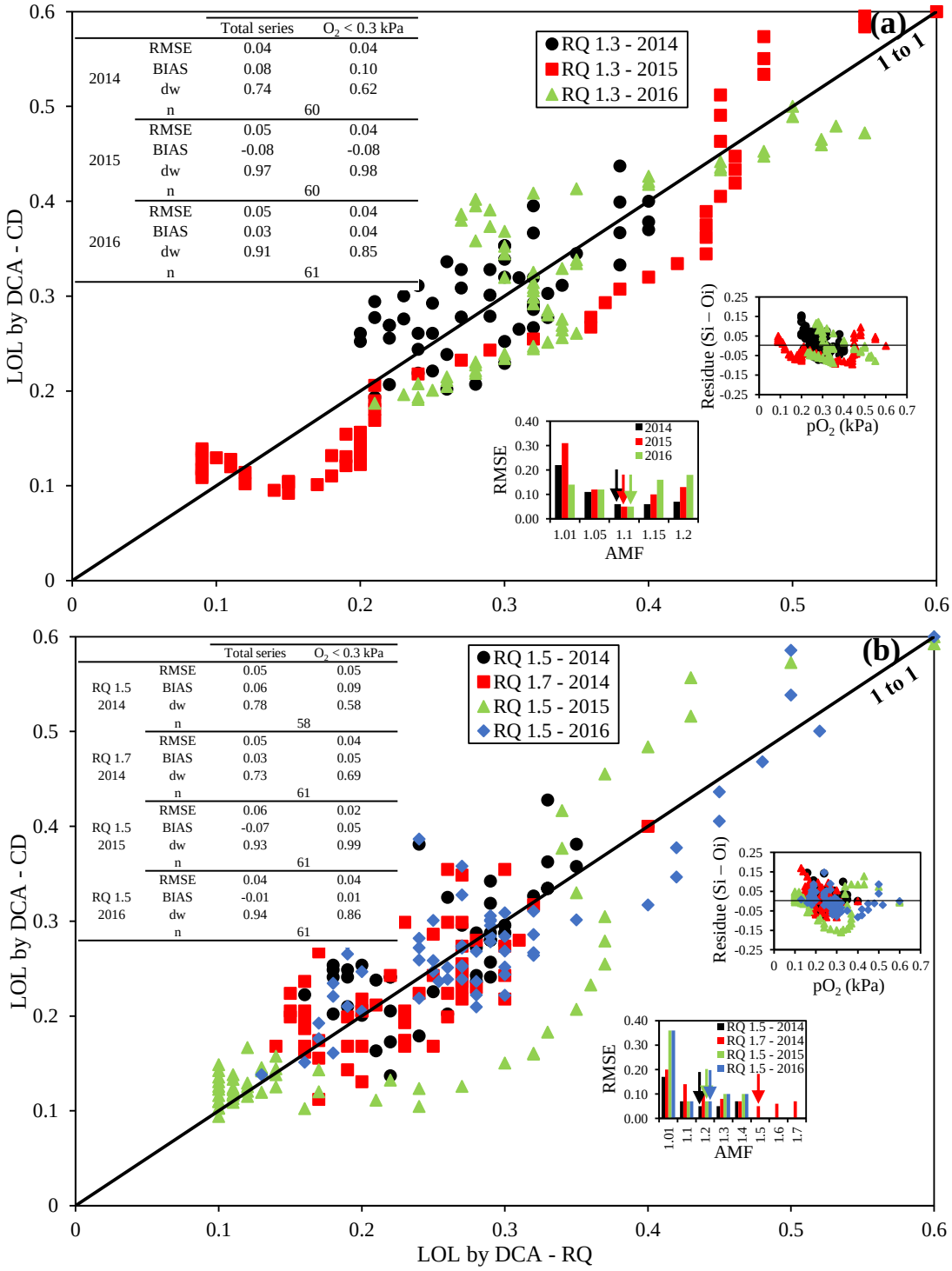


Figure 5. The estimated lower oxygen limit in kPa (LOL) by the new dynamic controlled atmosphere method based on carbon dioxide (DCA - CD) versus the LOL by respiratory quotient (DCA – RQ1.3, 1.5 and 1.7) for 'Pink Lady®' apple during 9 months of storage at 2.0 °C, relative humidity 94%. The solid line is the 1 to 1 line. Inserts are the residues, and root mean square error (RMSE) in several anaerobic metabolism factors (AMF) used to estimate the LOL by the DCA - CD method. RMSE: root mean square error, BIAS: BIAS

index, dw: index of agreement, n: number of LOL determinations during the 9 months. The arrows in the inserts represent the AMF that proportionated the lowest RMSE.

5.1.3.6. Monitoring the LOL by DCA – CD in 'Granny Smith' apple

The LOL estimated by the DCA – CD versus the one obtained by DCA – RQ for 'Granny Smith' apple are shown in figure 6. The RQs evaluated were 1.3, 1.5 and 1.7 in 2014 and 1.3 and 1.5 in 2015. To obtain these RQs, the AMF employed were 1.03, 1.1 and 1.3, respectively (Figure 6a and b). The RMSE during the two years evaluated ranged from 0.03 until 0.07 kPa, showing that, regardless the RQ, the DCA – CD had a good performance with an extremely low error. The highest over or underestimation of LOL by DCA – CD, according to the BIAS index, was observed for RQ 1.5 at the year 2015 (Figure 6b).

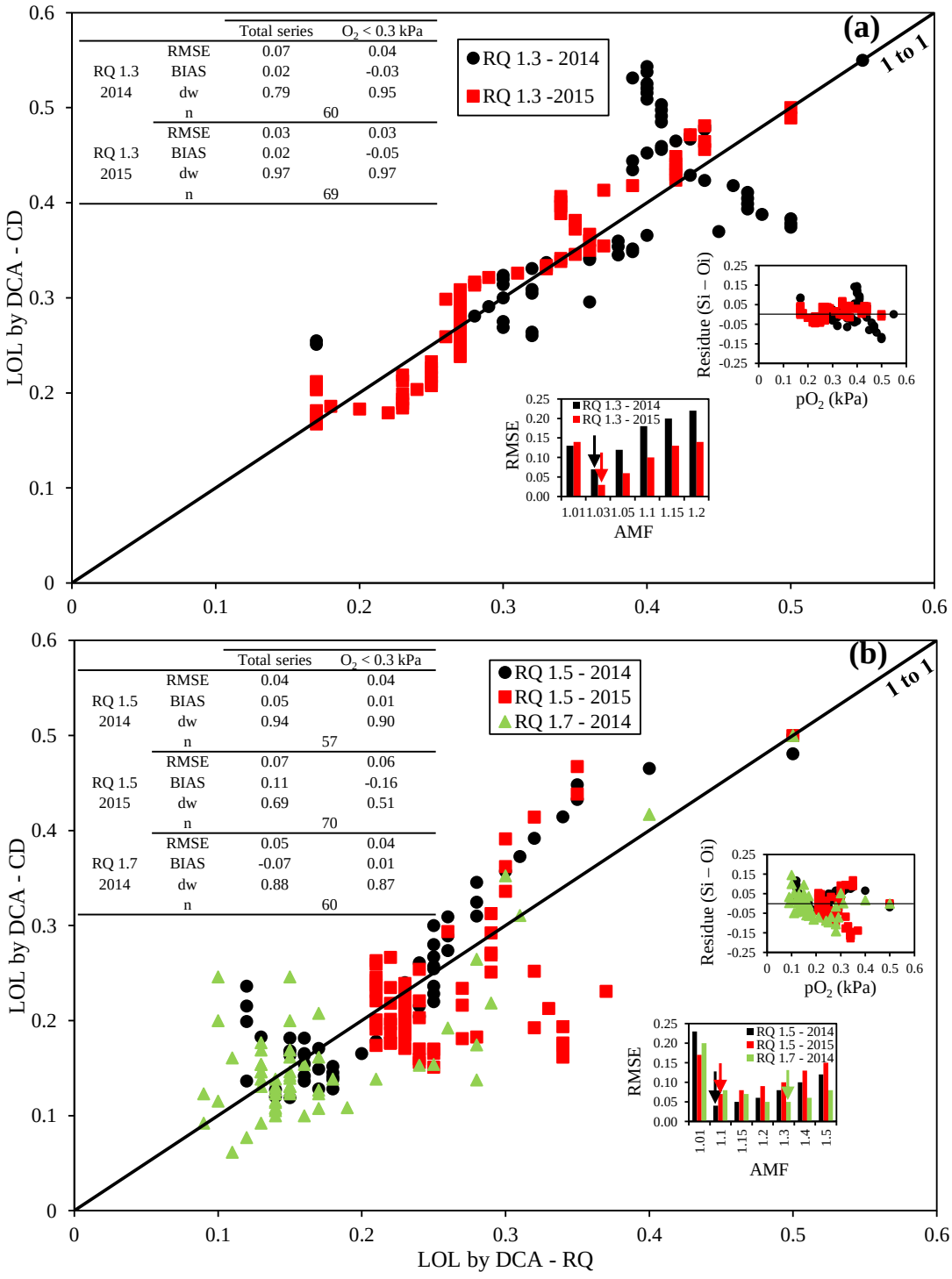


Figure 6. The estimated lower oxygen limit in kPa (LOL) by the new dynamic controlled atmosphere method based on carbon dioxide (DCA - CD) versus the LOL by respiratory quotient (DCA – RQ 1.3, 1.5 and 1.7) for 'Granny Smith' apple during 9 months of storage at 1.5 °C, relative humidity 94%. The solid line is the 1 to 1 line. Inserts are the residues, and root mean square error (RMSE) in several anaerobic metabolism factors (AMF) used to estimate the LOL by the DCA - CD method. RMSE: root mean square error, BIAS: BIAS

index, dw: index of agreement, n: number of LOL determinations during the 9 months. The arrows in the inserts represent the AMF that proportionated the lowest RMSE.

5.1.3.7. Monitoring the LOL by DCA – CD in 'Golden Delicious' apple

Fruit of 'Golden Delicious' apple were stored under three RQs (1.3, 1.5 and 1.7) (Figure 7). To obtain these RQs, the AMF used were 1.05, 1.1 and 1.35, respectively. The RMSE ranged from 0.03 to 0.10 kPa, with the highest error for RQ 1.3 (Figure 7). Nevertheless, the index of agreement (dw) was very high in all RQ evaluated, ranging from 0.81 to 0.97, this showed that the data had a good agreement with the 1:1 line, but the data had a high dispersion (high RMSE), especially for RQ 1.3 (Figure 7).

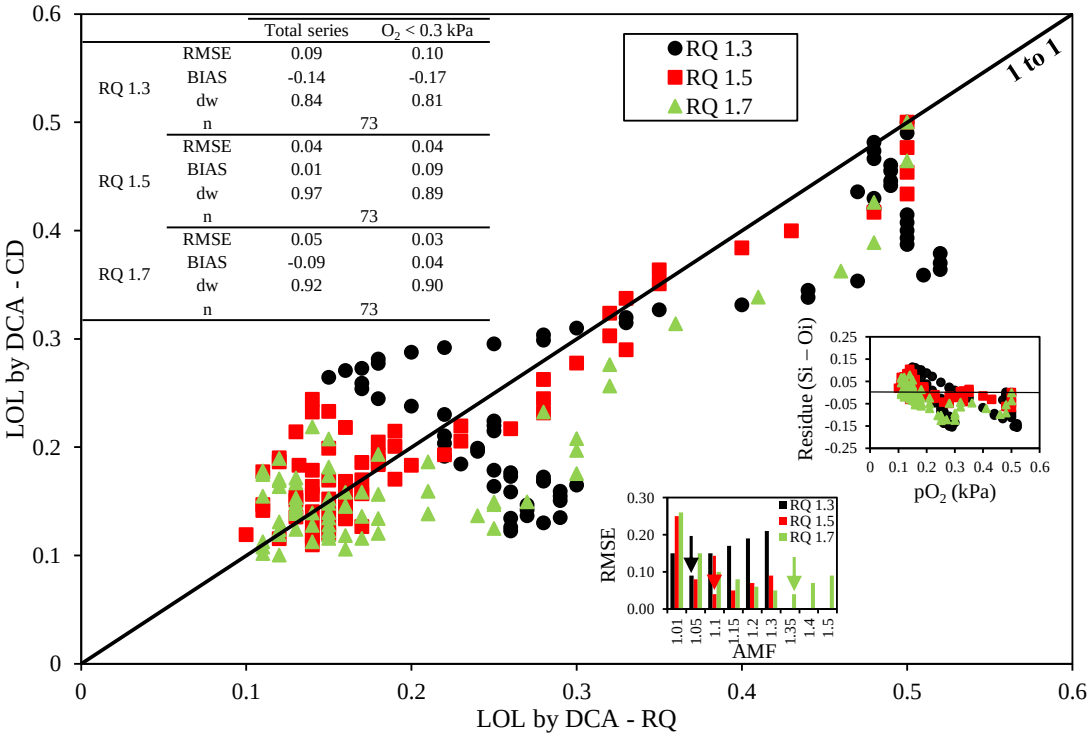


Figure 7. The estimated lower oxygen limit in kPa (LOL) by the new dynamic controlled atmosphere method based on carbon dioxide (DCA - CD) versus the LOL by respiratory quotient (DCA – RQ 1.3, 1.5 and 1.7 - 2016) for 'Golden Delicious' apple during 9 months of storage at 2.0 °C, relative humidity 94%. The solid line is the 1 to 1 line. Inserts are the residues, and root mean square error (RMSE) in several anaerobic metabolism factors (AMF) used to estimate the LOL by the DCA-CD method. RMSE: root mean square error, BIAS: BIAS index, dw: index of agreement, n: number of LOL determinations during the

9 months. The arrows in the inserts represent the AMF that proportionated the lowest RMSE.

5.1.3.8. Relationship between RQ variation × O_2 uptake, and RQ variation × CO_2 release

It was shown that the DCA – CD method had a good performance in the LOL estimation only by CO_2 release for several apple cultivars. This good performance of the new method occurs because the RQ variation during storage is much more correlated to the CO_2 release as to the O_2 uptake in experimental rooms, where the room leak is almost zero (Figure 8a and j). The Pearson correlation between CO_2 release and RQ variation was more than the double as compared to the correlation between O_2 uptake and RQ variation, for the cultivar Galaxy, Fuji and Pink Lady® (Figure 8a – f). Nevertheless, for the cultivar Granny Smith and Golden Delicious, the Pearson correlation between RQ variation and O_2 uptake was higher as compared to the other cultivars, but still remaining below the correlation between CO_2 release and RQ variation (Figure 8g and j). These results clearly show that the LOL can be measured accurately only by CO_2 release of fruit, which may facilitate the implementation of DCA in commercial storage room, once one of the biggest problems of DCA in commercial room is the measurement of O_2 uptake, because of chambers leakage.

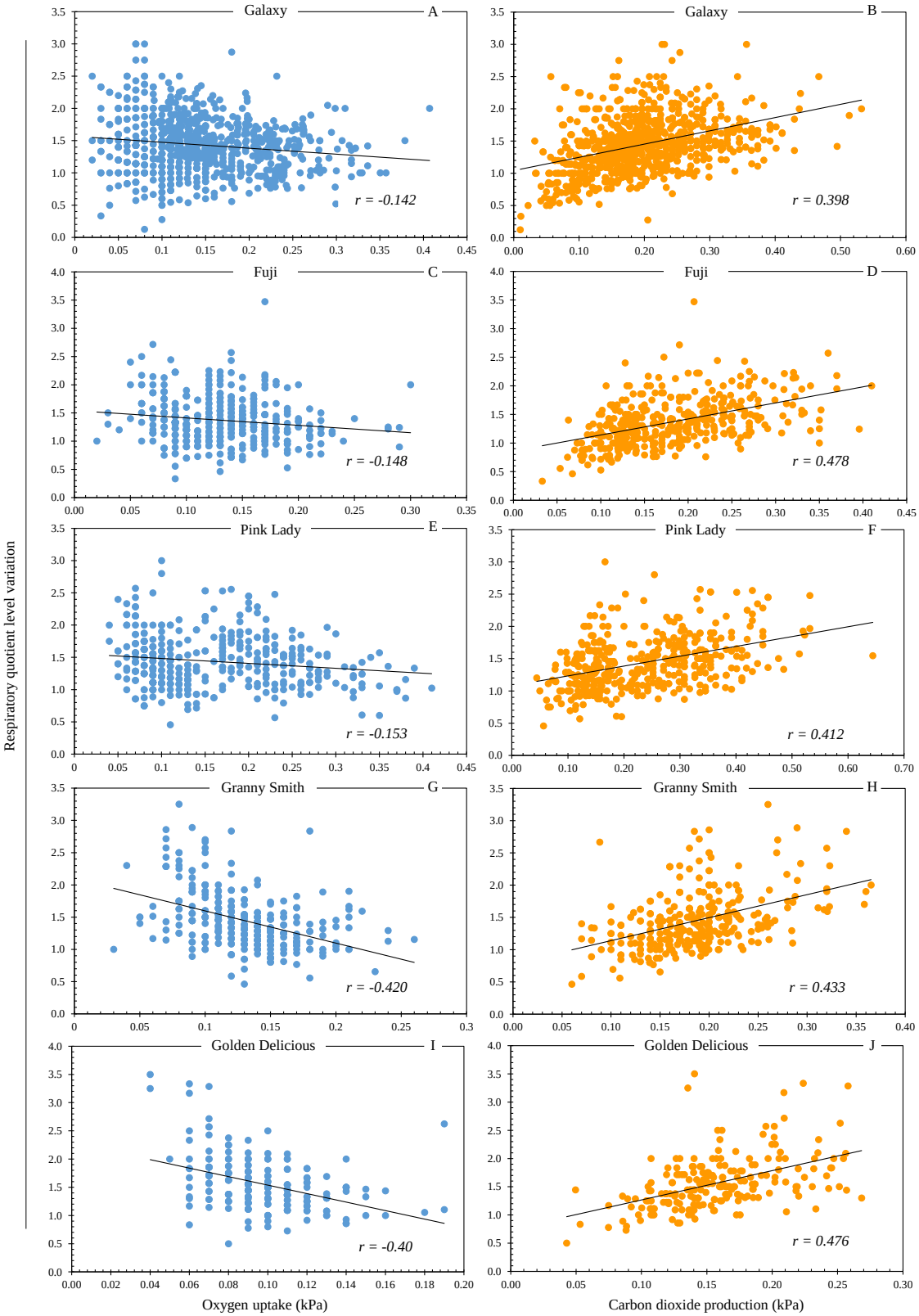


Figure 8. Pearson correlation between oxygen uptake × RQ level variation and carbon dioxide production × RQ level variation for 'Galaxy', 'Fuji', 'Pink Lady®', Granny Smith' and 'Golden Delicious' apple stored in dynamic controlled atmosphere.

5.1.3.9. Relationship between RQ and AMF

To obtain a determined RQ during storage, the AMF employed in DCA – CD is different between RQ levels and cultivars, but among years no differences were identified (Figure 9). The relationship between RQ and AMF is not linear, but as higher the RQ level higher the AMF in DCA – CD method. For 'Galaxy' apple, the AMF coincided with the RQ 1.1 and 2.0, but in intermediary RQ levels, i.e. 1.3 and 1.5, the AMFs are lower than the RQ level adopted (Figure 9). All the other cultivars had an AMF below the one obtained for 'Galaxy' apple, showing that the 'Galaxy' apple tolerated a lower pO_2 to obtain a determined RQ.

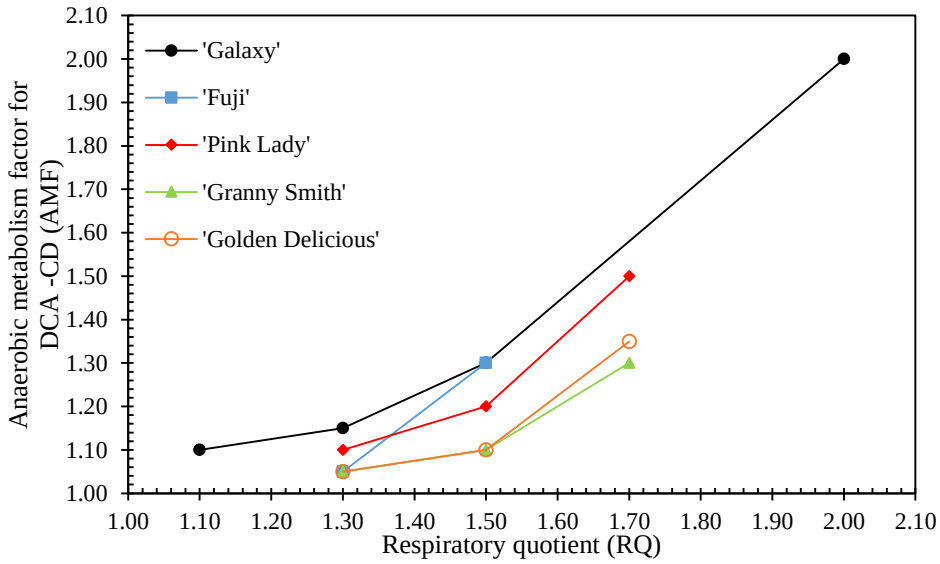


Figure 9. Relationship between RQ level and AMF for 'Galaxy', 'Fuji', 'Pink Lady®', Granny Smith' and 'Golden Delicious' apple stored in dynamic controlled atmosphere.

5.1.4. Discussion

In this study was demonstrated that the DCA – CD method has the capability to estimate the LOL during storage of apples under DCA. This method of LOL monitoring was based only on the CO_2 release by fruit in a determined CA room closousure for 13 - 14 h. The new method estimated the oxygen set point that the CA room should be kept to obtain some anaerobic metabolism with RQ above 1 and therefore maintain the highest fruit quality in terms of flesh firmness, volatile compounds, mealiness, despite other quality atributes. In several studies was observed that the highest fruit quality was maintained by the storage of apples with a RQ higher than 1.0 (RQ of 1.3 and 1.5) (Bessemans et al., 2016; Both et al., 2017; Thewes et al., 2017a; Thewes et al., 2017b; Weber et al., 2017).

The development of a DCA method based only on the CO_2 release is important in practical terms, because one of the main problems to apply the DCA in commercial storage rooms is the needs of additional devices (ethanol sensors, chlorophyll fluorescence sensors, savepods to determine the RQ) to determine the LOL accurately and safetly during the full storage period. The CO_2 release can be determined directly on the storage room, because there are little errors due to room sealing or gases leakage. According to Bessemans et al. (2016) and Bessemans et al., (2018), the RQ variation during the storage time occurs mainly due to the chamber leakage, which causes errors in the O_2 uptake measurement. These same authors evaluated the RQ of a specific chamber three times, twice when the atmospheric pressure was increasing and one when the atmospheric pressure was reducing, and they found a clear difference in RQ between these three situations. When the atmospheric pressure was increased, the RQ was generally overestimated or not valid (negative RQ), due to the error in O_2 uptake measurement, but when the atmospheric pressure decreased the RQ calculation was correct. This fact occurred because when the atmospheric pressure was increasing there is a trend to enter oxygen into the CA room, resulting in error on O_2 uptake measurement. Nevertheless, at the same three scenarios described above, the CO_2 release was not affected by the room leakage, so it could be used alone to estimate the LOL accurately, as we proposed in the present investigation.

In the literature, there are reports of some techniques to monitor the LOL throughout the storage period to change the pO_2 according to fruit metabolism. One of the first reports to store apple in a dynamic atmosphere was made by Wolfe et al. (1993), they developed a model to estimate the fruit respiration by the temperature, oxygen and carbon dioxide partial pressure that the fruit are stored. According to these authors, the model had the ability to "learn during storage" and so predict accurately the fruit respiration and, consequently, optimize the storage environment. Nevertheless, this method is not used in commercial storage rooms nowadays. Some years later, a method of DCA based on ethanol production by fruit was proposed by Schouten et al. (1997) and Veltman et al. (2003) and used only in some commercial rooms in Europe. More recently, the concept of DCA was put in practice by LOL monitoring with chlorophyll fluorescence sensors, which monitor the variation of chlorophyll fluorescence emission according to the pO_2 variation in the storage rooms (Prange et al., 2007; Wright et al., 2010; Wright et al., 2012; Tran et al., 2015). Another recent technology of DCA is based on RQ measurement, and the results are promising in superficial scald control (Bessemans et al., 2016), firmness maintenance (Weber et al., 2015; Bessemans et al., 2016; Both et al., 2017; Thewes et al., 2017a), higher volatile compounds (Both et al., 2017; Thewes et al., 2017b),

despite other benefits. Due to these positive results obtained experimentyally in DCA – RQ and lower feasibility to adopt in commercial rooms, in the present study we compared the LOL estimated by DCA – CD to the one obtained by DCA – RQ.

The DCA-CD had a good performance in the estimation of the LOL regardless of the cultivar, year, RQ, and growth regulator application, showing an error ranging between 0.01 until 0.10 kPa. This error is below the resolution of the major gas analyzers used in commercial storage rooms, which had generally a resolution of 0.1 kPa. Nevertheless, in an average the RMSE was 0.05 kPa. The performance of DCA – CD was better as the pO_2 was reduced in the storage room, where the anaerobic metabolism becomes important and the risk of physiological disorders development is high.

5.1.5. Conclusions

The DCA method based only on the CO_2 release (DCA – CD) for several apple cultivars has the capability to estimate accurately the LOL variation during the storage period, allowing the pO_2 variation according to fruit metabolism during storage for dynamically control the atmosphere.

The AMF used to obtain a determined RQ, i.e. a little of anaerobic metabolism (RQ between 1.1 and 2.0), was different between cultivars and RQ, but was not affected by the application of growth regulators and years of growing.

The relationship between RQ and AMF is not linear, so it should be calibrated for each cultivar that the DCA-CD method will be used.

The variation of RQ during the storage period was more correlated to the CO_2 release than to the O_2 uptake, which was the reason of the high reliability of DCA – CD in LOL estimation for all investigated apple cultivars.

Future studies should evaluate the effect of the novel DCA – CD method on the overall fruit quality and compare its quality maintenance to the fruit stored in other DCA methods, like DCA – RQ and DCA – CF.

5.1.6. References

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6. ARTIGO 4

6.1. DYNAMIC CONTROLLED ATMOSPHERE BASED ON CO_2 RELEASE (DCA – CD): IMPACTS ON ANAEROBIC METABOLISM, OVERALL QUALITY AND VOLATILE PROFILE OF 'ELSTAR' APPLES[4]

Abstract

The storage of apple fruit under very low oxygen partial pressures is becoming more important in commercial storage facilities. To reduce the oxygen at a safety level is necessary a method to detect the lowest oxygen limit (LOL). Nowadays, the LOL is generally monitored by chlorophyll fluorescence (DCA - CF) and respiratory quotient (DCA - RQ). We developed another method to determine the LOL in real time over the storage period, based only on the CO_2 released. Therefore, the objective of this study was to assess the effect of the new DCA method, based only on the CO_2 production (DCA - CD), and compare it with other DCA methods, such as DCA - CF and DCA - RQ, on fruit metabolism, overall quality and volatile compounds concentration. Additionally, we also aimed to compare the new DCA - CD method with the 1-MCP treatment on 'Elstar' apples after 6 and 9 months of storage plus 7 days of shelf life at 20°C. DCA - CD was able to predict accurately the LOL and induce anaerobic metabolism at safety levels in 'Elstar' apples. Fruit stored under DCA - CD had lower ACC oxidase, ethylene production, decay, flesh breakdown incidence and higher flesh firmness and healthy fruit amount as compared to DCA - CF and CA and, similar to DCA - RQ. 1-MCP application is not necessary when fruit were stored under DCA - CD or DCA - RQ. PDC enzyme activity is increased by the storage under DCA - RQ 1.5 and DCA - CD 1.3, i.e. extremely low oxygen partial pressures, but ADH enzyme not. Butyl acetate, 2-methybutyl acetate and hexyl acetate concentration are increased by the storage under DCA - CD, especially under CD 1.1, resulting in comparable concentrations as DCA - RQ and higher as compared with CA and DCA - CF. Moreover, fruit stored under DCA - CD had lower accumulation of ethanol and ethyl acetate, compounds correlated to off-flavour formation in apples, when compared with DCA - RQ.

Keywords: *Malus domestica;* apple fruit; DCA – RQ; DCA – CF; esters, alcohols.

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6.1.1. Introduction

Apples are fruit produced in temperate climate, with harvest restricted to some months of the year. However, its consumption is spread over the year and worldwide. In Germany, 'Elstar' is one of the main cultivars produced (Wapa 2018). To allow the delivery of fresh fruit over the year, they should be stored adequately. Nowadays almost all apples are stored under controlled atmosphere (CA), were the oxygen is lowered to partial pressures near 1.0 kPa and the carbon dioxide increased to partial pressures near 2.0 kPa and maintained constant over the storage period (Veltman et al., 2003; Brackmann et al., 2015; Köpcke, 2015). Nevertheless, even with the use of CA quality losses occur, especially because softening (Veltman et al., 2003; Zanella et al., 2008; Weber et al., 2019) and physiological disorders, like flesh breakdown and mealiness (Franck et al., 2007; Kittemann et al., 2015; Thewes et al., 2017a; Weber et al., 2017; Both et al., 2018).

Trying to solve these problems of CA, were performed several studies evaluating the effect of dynamic controlled atmosphere (DCA) on apple softening and overall quality (Veltmann et al., 2003; Gasser et al., 2008; Brackmann et al., 2015; Deuchande et al., 2016; Bessemans et al., 2016; Thewes et al., 2017; Weber et al., 2017). According to these studies, the storage under DCA resulted in fruit with better overall quality and lower physiological disorders, especially flesh breakdown. However, the DCA efficiency on quality maintenance is dependent on the species, cultivar, 1-methylcyclepropene (1-MCP) treatment and the DCA method used to monitor the LOL.

Nowadays, there are in the market three main methods to monitor fruit metabolism to detect the LOL, based on ethanol production (DCA – Eth) (Schouten, 1995; Veltman et al., 2003; Deuchande et al., 2016), chlorophyll fluorescence (DCA – CF) (Prange et al., 2007; Wright et al., 2010; Wright et al., 2012) and respiratory quotient (DCA – RQ) (Gasser et al., 2008; Brackmann, 2015; Weber et al., 2015; Bessemans et al., 2016). 'Elstar' apples stored under DCA based on ethanol production were firmer and had lower skin spots incidence as compared to CA (Veltman et al., 2003), but in 'Rocha' pears it resulted in higher flesh breakdown, when compared to DCA – CF (Deuchande et al., 2016). DCA – CF is the method most used commercially nowadays, it reduces the softening and superficial scald, maintaining higher overall quality as compared to CA (Zanella et al., 2008; Köpcke, 2015; Thewes et al., 2015; Tran et al., 2015), but results in fruit with lower volatile compounds concentration,

especially esters (Thewes et al., 2017b; Both et al., 2017). On the other hand, fruit stored under DCA - RQ had an increment of volatile compounds emission, reduction of physiological disorders and better fruit quality maintenance (Weber et al., 2015; Bessemans et al., 2016; Thewes et al., 2017b; Both et al., 2017), allowing the increase of the storage temperature without quality losses (Anese et al., 2018; Both et al., 2018).

All the three methods of DCA cited above need the addition of sensors and/or devices to determine the LOL in real time over the storage period. For DCA – Eth, ethanol sensors should be added to the storage room (Veltman et al., 2003). To perform DCA - CF, chlorophyll sensors should be added to the storage rooms (Prange et al., 2007). For DCA - RQ, should be added to the rooms safe pods (Schaefer and Bishop, 2012; Brackmann 2015). Trying to reduce the costs of DCA implementation in commercial rooms, we developed a method for LOL monitoring based on the CO_2 production rate only (DCA - CD), which could be determined directly on the commercial rooms, without any additional devices, as discussed in article 3. This is possible because the CO_2 accumulation inside the storage rooms has little influence of atmosphere pressure changes and room leakage (Bessemans et al., 2018). So far, there are no results in the literature evaluating the metabolism and quality of apples stored under DCA - CD and compare it to other DCA methods. Additionally, the new DCA method need to be compared to a worldwide used technology, such as 1-MCP, to prove its performance.

1-MCP is a compound that block the ethylene action, reducing fruit ripening and extending shelf life of fruit (Watkins, 2006; Lee et al., 2012). However, its effect on fruit quality maintenance is reduced under DCA storage, especially in DCA - RQ (Anese et al., 2018; Both et al., 2018). Additionally, the 1-MCP application result in fruit with lower volatile compounds emission (Streif et al., 2010, Lee et al., 2012; Yang et al., 2016; Thewes et al., 2017a). Thus, the development of storage technologies that reduces fruit metabolism similarly as 1-MCP treatment and maintain higher volatile compounds emission are very important, in order to offer high fruit quality to the consumers.

The objective of this study was to assess the effect of the new DCA method, based on CO_2 production only (DCA - CD), and compare it with other DCA methods, such as DCA - CF and DCA - RQ, on fruit metabolism, overall quality and volatile compounds concentration. Additionally, we also aimed to evaluate the efficacy of 1-MCP treatment to ‘Elstar’ apples in the new DCA – CD, another DCA methods and CA, after 6 and 9 months of storage plus 7 days of shelf life at 20°C.

6.1.2. Material and methods

6.1.2.1.Fruit harvest, sampling and treatment application

Apples of the cultivar Elstar were harvested at the optimal harvest maturity for long-term storage in a commercial orchard located at the Constance Lake region, Southwest Germany. Immediately after harvest, fruit were transported to the Competence Centre for Fruit Growing at Lake Constance (KOB), Germany. At KOB, the fruit were randomly sample to perform samples of 20 fruit each. The sampling was performed to homogenize fruit size and color.

After this process, the samples were placed into 250 L experimental gas-tight chambers. In each chamber were put 4 samples for evaluation after 6 months of storage and 4 for evaluation after 9 months of storage. The experimental chambers were allocated inside a cold room at temperature of 1 ± 0.2 °C. Each chamber was connected to an automatic CA control system (Isollcel®, Bolzano Italy), to continuously control de atmosphere inside the chambers. At the same day was undertaken an initial analysis to determine fruit quality at harvest, where the SPI was 2.9 (0 – unripe and 10 – overripe), firmness of 63.3 N, acidity 13.8 mEq 100 mL^{-1} and soluble solids of 12.3 °Brix.

6.1.2.2. Storage conditions evaluated

The atmosphere conditions evaluated were: [1] CA - 1.2 kPa O_2 + 2.0 kPa CO_2; [2] DCA - CF + 1.2 kPa CO_2; [3] DCA - RQ 1.3 + 1.2 kPa CO_2; [4] DCA - RQ 1.5 + 1.2 kPa CO_2; [5] DCA - CD 1.1 + 1.2 kPa CO_2; [6] DCA - CD 1.3 + 1.2 kPa CO_2. All fruit of the six storage conditions were without and with 1-MCP treatment.

6.1.2.3. 1-MCP treatment

In order to 1-MCP application fruit were put into the 250-L chamber together with a solution containing 0.650 µL L^{-1} 1-MCP (SmartFresh®, 0.14% of active ingredient). Thereafter, the chamber was hermetically closed during 24 hours. During this period, the air inside the chamber was homogenized with a fan. After, the fruit were removed from the 1-MCP application chamber and stored in CA and DCA conditions as reported in section 2.2.

6.1.2.4. Storage temperature and relative humidity

The storage temperature in the container was seated and maintained at 1.0 ± 0.1°C during the entirely storage period. Throughout the storage, the temperature was monitored daily with a mercury thermometer inserted in fruit flesh, to determine the pulp temperature. The relative humidity was seated and maintained at 94 ± 1% with aid of a psychrometer.

6.1.2.5. Methods to determine the optimal oxygen level for DCA

For chlorophyll fluorescence (DCA - CF), two batches of 6 fruit were monitored with a CF sensor in real time throughout the storage period as proposed by Prange et al. (2007). To perform the RQ method the experimental chambers were closed for 13 to 14h, and the pO_2 and pCO_2 measured before and after this period. The ratio between CO_2 release and O_2 uptake was calculated to give the RQ. Two RQ treatments: 1.3 and 1.5 were tested in this experiment, and the RQ was calculated two times a week. When the RQ was below the setpoint, the pO_2 was decreased, and if the RQ was above the setpoint, the pO_2 was increased, according to Weber et al. (2015). To monitor the optimal oxygen level based only on CO_2 production, the method described by Thewes et al. (2019) was used (article 3).

6.1.2.6. Metabolism, overall quality and volatile compounds evaluation

After 6 and 9 months of storage plus 7 days of shelf life, the fruit were assessed for metabolism, quality and volatile compounds characteristics, in order to show the effect of each storage condition, as described below.

6.1.2.6.1. ACC oxidase enzyme activity

ACC oxidase was evaluated by the method proposed by Bufler (1986) and results were expressed in ng kg^{-1} s^{-1}.

6.1.2.6.2. Ethylene production and respiration rate

Measured by placing a sample of about 0.8 to 1 kg of intact fruit into a 4.25 l jar. To measure ethylene, the jar was closed hermetically for 2 h. 1 mL of headspace gas from the jar

was injected into a Carlo Erba, Fractovap Series 2150 gas chromatograph, equipped with a flame ionization detector (FID) and a stainless steel column, 0.9m x 1/8" filled with 60-mesh activated alumina. Injector and oven temperature were 175 °C and 100 °C, respectively, and data were expressed in pg kg^{-1} s^{-1}. To determine the respiration rate, the air of the same jar was circulated throughout a gas analyzer (Hartmann and Braun GmbH, Germany) to measure CO_2 and results were expressed in μg kg^{-1} s^{-1}.

6.1.2.6.3. Decay, flesh breakdown and healthy fruit amount

The method proposed by Thewes et al. (2015) was used. In brief, decay incidence was evaluated by counting the fruit with fungal injury higher than 5 mm, in relation to the total number of fruit per replicate (20 fruit each replicate). Flesh breakdown incidence was evaluated by slicing the fruit on the equatorial region and visualization of any symptom of pulp browning. To determine the healthy fruit amount was taken in account the total number of fruit per replicate (20 fruit) minus fruit with any symptom of decay and/or physiological disorder. All results were expressed in percentage.

6.1.2.6.4. Electrolyte leakage

Twelve discs (5 mm thickness and diameter) from 12 fruit were taken. These discs were stowed into 20 mL of double distilled water during 1 hour (20 ± 1 °C), afterward the conductivity of the suspension was measured. The suspension was then placed into a water bath at 120 °C for 30 min and thereafter allowed to cool into a -30 °C freezer down to 20 °C, then conductivity was measured again and taken as total leakage. Results expressed as percentage.

6.1.2.6.5. Flesh firmness

Determined by the insertion of an 11 mm tip in the fruit flesh at the equatorial region, between the red and the green colored sides. At the region of measurement, the skin was previously removed (1mm depth). Results were expressed in Newton.

6.1.2.6.6. Pyruvate decarboxylase and alcohol dehydrogenase

The crude enzymes extracts were obtained from 100 mg of lyophilized powdered tissue taken from 15 apples by the homogenization in 500 µl of extraction solution containing 85 mM 2-(N-morpholino) ethane-sulfonic acid (MES) buffer, pH 7.5, 5 mM dithiothreitol (DTT), 1 % (w/v) polyvinyl polypyrrolidone (PVPP) and 20 µL triton X-100. The homogenate was kept below 4 °C for 20 min and stirred continuously. The samples were then centrifuged at 14,000 g for 20 min at 4 °C. These steps were repeated once, according to the method proposed by Saquet and Streif (2008). To assess alcohol dehydrogenase (ADH), a 150 µL aliquot of the crude extract was added to a reaction mixture consisting of 2.65 mL of a 0.15 mM NADH solution in MES buffer (pH 6.5) and 200 µL of 80 mM acetaldehyde. Pyruvate decarboxylase (PDC) activity was measured in a reaction mixture with 2.25 mL containing 5 mM thiamine pyrophosphate with 50 mM $MgCl_2$ in MES buffer (pH 6.5); 300 µL of 0.85 mM NADH and 180 units mg^{-1} ADH; and 300 µL of 50 mM Na-pyruvate. 150 µl of crude extract was added to this solution. For both PDC and ADH, activity was measured by following the spectrophotometric decrease in absorbance at 340 ηm over 160 s, due to NADH oxidation. Activity was defined as the decrease in absorbance at 340 ηm min^{-1}, and results expressed as U kg^{-1} s^{-1}.

6.1.2.6.7. Volatile compounds determination

The volatile compounds concentration were determined in juice obtained from 15 apples per replicate. To obtain the juice, the fruit were cooled to 0 °C before juice extraction. Immediately after the pulp cooling, horizontal slices of the equatorial region of each fruit were taken, discharged the seeds, and centrifuged with a Juicer (Philips Walita®) under low temperature, to avoid the chemical and enzymatic oxidation of samples (the maximum juice temperature during sample preparation was 5°C). The juice was placed inside vials and immediately frozen down to -30°C up to the volatile compounds analysis.

To quantify and identify the volatile compounds the method proposed by Both et al. (2014) was used. In brief, an aliquot of 10 mL of this juice was taken, mixed with 3g NaCl and 10 µL of 3-octanol standard solution (81.8 µg L^{-1}) inside a 20 mL vial that allowed hermetically sealing with a PTFE-coated silicone lid. The volatile compounds were extracted from the headspace of the juice via solid phase microextraction (HS-SPME) with a Divinylbenzene/carboxen/polydimethylsiloxane (DVB/Car/PDMS) fibre (Supelco, 50/30 µm × 20 mm) preconditioned following the manufacturer protocol. Before the fiber exposing, the vial

was submerged in a water bath at 35 °C during 5 min. After 5 min, the fiber was exposed to the headspace of the sample during 60 min under constant stirring at the same temperature.

The fiber was thermally desorbed into the injection port at a temperature of 250 °C for 10 min, in a split less mode. The compounds were separated in a polar fused silica capillary column (ZB - WAX, USA; 30 m × 0.25 mm × 0.25 μm). Helium was used as carrier gas at constant flow rate 1.2 mL min^{-1}. The initial column temperature was set to 35 °C and held for 3 min. Then, a temperature gradient was started of 2 °C min^{-1} until 80 °C, followed by a 5 °C min^{-1} increase until 230 °C, and maintained at isothermal conditions for 5 min. The detector was operated in the electron impact ionization mode with ionization energy of +70 eV and a scan mass range from 35 to 350 m/z, at a temperature of 230 °C. A series of homologous n-alkanes was analyzed under the same conditions to calculate the linear retention index (LRI). The concentration of volatile compounds were determined by internal standardization. The analytes were identified based on comparison with mass spectra available in the National Institute of Standards and Technology (NIST) library and by comparing the calculated LRI with those available in the scientific literature.

6.1.2.7. Statistical analysis

The experiment was conducted in a completely randomly scheme with a factorial arrangement. A variance analysis (ANOVA) at 5% of error probability was carried out ($p < 0.05$). Data that showed significant difference by ANOVA were subjected to the LSD-Fisher's test at 5% error probability. Additionally, all data were submitted to a Principal Component Analysis (PCA) using The Unscrambler® X software (version 9.7, CAMO A/S, Trondheim, Norway) to show an overview of the results. Before the PCA the data matrix was auto scaled for each variable in order to obtain the same weight for all variables (mean = 0 and variance = 1).

6.1.3. Results and discussion

6.1.3.1. Ethylene and respiration metabolism

The ethylene-forming enzyme (ACC oxidase) converts ACC to ethylene, being oxygen-depend, so its activity can be regulated by the oxygen partial pressure in the storage environment. In the present study, the ACC oxidase enzyme activity was reduced by the storage

under DCA, especially when the LOL was monitored by DCA - RQ and DCA - CD, after both 6 and 9 months of storage (Figure 1). Probably, this is a combined effect of the reduced oxygen partial pressure in storage room (Supplementary Figure 1) and the higher accumulation of ethanol (Figure 5). Several previously studies showed that the ethanol reduced the ACC oxidase enzyme activity (Asoda et al., 2009; Both et al., 2017; Thewes et al., 2017; Weber et al., 2019), but our study is the first presenting the effect of DCA - CD on this enzyme. A noteworthy fact is that the 1-MCP application had a higher effect on ACC oxidase activity when the fruit were stored under CA and DCA - CF, showing that at extremely low oxygen (Supplementary Figure 1), by the storage under DCA - RQ and DCA - CD, the effect of 1-MCP is reduced (Figure 1).

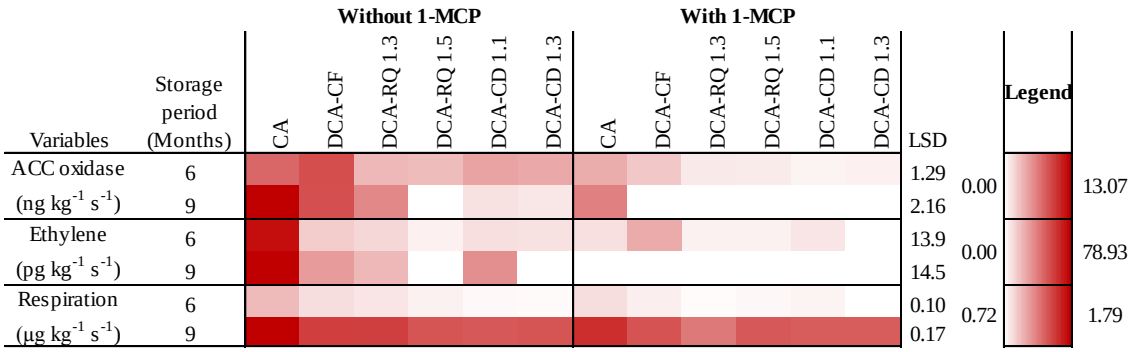
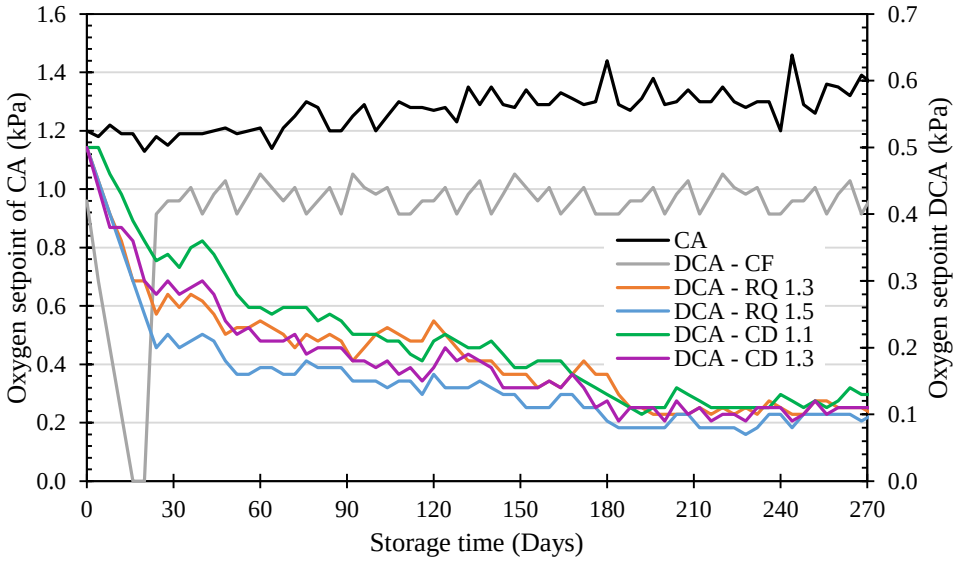


Figure 1. ACC oxidase enzyme activity, ethylene production and respiration rate of 'Elstar' apples after 6 and 9 months of storage under controlled atmosphere (CA) and dynamic controlled atmosphere monitored by chlorophyll fluorescence (DCA - CF), by respiratory quotient (DCA - RQ) and by carbon dioxide (DCA - CD), either without or with 1-MCP treatment (0.650 µL L-1), plus 7 d of shelf life at 20°C. LSD = Fisher's lowest significant difference ($p<0.05$).



Supplementary Figure 1. Oxygen setpoint variation of 'Elstar' apples stored under CA and DCA at 1 °C over 9 months.

Lower ACC oxidase enzyme activity in fruit stored under DCA - CF, DCA - RQ and DCA - CD resulted in lower ethylene production (Figure 1). After 9 months, fruit stored under DCA - RQ 1.5 and DCA - CD 1.3 had the lowest ethylene production, and the 1-MCP application had no effect on ethylene production in these storage conditions. The 1-MCP treatment had the highest effect on ethylene production when fruit were stored under CA (Figure 1). The higher ethylene production of fruit stored under CA without 1-MCP resulted in higher respiration rate after 6 and 9 months of storage (Figure 1). The respiration rate increased from 6 to 9 months of storage, showing that fruit metabolism is induced over the storage period. Storage under DCA - CD resulted in similar ethylene and respiration rate as compared to DCA - CF, DCA - RQ and 1-MCP application, showing that this new DCA technology is promising to be used in commercial storage rooms, especially because its easy implementation, without the need of additional devices/sensors to the storage room.

6.1.3.2. Overall quality assessments

In commercial storage rooms, one of the main reason of apple losses is the decay incidence, which could reach more than 35%, depending on the year (Corrent et al., 2009; Antoniolli et al., 2011). After 6 months, fruit stored under DCA - RQ 1.5, DCA - CD 1.1 and DCA - CD 1.3 had lower decay incidence, differing from DCA - CF and CA (Figure 2). However, after 9 months, all DCA methods resulted in lower decay incidence as compared to

CA. The 1-MCP application had no positive effect on decay incidence, resulting in higher decay incidence when fruit were stored under DCA - CF after 9 months of storage. These results evidence that the storage under DCA, especially DCA - CD and DCA - RQ, results in lower losses due to decay as compared to 1-MCP treatment, which is a very important practical result. Probably, the lower decay in fruit stored under DCA - RQ and DCA - CD is a result of higher anaerobic metabolism compounds accumulation, especially acetaldehyde and ethanol (Figure 5 and 6). In sweet cherries, table grapes and melons, the ethanol treatment reduced the decay incidence (Bai et al., 2011; Candir et al., 2012; Jin et al., 2013), corroborating the results obtained at the present study, but we induced the ethanol production by the own fruit.

Another problem during the apple storage period is the incidence of physiological disorders, such as flesh breakdown, characterized as flesh browning. After 6 months of storage, there was no incidence of flesh breakdown, regardless the atmosphere conditions and 1-MCP application (Figure 2). Nevertheless, after 9 months, fruit stored under CA and DCA - CF had higher flesh breakdown as compared to fruit under DCA - RQ and DCA - CD, which had no incidence (Figure 2). The flesh breakdown is a result of cell compartmentalization loss, resulting in phenol oxidation. Compartmentalization loss is due to damages on the cell membrane, resulting in electrolyte leakage (Saquet et al., 2000; Franck et al., 2007; Gago et al., 2015). Thus, we evaluate the electrolyte leakage as a measurement of cell membrane integrity (Figure 2), and there was observed that the higher flesh breakdown incidence in fruit stored under CA and DCA - CF is due to higher electrolyte leakage (Figure 2).

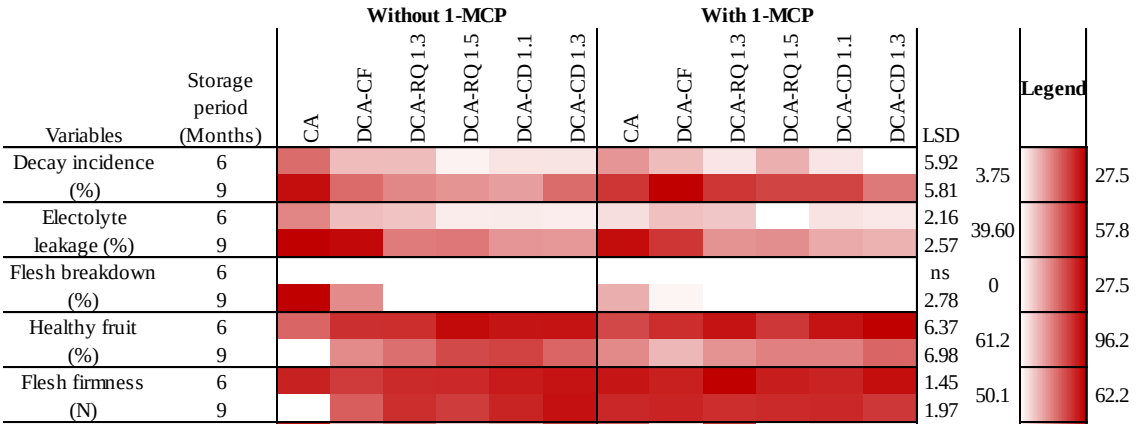


Figure 2. Quality of 'Elstar' apples after 6 and 9 months of storage under controlled atmosphere (CA) and dynamic controlled atmosphere monitored by chlorophyll fluorescence (DCA - CF), by respiratory quotient (DCA - RQ) and by carbon dioxide (DCA - CD), either without

or with 1-MCP treatment (0.650 μL L-1), plus 7 d of shelf life at 20°C. LSD = Fisher's lowest significant difference ($p<0.05$).

Flesh breakdown incidence was reduced by 1-MCP application, when fruit were stored under CA and DCA - CF (Figure 2). This shows that 1-MCP application results is benefits on quality maintenance when fruit were stored in higher oxygen partial pressures (Supplementary Figure 1). Probably, the lower flesh breakdown incidence is a result of lower ethylene production/action, delaying fruit ripening and consequently the cell compartmentalization loss. In the literature, the effect of 1-MCP on flesh breakdown is not clear, and it depends on several factors, such as maturity (Gago et al., 2015; Thewes et al., 2017a), oxygen partial pressure (Thewes et al., 2017a; Watkins and Nock, 2012), temperature (Jung and Watkins, 2011; Anese et al., 2018; Both et al., 2018), despite other factors. At the present study, for 'Elstar' apples, there are an interection between storage conditions and 1-MCP application, in the flesh breakdown incidence, with no effect of 1-MCP when fruit were stored at extremely low oxygen (Figure 2 and Supplementary Figure 1).

Taken in account the incidence of decay and physiological disorders, we calculate the healthy fruit amount, which is one of the most important parameter to select optimal storage conditions. After both 6 and 9 months of storage, fruit stored under CA had the lowest healthy fruit amount, when compared to DCA (Figure 2). The storage under DCA - CD 1.1 and DCA - RQ 1.5 resulted in the highest healthy fruit amount without 1-MCP treatment (Figure 2). When fruit were stored under DCA, the 1-MCP application had no positive effect, but under CA, it resulted in benefits on healthy fruit amount. These results show that the new DCA method (DCA - CD) results in similar quality maintenance as compared to DCA – RQ and 1-MCP application, and better as compared to CA and DCA - CF. The better results of the storage under DCA - RQ and DCA - CD is due to the extremely low oxygen partial pressure employed during storage (Supplementary Figure 1), which inhibited the fruit metabolism reducing decay and physiological disorders incidence.

Concerning the flesh firmness, after 6 months, they were little difference between atmosphere conditions and 1-MCP treatment (Figure 2). However, after 9 months of storage, fruit under CA without 1-MCP had the lowest firmness retention (Figure 2). Fruit stored under DCA - CD and DCA - RQ retained the highest flesh firmness, differing from fruit under CA and DCA - CF. 1-MCP treatment had effect on firmness retention when fruit were stored under CA only (Figure 2). The higher firmness retention when fruit were stored under DCA - CD, DCA - RQ and CA + 1-MCP is a result of lower ethylene production/action (Figure 1). Ethylene

is necessary to start the cell-wall degrading enzymes, so as lower the ethylene production/action the lower fruit softening (Prasanna et al., 2007; Payasi et al., 2009; Gwanpua et al., 2014; 2016). Additionally, the accumulation of anaerobic metabolism compounds (acetaldehyde and ethanol) also suppresses the cell-wall enzymes activity (Weber et al., 2019).

6.1.3.3. Volatile compounds concentration

The headspace micro extraction HS-SPME-GC/MS detected more than 100 volatile compounds, from which 59 were identified in all samples after both 6 and 9 months of storage plus 7 days of shelf life. Identified compounds comprised, 20 esters, 17 alcohols, 8 aldehydes, 10 acids and 4 ketones.

6.1.3.3.1. Accumulation of anaerobic metabolism compounds

The principle of DCA is to detect the LOL of the fruit during storage (Veltman et al., 2003; Prange et al., 2007) and induction of anaerobic metabolism at safety levels is beneficial (Brackmann 2015). Thus, to evaluate the performance of the DCA - CD method, anaerobic metabolism compounds are important indicators if this new method is able to detect the LOL. When the oxygen partial pressure is reduced below the LOL over the storage period, the flow of pyruvate is changed to fermentative pathway (Ke et al., 1994). The conversion of pyruvate to acetaldehyde is performed by the pyruvate decarboxylase enzyme (PDC), being the first step of alcoholic fermentation.

The PDC enzyme activity was higher when fruit were stored under DCA - RQ 1.5 and without -1-MCP treatment after both outturn evaluation times (Figure 3). Fruit stored under CA, DCA - CF and DCA - CD 1.1 had the lowest PDC enzyme activity after both evaluations times. When the fruit were stored under DCA - CD 1.3, the PDC enzyme activity increased as compared to DCA - CD 1.1 (Figure 3), but the oxygen partial pressure of these two conditions was very similar (Supplementary Figure 1), in average 0.21 kPa O_2 for DCA - CD 1.1 and 0.18 kPa O_2 for DCA - CD 1.3. These results show that the switch between aerobic and anaerobic metabolism is efficiently detected by the new DCA method (DCA - CD), showing that CD 1.1 did not start significantly PDC enzyme activity, but CD 1.3 started the PDC activity.

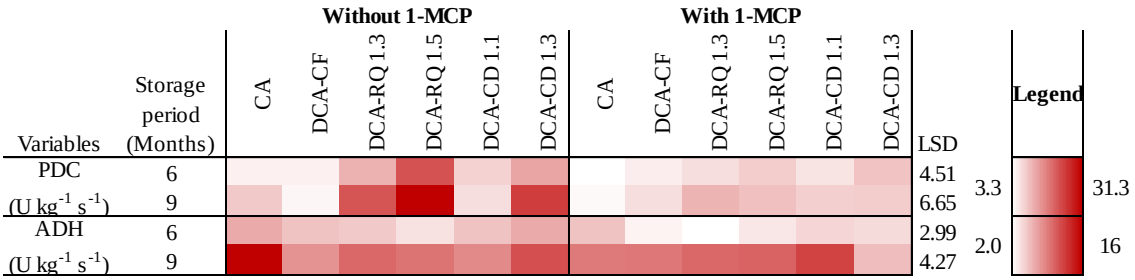


Figure 3. Pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) enzymes activity in 'Elstar' apples after 6 and 9 months of storage under controlled atmosphere (CA) and dynamic controlled atmosphere monitored by chlorophyll fluorescence (DCA - CF), by respiratory quotient (DCA - RQ) and by carbon dioxide (DCA - CD), either without or with 1-MCP treatment (0.650 µL L-1), plus 7 d of shelf life at 20°C. LSD = Fisher's lowest significant difference ($p<0.05$).

The first compound produced during ethanolic fermentation is the acetaldehyde (Ke et al., 1994). After 6 months, fruit stored under DCA - RQ 1.5 and DCA - CD 1.1 had the highest concentration of acetaldehyde and, at 9 months also DCA - RQ 1.3 had high acetaldehyde concentration (Figure 6). Analyzing the PDC enzyme activity (Figure 3) and acetaldehyde concentration (Figure 6), is verified that not always high PDC enzyme activity is necessary to obtain high acetaldehyde. According to Boeckx et al. (2018; 2019), this is because when anaerobic metabolism is only slightly induced, the PDC enzyme is regulated via cofactor, especially when apples were stored under low temperature, and this cofactor regulation is not detected by our method to determine PDC. However, the accumulation of acetaldehyde, when fruit were stored under DCA - CD 1.1, can be a result of low ADH enzyme activity (Figure 3), which inhibit the conversion of acetaldehyde to ethanol (Figure 5).

Analyzing the enzyme ADH and ethanol concentration together is noteworthy that its activity had low correlation with the ethanol accumulation in the fruit (Figure 9). Fruit stored under DCA - RQ 1.5 had lower ADH enzyme activity after 6 months of storage without 1-MCP (Figure 3), the treatment with the lowest oxygen partial pressure (Supplementary Figure 1). This can be a negative feedback of the high ethanol concentration (Figure 5) on ADH enzyme (Lara et al., 2003). After 9 months, CA stored fruit had higher ADH enzyme activity in fruit without 1-MCP, in this case, probably because the higher ethylene concentration when fruit were stored under CA (Harb et al., 2011). However, fruit stored under CA had lower ethanol concentration (Figure 5), showing that the ADH enzyme activity had little influence on the ethanol accumulation. Early studies showed that the alcohol accumulation is directly correlated

to precursor concentration, with low effect of ADH enzyme activity (Lara et al., 2003; Echeverría et al., 2004; Thewes et al., 2018), corroborating our study. The 1-MCP application reduced ADH activity after 6 months of storage, when fruit were stored under DCA, regardless the method (Figure 3). On the other hand, after 9 months, the 1-MCP application increased the ADH enzyme activity when fruit were stored under DCA - CD 1.1. According to Yang et al. (2016), the 1-MCP application increased the expression of *MdADH1* and suppressed the expression of *MdADH2* and *MdADH3*, showing that ADH1 is not ethylene-dependent and ADH 2 and 3 are ethylene-dependent.

When the oxygen partial pressure is reduced below the LOL, the fruit accumulate off-flavors, such as ethyl esters (Raffo et al., 2009; Wright et al., 2015). At the present study three ethyl ester were detected (ethyl acetate, ethyl propanoate and ethyl butanoate) (Figure 4). After 6 months, fruit stored under DCA - RQ and DCA - CD without 1-MCP had higher ethyl acetate as compared to CA and DCA - CF (Figure 4). With 1-MCP, fruit stored under DCA - RQ and DCA - CD 1.3 had higher ethyl acetate. The storage under DCA - RQ induced higher accumulation of ethyl acetate after 9 months of storage without 1-MCP (Figure 4). When fruit were treated with 1-MCP, the storage under DCA - RQ 1.5 and DCA - CD 1.1 resulted in the highest ethyl acetate concentrations. Ethyl acetate accumulation is a result of higher ethanol (Figure 5) and ethanoic acid accumulation (Figure 7), corroborating early studies (Echeverría et al., 2004; Thewes et al., 2018; Anese et al., 2018). A noteworthy fact is that no treatment resulted in accumulation of ethyl acetate higher than 13,500 μg L^{-1}, which is its odor threshold (Lopéz et al., 2007).

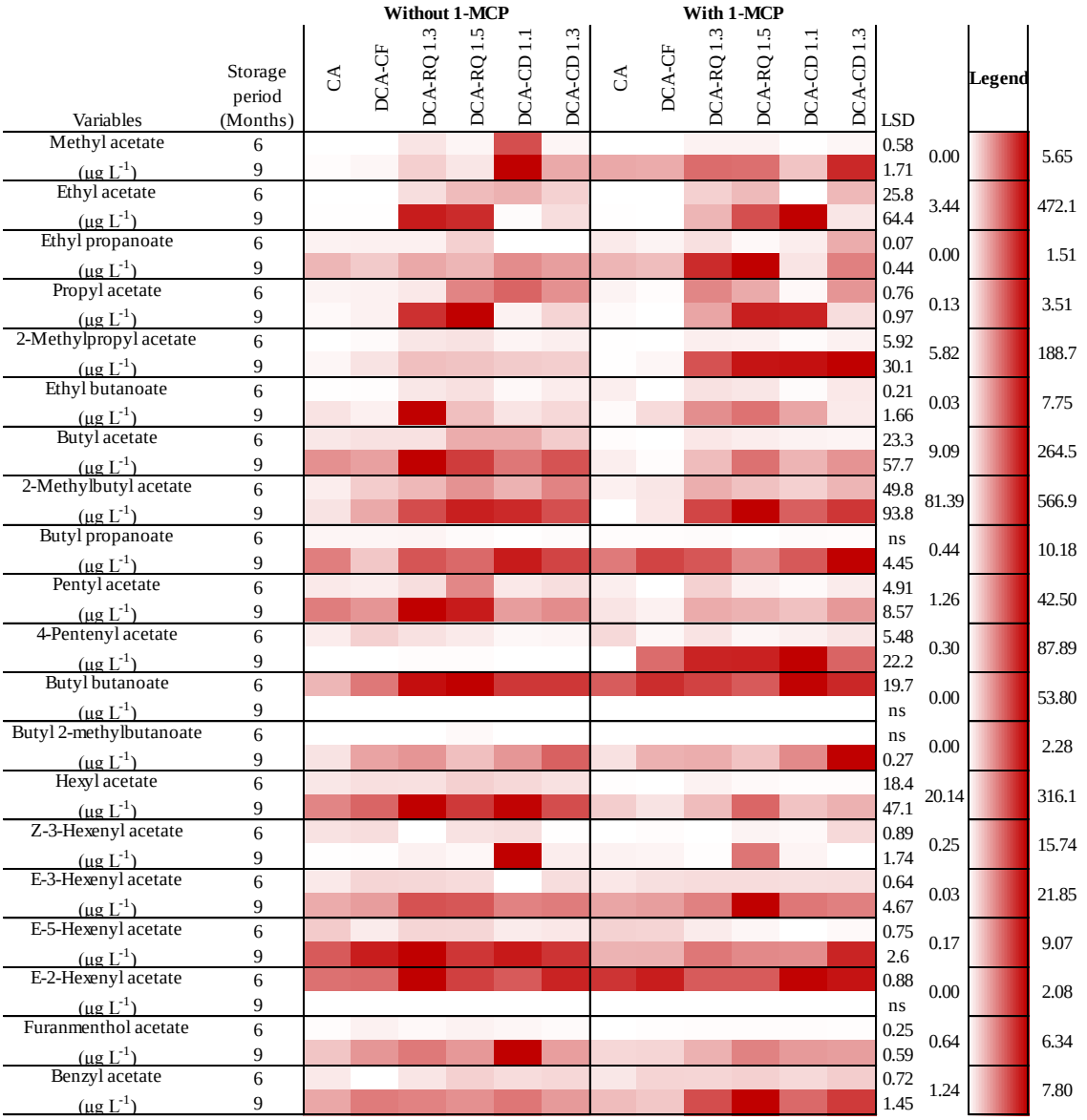


Figure 4. Ester concentration in 'Elstar' apples after 6 and 9 months of storage under controlled atmosphere (CA) and dynamic controlled atmosphere monitored by chlorophyll fluorescence (DCA - CF), by respiratory quotient (DCA - RQ) and by carbon dioxide (DCA - CD), either without or with 1-MCP treatment (0.650 µL L-1), plus 7 d of shelf life at 20°C. LSD = Fisher's lowest significant difference ($p<0.05$).

Ethyl propanoate was higher in fruit stored under DCA - RQ 1.5 after 6 months of storage without 1-MCP (Figure 4). When fruit were treated with 1-MCP, the storage under DCA - CD 1.3 resulted in higher ethyl propanoate. After 9 months of storage, fruit under DCA - RQ + 1-MCP application had higher ethyl propanoate concentration. With regards to ethyl butanoate, fruit under DCA - RQ 1.3 without 1-MCP had the highest concentration after 9 months of storage (Figure 4). This is a result of higher ethanol concentration in this storage

condition (Figure 5). Among all anaerobic metabolism compounds, only ethyl butanoate concentration was higher its odor threshold (1 μg L^{-1}), which confers to the fruit a fruity apple-like flavor. (Echeverría et al., 2003; Lopéz et al., 2007).

Analyzing the oxygen partial pressure variation over the storage period (Supplementary Figure 1) and the accumulation of anaerobic metabolism compounds (Figure 4, 5 and 6), is noteworthy that the new DCA method (DCA - CD) is efficient to LOL determination. These results show that the LOL could be monitored adequately by CO_2 production rate allowing the induction of anaerobic metabolism at safety levels by the employment of anaerobic metabolism factors higher than one, at the present study were tested two factors (CD 1.1 and CD 1.3). Anaerobic metabolism induction is very important in order to allow the accumulation of ethanol in pulp, which reduces ethylene production (Weber et al., 2019), increase the volatile compounds emission (Thewes et al., 2017a, b; Both et al., 2017), despite other benefits on overall fruit quality.

6.1.3.3.2. Main esters concentration

The main volatile compounds that confers to apple a fruity aroma are esters (Khomthong et al., 2006; Mehinagic et al., 2006; Lumpkin et al., 2015). Among all esters, butyl acetate, 2-methylbutyl acetate and hexyl acetate are the one that contribute most significantly to apple aroma (Young et al., 1996; Salazar and Orozco, 2011). The storage under DCA - CD 1.1 and DCA - RQ 1.5 resulted in higher butyl acetate concentration after 6 months when fruit were stored without 1-MCP (Figure 4). When fruit were treated with 1-MCP, there were no differences between storage conditions after 6 months. The higher butyl acetate in fruit stored under DCA - CD 1.1 and DCA - RQ 1.5 is a result of higher ethanoic acid concentration (Figure 7) and butanol in DCA - RQ 1.5 (Figure 5). A previous study showed that the ester formation is mainly dependent on its precursor concentration (Echeverría et al., 2004).

After 9 months, fruit stored under DCA - RQ and DCA - CD 1.3 without 1-MCP had the highest butyl acetate concentration, differing from the one stored under CA and DCA - CF (Figure 4). When fruit were treated with 1-MCP, again the storage under DCA - RQ and DCA - CD resulted in higher butyl acetate concentration, resulting in higher butyl acetate concentration as compared to CA without 1-MCP. A noteworthy fact is that the storage under DCA – CF resulted in the lowest butyl acetate concentration (Figure 4), corroborating early studies (Aubert et al., 2015; Thewes et al., 2017b; Both et al., 2017). Regardless the moment of evaluation and 1-MCP treatment, fruit stored under DCA - RQ and DCA - CD had better aroma

quality as compared to DCA - CF. The higher accumulation of butyl acetate is a due to higher butanol (Figure 5) and ethanoic acid concentration (Figure 7). A remarkable fact is that the oxygen partial pressures lowering to levels below 0.4kPa (Supplementary Figure 1) stimulates the accumulation of butyl acetate, probably because of the anaerobic metabolism raise. Thewes et al. (2017a) also observed that the induction of anaerobic metabolism is beneficial to butyl acetate accumulation. However, in the literature, almost all studies show that the oxygen lowering reduced the main ester accumulation (Lopéz et al., 2007; Raffo et al., 2009; Lumpkin et al., 2015).

2-Methylbutyl acetate is an important ester and its concentration is generally not negatively affected by the oxygen lowering (Both et al., 2014), because its precursors are amino acids and not lipids. When fruit were stored without 1-MCP, DCA RQ 1.5 and DCA - CD 1.3 had the highest concentration of 2-methylbutyl acetate after 6 months (Figure 4). On the other hand, after 9 months of storage, fruit under DCA - RQ 1.5 and DCA - CD 1.1 had higher concentration of 2-methylbutyl acetate, when compared to CA and DCA - CF (Figure 4). These results show that the storage under DCA, based on RQ or CD, results in fruit with higher 2-methylbutyl acetate as compared to DCA - CF, which is the DCA method most used nowadays (Figure 4). The concentration of 2-methylbutyl acetate is correlated to high amount of ethanoic acid accumulation (Figures 9 and 10), corroborating early studies (Both et al., 2014; Thewes et al., 2017b).

Lowering the oxygen partial pressure reduces more the straight-chain ester as compared to the branched-chain one (Brackmann et al. 1993; Both et al., 2014), being hexyl acetate the straight chain ester that generally is more affected by low oxygen storage (<1.0 kPa). After 6 months, there were little differences among CA and DCA methods, but 1-MCP application reduced it concentration in all storage conditions (Figure 4). The negative effect of 1-MCP on hexyl acetate is widely reported (Raffo et al., 2009; Yang et al., 2016). On the other hand, after 9 months, fruit stored under DCA - RQ 1.3 and DCA - CD 1.1 had the highest hexyl acetate when without 1-MCP. When fruit were treated with 1-MCP, storage under DCA - RQ 1.5 resulted in the highest and DCA - CF in the lowest hexyl acetate concentration (Figure 4). These results can be explained by the higher accumulation of 1-hexanol when fruit were stored under DCA - CD 1.1 and DCA - RQ 1.3 (Figure 5), which is a hexyl ester precursor (Holland et al., 2005; Souleyre et al., 2005).

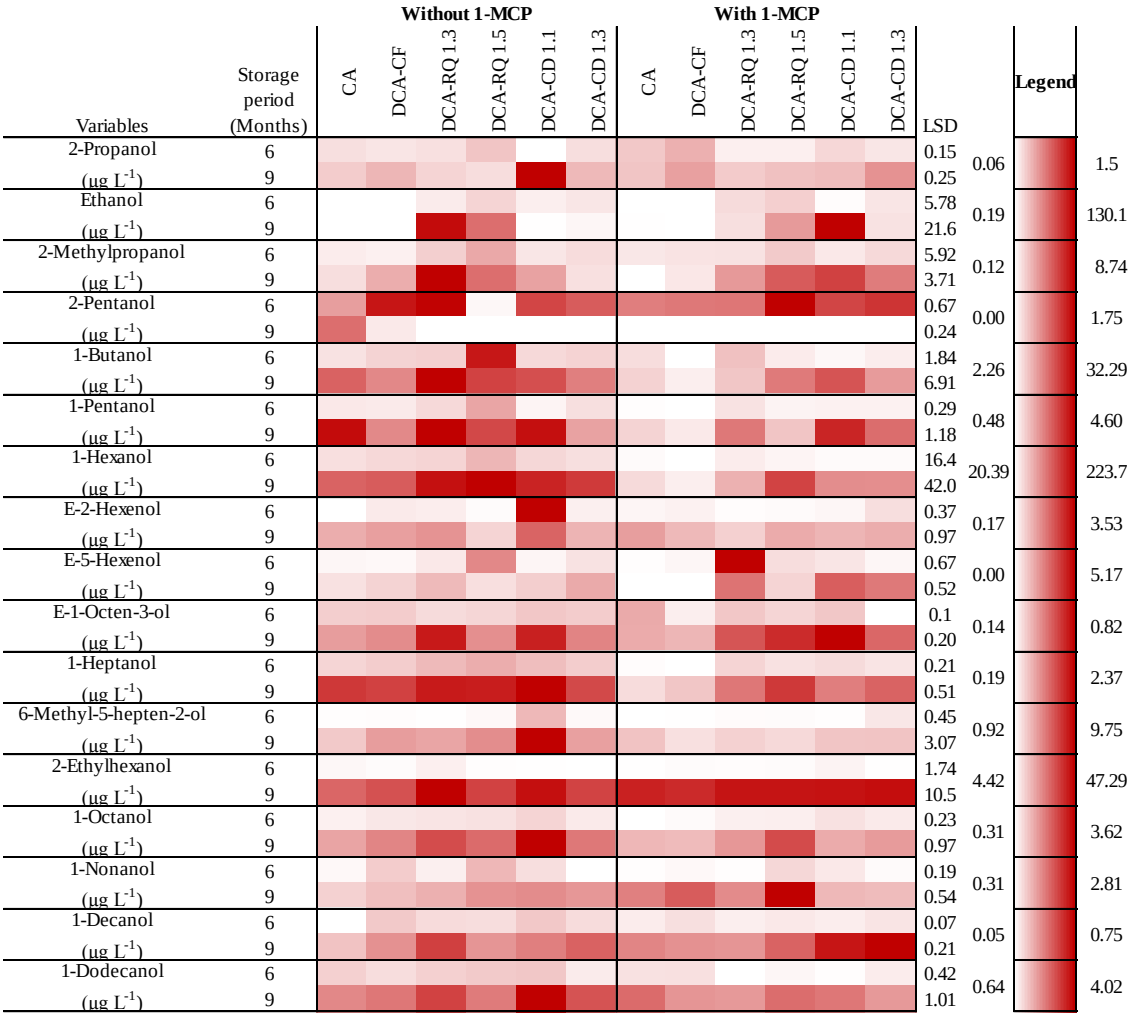


Figure 5. Alcohol concentration in 'Elstar' apples after 6 and 9 months of storage under controlled atmosphere (CA) and dynamic controlled atmosphere monitored by chlorophyll fluorescence (DCA - CF), by respiratory quotient (DCA - RQ) and by carbon dioxide (DCA - CD), either without or with 1-MCP treatment (0.650 µL L-1), plus 7 d of shelf life at 20°C. LSD = Fisher's lowest significant difference ($p<0.05$).

Analyzing the three main esters (butyl acetate, 2-methylbutyl acetate and hexyl acetate), is noteworthy that DCA - CD and DCA - RQ methods results in fruit with higher key aroma compounds when compared to CA and DCA - CF storage. These results showed that it is possible to implement DCA in commercial rooms without any additional device, using DCA - CD, and obtain similar results as DCA - RQ and much better than CA and DCA - CF stored fruit. In practical terms, it results in benefits for the storage companies, due to the reduced cost for DCA - CD implementation, and for consumers because the delivery of fruit with more aroma.

6.1.3.3.3. Other esters as influenced by the DCA storage methods

Methyl acetate concentration was higher when fruit were stored under DCA - CD 1.1 without 1-MCP (Figure 4). On the other hand, when fruit were treated with 1-MCP, DCA - CD 1.3 had higher methyl acetate concentration after 9 months. The accumulation of methyl acetate is generally associated with flesh breakdown (Lee et al., 2012), but on our study this was not verified, probably the association between methyl esters and flesh breakdown incidence is also cultivar-dependent. DCA - CD 1.1 stored fruit had higher propyl acetate when compared to CA and DCA - CF after 6 months of storage without 1-MCP (Figure 4). After 9 months, fruit stored under DCA - RQ without 1-MCP had higher propyl acetate, but when fruit were treated with 1-MCP, DCA - RQ 1.5 and DCA - CD 1.1 had higher propyl acetate.

With regards the other esters, in general its concentrations increase with the storage time, with exception of butyl butanoate, Z-3-hexenyl acetate, E-2-hexenyl acetate and 4-pentenyl acetate in fruit without 1-MCP (Figure 4). These results are in agreement with the literature, which report an increment of the ester with ripening advance (Brackmann et al., 1993; Echeverria et al., 2004; Bangerth et al., 2012; Thewes et al., 2017). Generally, the fruit stored under DCA - RQ and/or DCA - CD had higher ester concentration when compared to CA and DCA - CF.

6.1.3.3.4. Effects of DCA on the alcohol, aldehydes and acids accumulation

The most abundant alcohols detected in 'Elstar' apples were 1-hexanol, 1-butanol, ethanol and 2-ethylhexanol (Figure 5). Among these alcohols, 1-hexanol and 1-butanol contribute positively to the apple flavor (Echeverria et al., 2003; Khomthong et al., 2006; Mehinagic et al., 2006). 1-Hexanol concentration was higher when fruit were stored under DCA - RQ and DCA - CD over 9 months without 1-MCP, differing from fruit stored under CA and DCA - CF (Figure 5). When fruit were treated with 1-MCP, the lowest 1-hexanol was observed under DCA - CF. With regard to 1-butanol, fruit stored under DCA - RQ 1.5 had higher concentration after 6 months, but after 9 months fruit under DCA - RQ and DCA - CD 1.1 had higher concentration as compared to DCA - CF. When fruit were treated with 1-MCP, the storage under DCA - CD 1.1 resulted in higher 1-butanol concentration. These results contradict the literature, which report reduction of 1-hexanol and 1-butanol concentrations with low oxygen storage (Echeverría et al., 2008; Raffo et al., 2009; Both et al., 2014). Nevertheless, Thewes et al. (2017a) found an increase in 1-hexanol and 1-butanol by the storage of 'Galaxy'

apple under DCA - RQ 1.5, corroborating our results. A noteworthy fact is that when fruit were treated with 1-MCP, the storage under DCA - RQ and DCA - CD results in higher 1-hexanol and 1-butanol, showing that the increment of these alcohols has a relationship with the anaerobic metabolism induction. According to several early studies, the 1-MCP application reduced the production of these alcohols (Lee et al., 2012; Yang et al., 2016; Thewes et al., 2017). Additionally, our results show that under extremely low oxygen (Supplementary Figure 1) the accumulation of these alcohols is not ethylene-dependent (Figure 1 and 5).

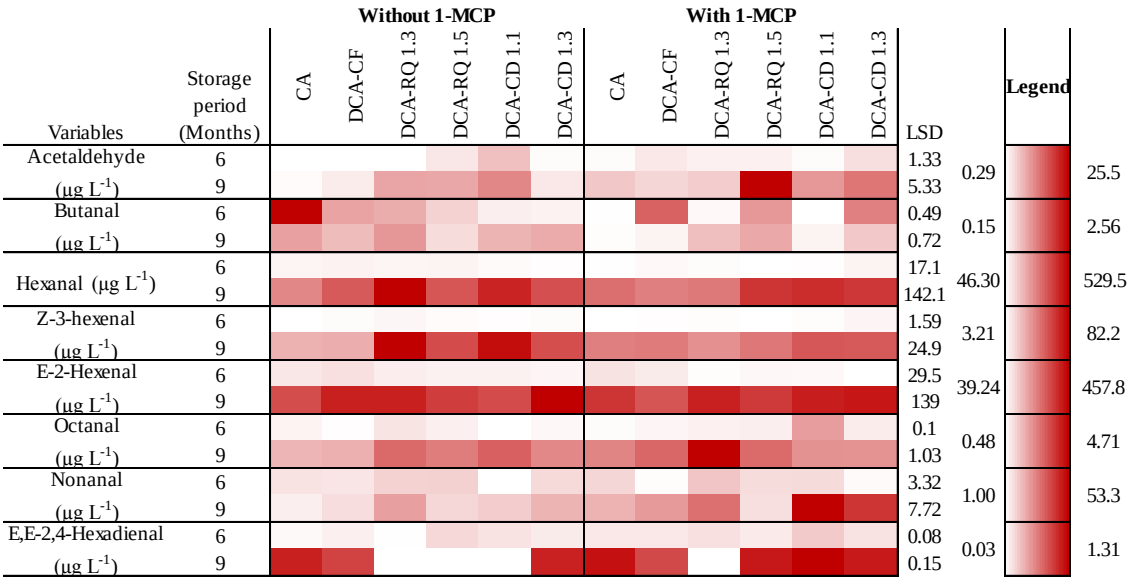


Figure 6. Aldehyde concentration in 'Elstar' apples after 6 and 9 months of storage under controlled atmosphere (CA) and dynamic controlled atmosphere monitored by chlorophyll fluorescence (DCA - CF), by respiratory quotient (DCA - RQ) and by carbon dioxide (DCA - CD), either without or with 1-MCP treatment (0.650 µL L-1), plus 7 d of shelf life at 20°C. LSD = Fisher's lowest significant difference ($p<0.05$).

Hexanal and E-2-Hexenal were the aldehydes with higher concentration after 9 months of storage (Figure 6). Fruit stored under DCA - RQ 1.3 and DCA - CD 1.1 over 9 months had higher hexanal concentration as compared to the other storage conditions, without 1-MCP. When fruit were treated with 1-MCP, the storage under DCA - CD and DCA - RQ 1.5 resulted in higher hexanal. E-2-hexenal was increased by the storage under DCA - CD 1.3 without 1-MCP application after 9 months. These compounds confers to the fruit an apple-green flavor (Khomthong et al., 2006; Mehinagic et al., 2006). Ketone concentration are affected by the storage conditions and their concentrations were higher after 9 months of storage (Figure 8).

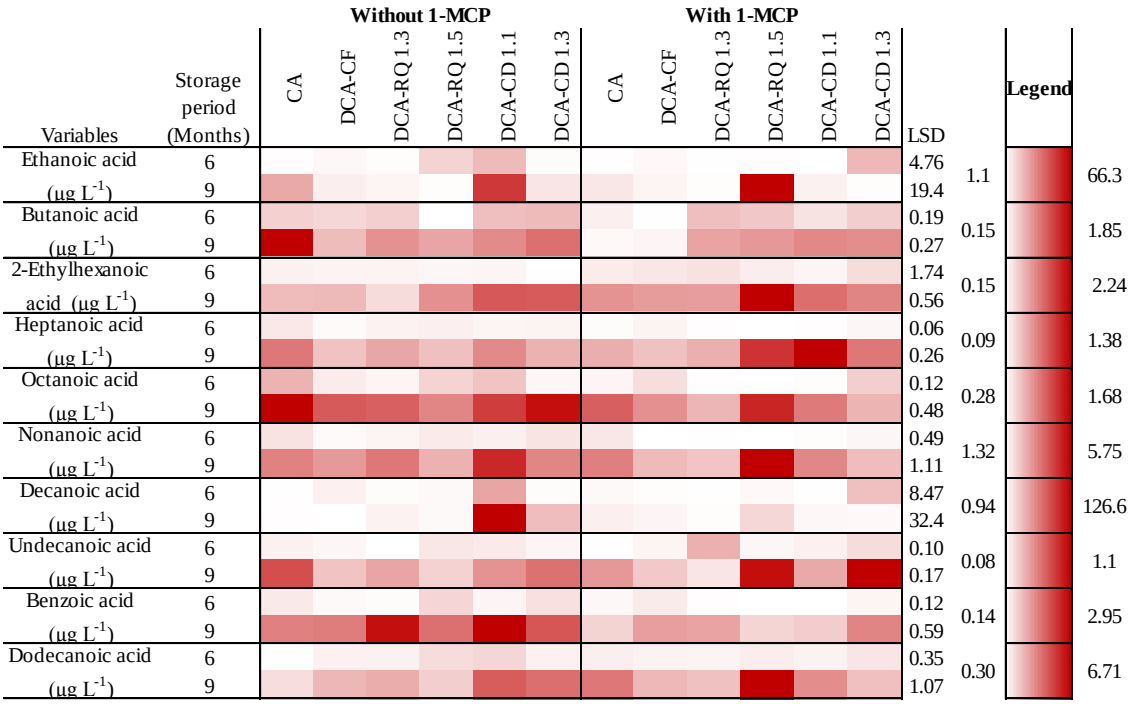


Figure 7. Volatile acids concentration in 'Elstar' apples after 6 and 9 months of storage under controlled atmosphere (CA) and dynamic controlled atmosphere monitored by chlorophyll fluorescence (DCA - CF), by respiratory quotient (DCA - RQ) and by carbon dioxide (DCA - CD), either without or with 1-MCP treatment (0.650 µL L-1), plus 7 d of shelf life at 20°C. LSD = Fisher's lowest significant difference ($p<0.05$).

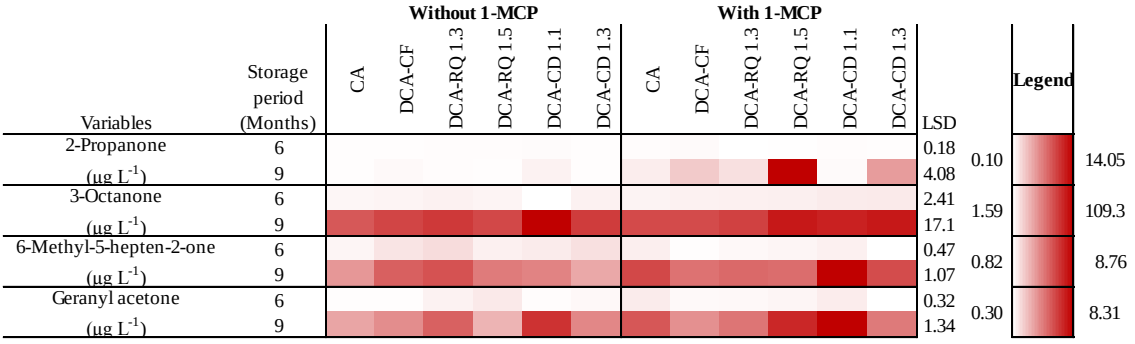


Figure 8. Ketone concentration in 'Elstar' apples after 6 and 9 months of storage under controlled atmosphere (CA) and dynamic controlled atmosphere monitored by chlorophyll fluorescence (DCA - CF), by respiratory quotient (DCA - RQ) and by carbon dioxide (DCA - CD), either without or with 1-MCP treatment (0.650 µL L-1), plus 7 d of shelf life at 20°C. LSD = Fisher's lowest significant difference ($p<0.05$).

6.1.3.4.Overview of the results – Principal component analysis (PCA)

To show an overview and the relationships between the variables evaluated, were undertaken two PCA analysis, one after 6 months of storage and another after 9 months of storage (Figure 9 and 10). Principal component one (PCI) and PCII explained together 44.8 % and 49% of the overall variable variation after 6 and 9 months of storage respectively. Along PCI were separated fruit stored under CA, DCA – RQ and DCA – CD after 6 and 9 months of storage (Figures 9 and 10). Alongside PCII were separated fruit without and with 1-MCP treatment (Figures 9 and 10). According to the results, the main volatile compounds concentrations were correlated to fruit stored under DCA – RQ 1.5 and DCA – CD 1.1 after 6 months of storage, but after 9 months higher volatile compounds were correlated to DCA – RQ1.3, DCA – RQ 1.5 and DCA – CD 1.1.

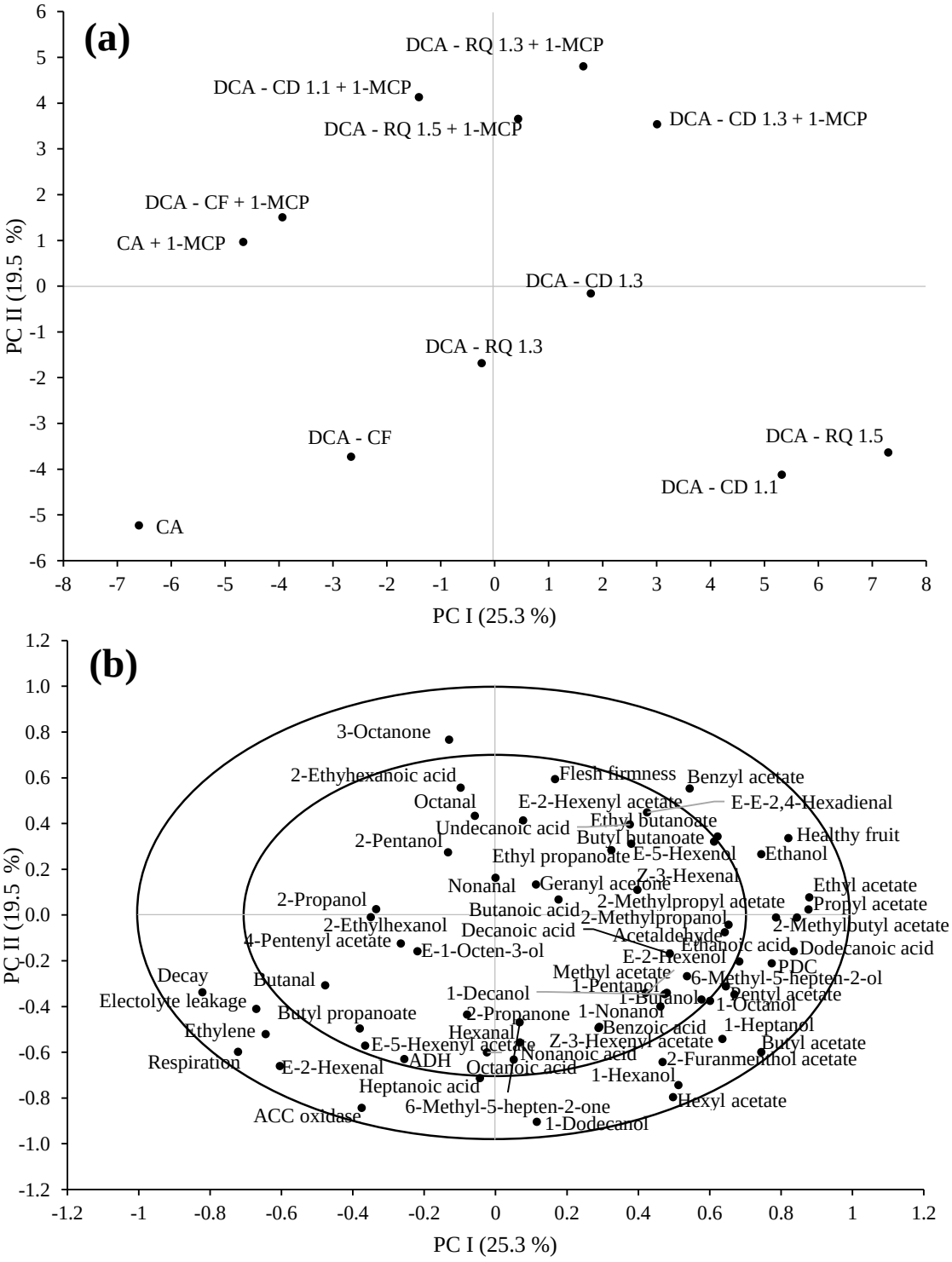


Figure 9. Principal component analysis (PCA) in 'Elstar' apples after 6 months of storage under controlled atmosphere (CA), dynamic controlled atmosphere monitored by chlorophyll fluorescence (DCA – CF), by respiratory quotient (DCA – RQ) and by carbon dioxide (DCA – CD), either without or with 1-MCP treatment (0.650 μL L^{-1}), plus 7 d of shelf life at 20°C.

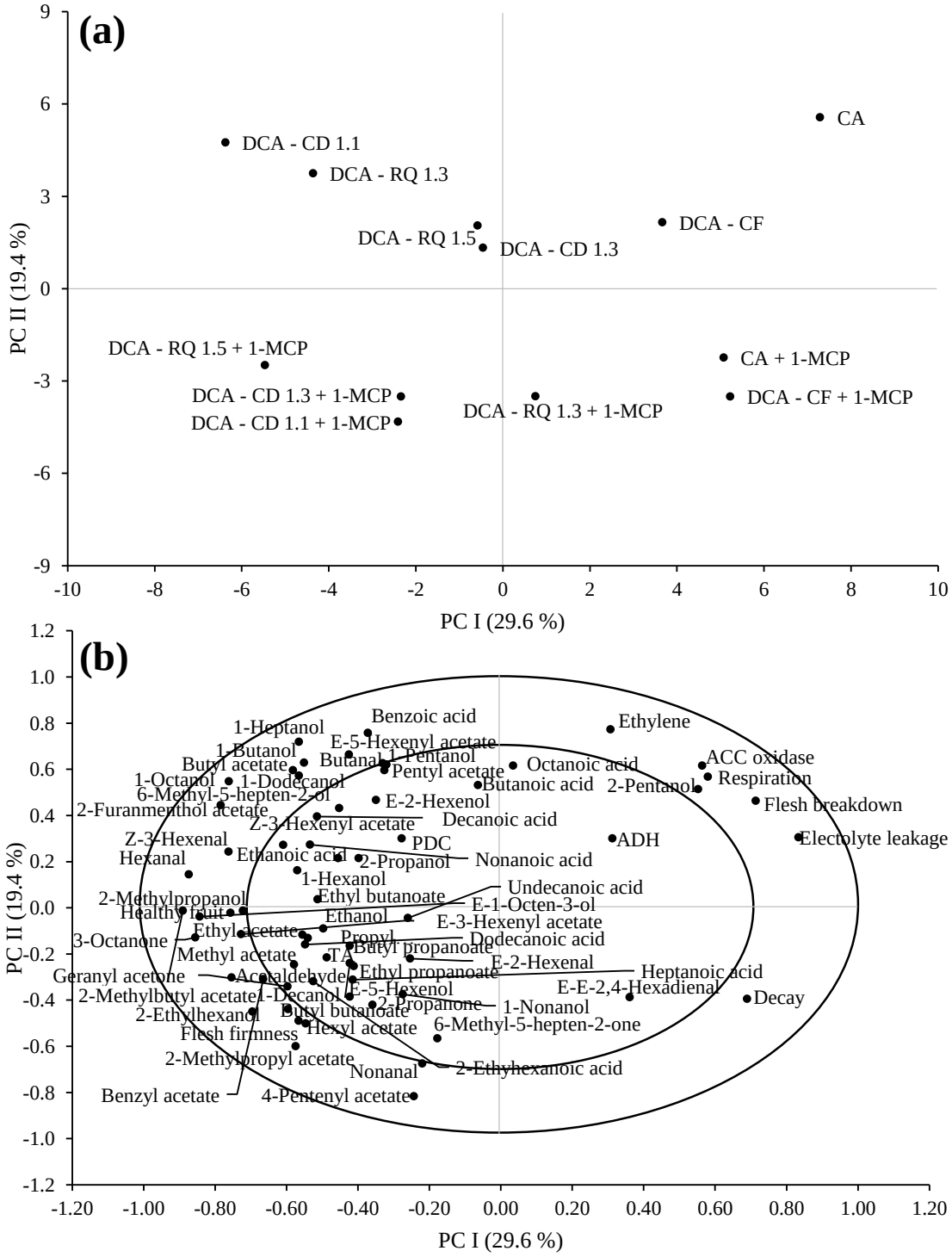


Figure 10. Principal component analysis (PCA) in 'Elstar' apples after 9 months of storage under controlled atmosphere (CA), dynamic controlled atmosphere monitored by chlorophyll fluorescence (DCA – CF), by respiratory quotient (DCA – RQ) and by carbon dioxide (DCA – CD), either without or with 1-MCP treatment (0.650 µL L^{-1}), plus 7 d of shelf life at 20°C.

Fruit stored under CA and DCA – CF were more correlated to ethylene production, respiration rate, decay incidence, flesh breakdown and electrolyte leakage, showing that fruit under these conditions were riper and had lower quality as compared to DCA – RQ and DCA – CD (Figures 9 and 10). 1-MCP conferred to the fruit a firmer pulp, but with lower volatile compounds accumulation, especially esters (Figure 9 and 10). These results evidence that the storage under DCA – CD and DCA – RQ result in fruit with flesh firmness similar as 1-MCP treatment, but with a more aromatic odour. This shows that these two DCA technologies are promising to allow firmness maintenance and a high concentration of aroma compounds, especially butyl acetate, 2-methylbutyl acetate and hexyl acetate, the main volatile of 'Elstar' apples. These results corroborate several early studies that reported higher key volatile compounds under DCA – RQ (Thewes et al., 2017b; Both et al., 2017).

6.1.4. Conclusions

A new type of dynamic controlled atmosphere based on CO_2 production (DCA - CD) was applied to store 'Elstar' apples over 9 months. The DCA - CD was able to predict accurately the LOL and induce anaerobic metabolism at safety levels, allowing the storage of 'Elstar' apples in oxygen partial pressures below 0.4 kPa.

The storage under DCA - CD resulted in fruit with lower ACC oxidase, ethylene production, decay, flesh breakdown incidence and higher flesh firmness and healthy fruit amount as compared to DCA - CF and CA and similar to DCA - RQ. 1-MCP application is not necessary when fruit were stored under DCA - CD or DCA - RQ.

PDC enzyme activity is increased by the storage under DCA - RQ 1.5 and DCA - CD 1.3, i.e. extremely low oxygen partial pressures, but ADH enzyme not. 1-MCP treatment inhibit PDC enzyme activity under DCA. Additionally, the accumulation of anaerobic metabolism compounds is little influence by ADH enzyme activity.

Concentration of butyl acetate, 2-methybutyl acetate and hexyl acetate, the most important ester of apple aroma, increased by the storage under DCA - CD, especially CD 1.1, and are comparable to DCA – RQ storage.

6.1.5. References

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7. ARTIGO 5

7.1. METABOLIC PROFILING OF ‘ELSTAR’ AND ‘NICOTER’ APPLES: IMPACT OF STORAGE TIME, DYNAMIC CONTROLLED ATMOSPHERE METHOD AND 1-MCP TREATMENT[5]

Abstract

The aim of this work was to evaluate the effect of controlled atmosphere (CA), dynamic controlled atmosphere based on chlorophyll fluorescence (DCA – CF), respiratory quotient (DCA – RQ) and CO_2 release (DCA – CD) on sugars, tricarboxylic acid cycle (TCA), anaerobic metabolism and some volatile compounds of ‘Elstar’ and ‘Nicoter’ apples after harvest, 6 and 9 months of storage plus 7 days of shelf life at 20 °C. We also aimed to evaluate the effect of ethylene action blocking by 1-MCP in order to understand the effect of ethylene on these variables when fruit were stored under CA and DCA. The storage conditions tested for both cultivars were: [1] CA; [2] DCA – CF; [3] DCA – RQ 1.3; [4] DCA – RQ 1.5; [5] DCA – CD 1.1; [6] DCA – CD 1.3. All storage conditions were without and with 1-MCP treatment (0.650 ppm). The lowest oxygen limit (LOL) was higher for ‘Nicoter’ apples and the three DCA methods were able to detect this difference between cultivars, but LOL estimation was lower when fruit were stored under DCA – RQ and DCA – CD, due to the anaerobic metabolism induction. Sorbitol had a trend of accumulation when fruit were stored under DCA – RQ and DCA – CD, especially in higher RQ and CD with higher anaerobic metabolism factor (AMF), showing a negative Pearson correlation with the oxygen partial pressure over the storage period. 1-MCP treatment induced sorbitol accumulation even when fruit were stored under CA. TCA intermediaries, such as citrate, 2-oxoglutarate, succinate, fumarate and oxaloacetate, are the most affected by the atmosphere conditions and 1-MCP treatment for both cultivars. These acids generally have a positive Pearson correlation among each other. Malic acid is more affected by the storage time than by the storage conditions. Succinate and fumarate have an accumulation trend when fruit are stored under DCA – RQ, i.e., lowest oxygen partial pressures.

Keywords: *Malus domestica;* anaerobic metabolism; sugar metabolism; acids metabolism.

[5] Artigo formatado de acordo com as normas da revista Postharvest Biology and Technology.

7.1.1. Introduction

During postharvest life of apple fruit, the respiratory process is important to maintain fruit energy pool and cellular activity (Mbong Victor et al., 2017; Steffens et al., 2007). However, if it is too high, fruit quality will be reduced due to reserves starvation, such as sugars and organic acids (Mbong Victor et al., 2017). To decrease fruit metabolism, the storage temperature is reduced and gaseous atmosphere changed resulting in a so-called controlled atmosphere storage (CA). Under CA storage, the oxygen partial pressure is reduced near 1.0 kPa and carbon dioxide is increase to partial pressures near 2.0 kPa, depending on the cultivar (Brackmann et al., 2015; Lumpkin et al., 2014). The change in temperature and atmosphere composition affects on electron transport chain, tri-carboxylic acid cycle (TCA), sugars and anaerobic metabolism, suppressing overall fruit metabolism and extending postharvest life of fruit.

Oxygen partial pressure reduction is effective to decrease sugar and acids consumption until a certain level, which is the anaerobic compensation point (ACP), point with minimal CO_2 release (Boersig et al., 1988; Wright et al., 2015). If O_2 partial pressure is reduced below ACP, anaerobic metabolism is started, increasing glycolysis speed and consequently sugar consumption (Thewes et al., 2019), and production of anaerobic metabolism compounds, such as acetaldehyde and ethanol (Boeckx et al., 2019; Gasser et al., 2008; Thewes et al., 2017b; Weber et al., 2017), which can cause damages to the fruit. Thus, is important the use of methods to detect the lowest oxygen limit (LOL) during the storage under oxygen partial pressures below 1.0 kPa, in order to avoid low O_2 damages and changes in sugar, TCA and anaerobic metabolism.

The LOL can be monitored in real time during storage based on several fruit physiological responses. There are methods monitoring the production and accumulation of anaerobic metabolism compounds (Deuchande et al., 2016; Veltman et al., 2003), chlorophyll fluorescence (DCA – CF) (Wright et al., 2012), respiratory quotient (DCA – RQ) (Gasser et al., 2008; Weber et al., 2015) and, more recently, based on CO_2 release of fruit (DCA – CD) (Thewes et al., 2019). However, there are no reports in the literature evaluating the effect of these technologies, especially DCA – RQ and DCA – CD, on the dynamics of TCA acids, sugars and the switch between aerobic and anaerobic metabolism after long-term storage. In the literature, it is showed that the accumulation of TCA and derived acids, such as glutamate and succinate, are induced by the use of high CO_2 partial pressure (3.0 kPa) (Bekele et al., 2016),

but little is known about the effect of extremely low oxygen (<0.4 kPa) under DCA. Additionally, with the new DCA method based only on CO_2 release (DCA – CD), it is possible to modulate the CO_2 release of fruit to a minimal level using a low anaerobic metabolism factor (Thewes et al., 2019) and, consequently, maintain the oxygen partial pressure as close as possible to the ACP. With this method, it could be possible to reduce the aerobic metabolism to the lowest level possible, inducing anaerobic metabolism of fruit, and consequently reducing TCA and sugar consumption to minimal.

Another technology worldwide used to further reduce fruit metabolism is the ethylene action blocking by 1-methylcyclopropene (1-MCP) (Lee et al., 2012; Sisler and Serek, 1997; Watkins, 2006). According to Bekele et al. (2015), the 1-MCP application effects on sugars and organic acids metabolism, delaying the consumption of some TCA intermediaries, when 'Jonagold' apples were stored under regular atmosphere and CA. However, under extremely low oxygen partial pressures, as in DCA, the effect of 1-MCP on sugar and TCA metabolism is still not clear. Early studies showed that 1-MCP application suppresses the anaerobic metabolism of 'Galaxy', 'Pink Lady' and 'Fuji' apples stored under DCA – RQ 1.5 (oxygen below 0.3 kPa) (Thewes et al., 2018), but those work did not specified to what pathway the fruit metabolism is switch when treated with 1-MCP. According to Busatto et al. (2018), the 1-MCP application effects on sugar metabolism of 'Granny Smith' apples, especially sorbitol, which is increased when fruit were treated with 1-MCP and stored under low temperature conditions. Thewes et al., (2019) verified that sorbitol also accumulate when fruit were stored under extremely low oxygen partial pressures (DCA - RQ), they hypothesized that sorbitol accumulation could be an additional way for nicotinamide adenine dinucleotide (NADH) oxidation and protect the cell membrane from leakage. Thus, the evaluation of a combined effect of DCA plus 1-MCP treatment would be helpful to understand how fruit metabolism runs under extremely low oxygen partial pressures and, the effect of ethylene action under these conditions.

The aim of this work was to evaluate the effect of CA, DCA – CF, DCA – RQ and DCA – CD on sugars, TCA, anaerobic metabolism and some volatile compounds of 'Elstar' and 'Nicoter' apples after harvest, 6 and 9 months of storage plus 7 days of shelf life at 20 °C. We also aimed to evaluate the effect of ethylene action blocking by 1-MCP in order to understand the effect of ethylene on these variables when fruit were stored under CA and DCA.

7.1.2. Material and methods

7.1.2.1. Fruit harvest and storage conditions

Two experiments were performed over the season 2017 – 2018 at Competence Centre for Fruit Growing at Lake Constance (KOB) Germany, with apples of the cultivars Elstar and Nicoter. The fruit were harvest at optimal maturity for long-term storage. At harvest, the fruit had a Streif index of 1.79 for 'Elstar' and 1.76 for 'Nicoter' apples. Immediately after harvest, the fruit were submitted to a selection process, and performed samples of 20 fruit each. Four samples were used in each treatment.

After the sampling process, fruit were cooled to 5 °C, maintained during one week, and thereafter cooled down to the storage temperature, 3 °C for 'Nicoter' and 1 °C for 'Elstar'. Fruit were stored in chambers of 250 L. These chambers were gas-tight, to allow the application of CA and DCA conditions. The storage conditions tested for both cultivars were: [1] CA; [2] DCA – CF; [3] DCA – RQ 1.3; [4] DCA – RQ 1.5; [5] DCA – CD 1.1; [6] DCA – CD 1.3. All storage conditions were without and with 1-MCP treatment (0.650 ppm). The CO_2 concentration was 2.0 and 1.0 kPa for CA stored 'Elstar' and 'Nicoter' apples, respectively. For DCA, the CO_2 partial pressure was 1.2 and 1.0 kPa for 'Elstar' and 'Nicoter' apples, respectively. Each chamber was connected to an automatic CA control system (Isollcel®, Bolzano Italy), to continuously control of atmosphere inside the chambers. The oxygen partial pressure variation for both cultivars and storage conditions are shown in Figure 1.

7.1.2.2. 1-MCP treatment

To treat fruit with 1-MCP, they were put into a chamber together with a solution containing 1-MCP (SmartFresh®, 0.14% of active ingredient). The dose used was 0.650 ppm. Fruit were exposed to 1-MCP treatment during 24 h at the temperature 5°C and thereafter stored in the atmosphere condition described in section 7.1.2.1.

7.1.2.3. Storage temperature and relative humidity

Over the nine months of storage, the temperature was monitored daily with the aid of mercury thermometers. It was maintained at 3 ± 0.1 °C for 'Nicoter' and 1 ± 0.1 °C for 'Elstar' apples. The relative humidity was set at 94 ± 2 % by adding a container with calcium chloride (7.5 g kg^{-1} of fruit) in each chamber.

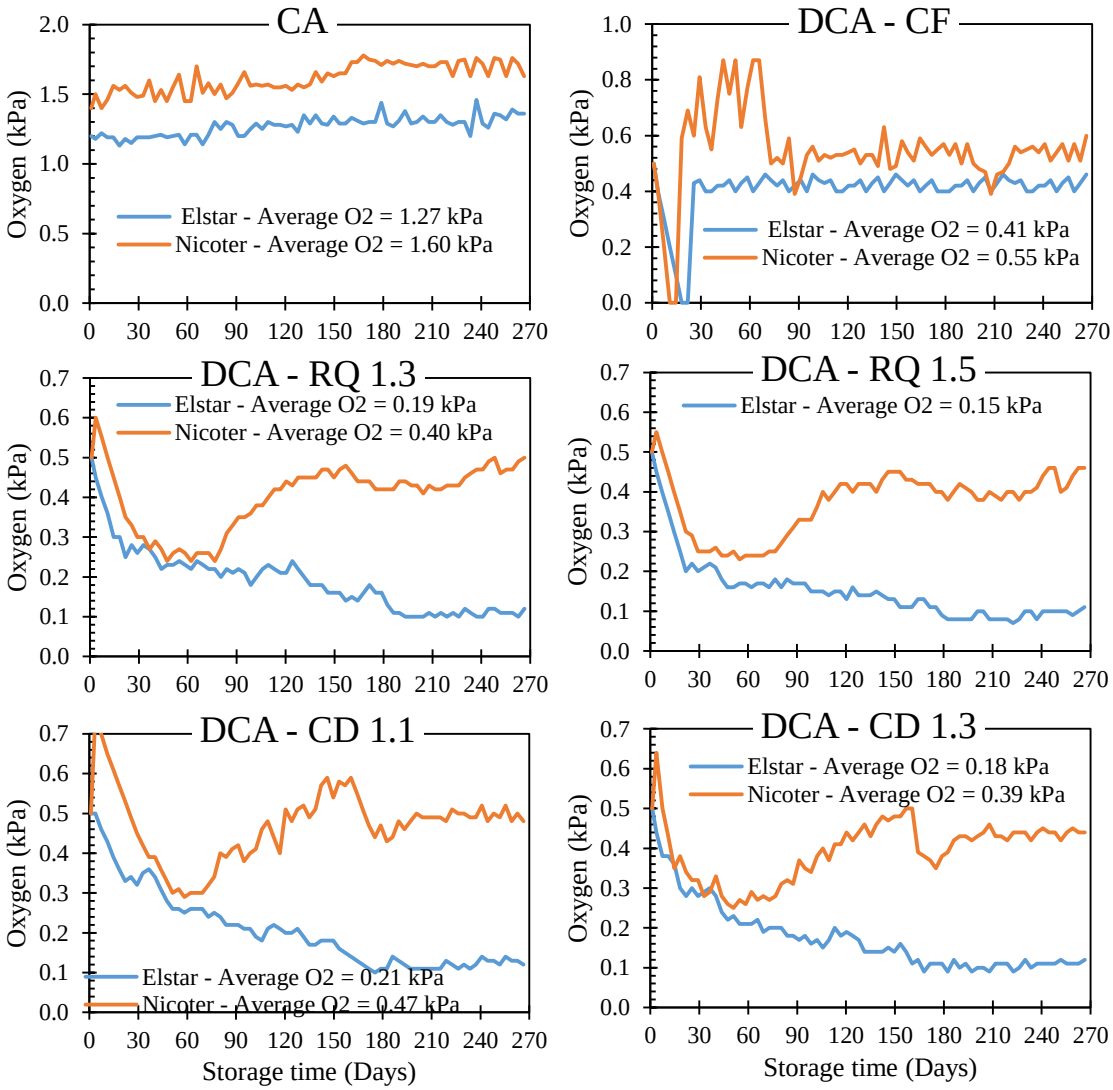

Figure 1. Oxygen set point variation for fruit stored under controlled atmosphere (CA) and dynamic controlled atmosphere based on chlorophyll fluorescence (DCA – CF), on respiratory quotient (DCA – RQ) and on carbon dioxide (DCA – CD) over 9 months at a temperature of 1 °C for 'Elstar' and 3 °C for 'Nicoter'.

7.1.2.4. Monitoring fruit metabolism

Fruit bio-response to low oxygen partial pressure was monitored based on chlorophyll fluorescence (DCA – CF), respiratory quotient (DCA – RQ) and CO_2 release (DCA – CD). For DCA – CF two batches of 6 fruit were monitored with a CF sensor in real time throughout the storage period (Wright et al., 2010). To perform the DCA – RQ method, the experimental chambers were closed for 13 to 14h, and the O_2 and CO_2 concentration measured before and after this period. The ratio between CO_2 release and O_2 uptake was calculated to give the RQ

(Weber et al., 2015). Two RQ treatments: 1.3 and 1.5 were tested in this experiment, and the RQ was calculated two times a week. When the RQ was below the setpoint, the pO_2 was decreased, and if the RQ was above the setpoint, the pO_2 was increased. To monitor the optimal oxygen partial pressure based only on CO_2 production, the method described by Thewes et al. (2019) was used (article 3).

7.1.2.5. Moments of metabolite evaluation

The ethylene production, respiration rate, sugars, TCA acids, anaerobic metabolism and some volatiles were evaluated after harvest, 6 and 9 months of storage plus 7 days of shelf life at 20 °C, as described below.

7.1.2.5.1. Ethylene production and respiration rate

Ethylene production and respiration rate were determined on eight fruit per replicate. Each replication was enclosed in an airtight glass container (4.25 L) for 2 h. Afterwards, 1 mL of headspace gas was injected into a gas chromatograph (Carlo Erba, Fractovap Series 2150, Electrometer, Mod. 180, Milano, Italy) equipped with a flame ionization detector and a stainless-steel column of 0.9 m × 1/8 in. packed with activated aluminium oxide (60 mesh). The oven temperature was set to 100 °C and the injector temperature to 175 °C. Nitrogen was used as carrier gas. Ethylene production was calculated based on a standard, and results showed as ng kg^{-1} s^{-1}. The respiration rate was measured with an infrared gas analyser (Hartmann and Braun GmbH, Germany) with a flow rate of 17 L h^{-1} using the same experimental setup as already described for ethylene. Results expressed in µg kg^{-1} s^{-1}.

7.1.2.5.2. Sample preparation for metabolite evaluation

All metabolites were evaluated in juice obtained from 20 apples per replicate. Before juice extraction, fruit were cooled to 0 °C. Immediately after pulp cooling, horizontal slices of the equatorial region of fruit were taken, discharged the seeds, and centrifuged under low temperature, to avoid the chemical and enzymatic oxidation of samples (the maximum juice temperature during sample preparation was 5 °C). For sugars and TCA intermediaries´ evaluation, 500 µL of juice were put into tubs of 2 mL, and mixed with 1500 µL of cold bi-distillated water. This solution was filtered with a 45-µm nylon filter, frozen in liquid nitrogen

and stored under -30 °C until analyses. To evaluate the anaerobic metabolism compounds and volatiles, 10 mL juice samples were taken and immediately put into 20 mL vials and frozen to -30 °C until analysis.

7.1.2.5.3. Individual sugars measurement

Determined with high performance liquid chromatography (HPLC, Bischoff, Germany), adapted method from Wang et al. (2010). Sugars were analyzed isocratically on the 305 × 7.8 mm HC-75 Ca^{2+} form cation exchange column (Hamilton, USA). The mobile phase was Bi-distillated water at constant flow of 0.4 mL min^{-1}. The temperature of the column was isothermal at 80 °C. A reflectance index detector (Bischoff, Germany) was used to determine the concentration of each sugar. The time for each sample run was 32 min. The individual sugars (sucrose, glucose, fructose and sorbitol) were identified and quantified by the comparison of retention times and the area of standards for each compound.

7.1.2.5.4. Organic acid determination

Evaluated with high performance liquid chromatography (HPLC, Bischoff, Germany), with a method adapted from Wang et al. (2010). Acids were determined on a Rezex RDA-organic acid H^+ column (Phenomenex, USA), 7.8 mm × 300 mm. The mobile phase was H_2SO_4 (50 mmol L^{-1}) at constant flow of 0.5 mL min^{-1}. The temperature of the column isothermal was 70 °C. A UV detector at 210 nm (Bischoff, Germany) was used to determine the acids concentrations. Acids were identified and quantified by comparison of the retention time and area of standards. The time for each sample run was 18 min.

7.1.2.5.5. Volatile metabolites evaluation

The vial containing 10 mL of this juice was taken, mixed with 3 g NaCl and 10 µL of 3-octanol standard solution (81.8 µg mL^{-1}) inside a 20 mL vial that was hermetically sealing with a PTFE-coated silicone lid. From this solution, the metabolites were extracted from the headspace via solid phase microextraction (HS-SPME). A Divinylbenzene/carboxen/polydimethylsiloxane (DVB/Car/PDMS) fibre (Supelco, 50/30 µm × 20 mm) was preconditioned following the manufacturer protocol. Before the fiber exposing, the vial was submerged into a water bath at 35 °C during 5 min. After 5 min, the fiber was

exposed to the headspace of the sample during 60 min under constant stirring at the same temperature.

Metabolites were quantify and identify on a Shimadzu QP2010 Plus gas chromatography coupled to mass spectrometry (GC/MS; Shimadzu Corporation, Kyoto, Japan). The fiber was thermally desorbed into the injection port at a temperature of 250 °C for 10 min, in a split less mode. The compounds were separated in a polar fused silica Zebron capillary column; ZB-WAX, 30 m × 0.25 mm i.d., 0.25 μm film thickness (Phenomenex, Aschaffenburg, Germany). Helium was used as a carrier gas at a constant flow rate 1.2 mL min^{-1}. The initial column temperature was set at 35 °C and held for 3 min. Then, a temperature gradient of 2 °C min^{-1} was started until 80 °C, followed by a 5 °C min^{-1} increase until 230 °C, and maintained at isothermal conditions for 5 min. The detector was operated in the electron impact ionization mode with an ionization energy of +70 eV and a scan mass range from 35 to 350 m/z, at a temperature of 230 °C. A series of homologous saturated n-alkanes (C7 – C30) was analyzed under the same conditions to calculate a linear retention index (LRI). The concentration of all compounds were determined by internal standardization, according to the method proposed by Both et al. (2014).The analytes were identified based on comparison with standards, mass spectra available in the National Institute of Standards and Technology (NIST) library and by comparing the calculated LRI with those available in the scientific literature.

7.1.2.6. Statistical analysis

A variance analysis (ANOVA) at 5% of error probability was carried out. Data that showed significant difference by ANOVA were subjected to the LSD test at 5% error probability. The experiment was conducted in a completely randomized scheme with a factorial arrangement. Additionally, a Pearson correlation network analyses was carried out, in order to show the linear relationships between the variables evaluated. This analysis was performed with CytoScape® using the app MetScape® (Karnovsky et al., 2012).

7.1.3. Results and discussion

7.1.3.1. Oxygen set point variation over the storage period

Over the 9 months of storage, the fruit response to low oxygen stress was monitored based on three methodologies, DCA – CF, DCA – RQ and DCA – CD, and the results are shown

in figure 1. All three DCA methods resulted in fruit with lower oxygen partial pressure as compared to CA stored one (Figure 1). A noteworthy fact is that the two cultivars had a completely different response to low oxygen partial pressure, with higher LOL for ‘Nicoter’ apples. Probably, the higher oxygen setpoint of ‘Nicoter’ apples is because this cultivar does not adapt to extremely low oxygen partial pressure (Thewes et al., 2019) and has low skin permeability (Wang et al., 2020). The oxygen setpoint behavior was similar when fruit were stored under DCA – RQ and DCA – CD for both cultivars (Figure 1). These results are extremely important, because they show that the new DCA method (DCA - CD) had the capability to detect accurately the LOL over the storage period in a similar way as compared to DCA – RQ. Additionally, our results evidenced that the LOL can be accurately monitored by measuring only the CO_2 release rate of fruit by DCA – CD. In practical terms, this is very important because the CO_2 release rate can be measured in rooms without any additional device.

7.1.3.2. Ethylene production and respiration rate of fruit

Ethylene production increased from harvest to 9 months of storage, when fruit were stored under CA without 1-MCP for both cultivar (Figure 2). However, when fruit were stored under DCA or treated with 1-MCP, the ethylene production increased less for ‘Elstar’ and reduced for ‘Nicoter’ apples. Probably, the lower ethylene production when fruit were stored under DCA is a result of low oxygen partial pressure during storage (Figure 1). This happen because the oxygen is necessary to produce ethylene from ACC (Both et al., 2017; Thewes et al., 2017b; Weber et al., 2017; Yang and Hoffman, 1984). On the other hand, the effect of DCA on ethylene production can be a result of ethanol accumulation (Jin et al., 2013; Liu et al., 2012; Pesis, 2005; Weber et al., 2020, 2016). A noteworthy fact is that the 1-MCP application had no effect on ethylene production when fruit were stored under DCA – RQ 1.5 and DCA – CD 1.3 for ‘Elstar’ apples and all DCA conditions for ‘Nicoter’ apples. For ‘Elstar’ apples stored under CA, DCA – CF, DCA – RQ 1.3 and DCA – CD 1.1, the 1-MCP application reduced ethylene production, especially after 9 months of storage (Figure 2).

To evaluate if a storage condition is effective to reduce fruit metabolism is important to evaluate respiration rate (CO_2 release) during shelf life (Steffens et al., 2007). The storage under DCA was more effective in fruit metabolism reduction from harvest to 6 months of storage as compared to CA, but respiration rate increased from 6 to 9 months of storage, evidencing an increase in fruit metabolism, regardless 1-MCP treatment (Figure 2). The lower respiration of fruit stored under DCA, with the exception of DCA – RQ 1.5 for ‘Nicoter’ apples, is due to

lower oxygen partial pressure storage (Figure 1). Several early studies reported a reduced respiration rate by the storage under DCA (Both et al., 2017; Thewes et al., 2017b; Weber et al., 2015). Nevertheless, the three DCA methods effects differently on the switch between aerobic and anaerobic metabolism as discussed below, and the storage of 'Nicoter' apples under DCA – RQ 1.5 increased the respiration rate due to high anaerobic metabolism after 6 months of storage (Figure 4), corroborating the results of the literature (Thewes et al., 2019; Weber et al., 2019). A noteworthy fact is that the 1-MCP treatment had no effect on respiration rate during shelf life, regardless the storage condition, showing that its applications did not reduce the respiration further when 'Nicoter' apple were stored under low oxygen partial pressures (Figure 1).

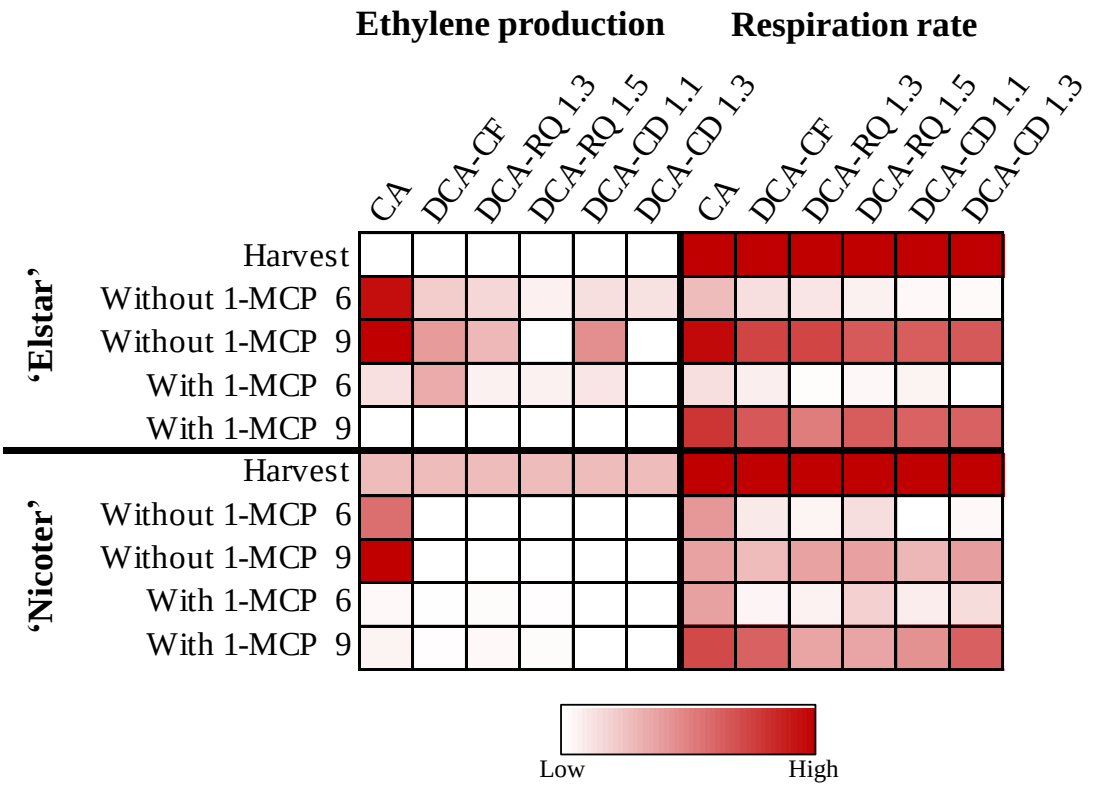

Figure 2. Ethylene production and respiration rate of 'Elstar' and 'Nicoter' apples after harvest, 6 and 9 months of storage under controlled atmosphere (CA) and dynamic controlled atmosphere monitored by chlorophyll fluorescence (DCA – CF), by respiratory quotient (DCA – RQ) and by carbon dioxide (DCA – CD), either without or with 1-MCP treatment (0.650 µL L^{-1}), plus 7 d of shelf life at 20°C.

7.1.3.3. Effect of CA, DCA and 1-MCP on metabolite change over the storage period

CA and DCA storage conditions changed the metabolism (CO_2 release) of apples over the storage period (Figure 2), but little is known about the dynamics of sugars, acids and anaerobic metabolism compounds when fruit were stored under DCA, especially DCA – RQ and DCA – CD. Thus, we evaluate the effect of CA and DCA, without and with 1-MCP, on the dynamics of sugars, TCA and anaerobic metabolites (Figures 3 and 4). Sucrose concentration increased from harvest to 6 months of storage, regardless of the atmosphere condition and 1-MCP treatment, remaining constant thereafter for 'Elstar' apples. However, for 'Nicoter' apples, the sucrose concentration increase from harvest to 6 months of storage and thereafter decrease at the evaluation made after 9 months of storage (Figure 4). In the literature there are studies reporting that sucrose content reduces (Wang et al., 2010) or increase during fruit ripening (Doerflinger et al., 2015) in normal atmosphere. We showed that in CA and DCA storage the sucrose content increase from harvest to 6 months of storage, either with or without ethylene action blocking with 1-MCP. According to Doerflinger et al. (2015), this sucrose increase is due to starch degradation, which release glucose, fructose and sucrose.

Sucrose is a disaccharide composed by fructose and glucose. Glucose concentrations were higher at harvest for both cultivars (Figure 3 and 4). These results corroborate (Thewes et al., 2019), which found a reduction of glucose from harvest until the end of storage. From 6 to 9 months of storage, the glucose concentration remained almost constant, with some influence of the storage condition. For 'Elstar' apples stored under DCA – CD, after 6 and 9 months, and DCA – RQ 1.5 after 9 months, the 1-MCP treatment resulted in higher glucose content, when compared to fruit without 1-MCP (Figure 3). When fruit were treated with 1-MCP, the storage under DCA – CD over 6 months and DCA – RQ 1.5 over 9 months, resulted in higher glucose content as compared to the other treatments (Figure 3). In 'Jonagold' apples, the 1-MCP treatment resulted in accumulation of glucose over the storage period in normal atmosphere (20.9 kPa O_2 + 0.04 kPa CO_2) (Bekele et al., 2015). However, 'Nicoter' stored under DCA – CF, DCA – RQ 1.3 and DCA – CD had higher glucose content after 9 months of storage without 1-MCP, differing from CA and DCA – RQ 1.5 (Figure 4). The 1-MCP treatments had no effect on glucose content for 'Nicoter' apples (Figure 4). Thewes et al. (2019) reported that apples stored under DCA consumed first glucose and thereafter fructose, corroborating the present results.

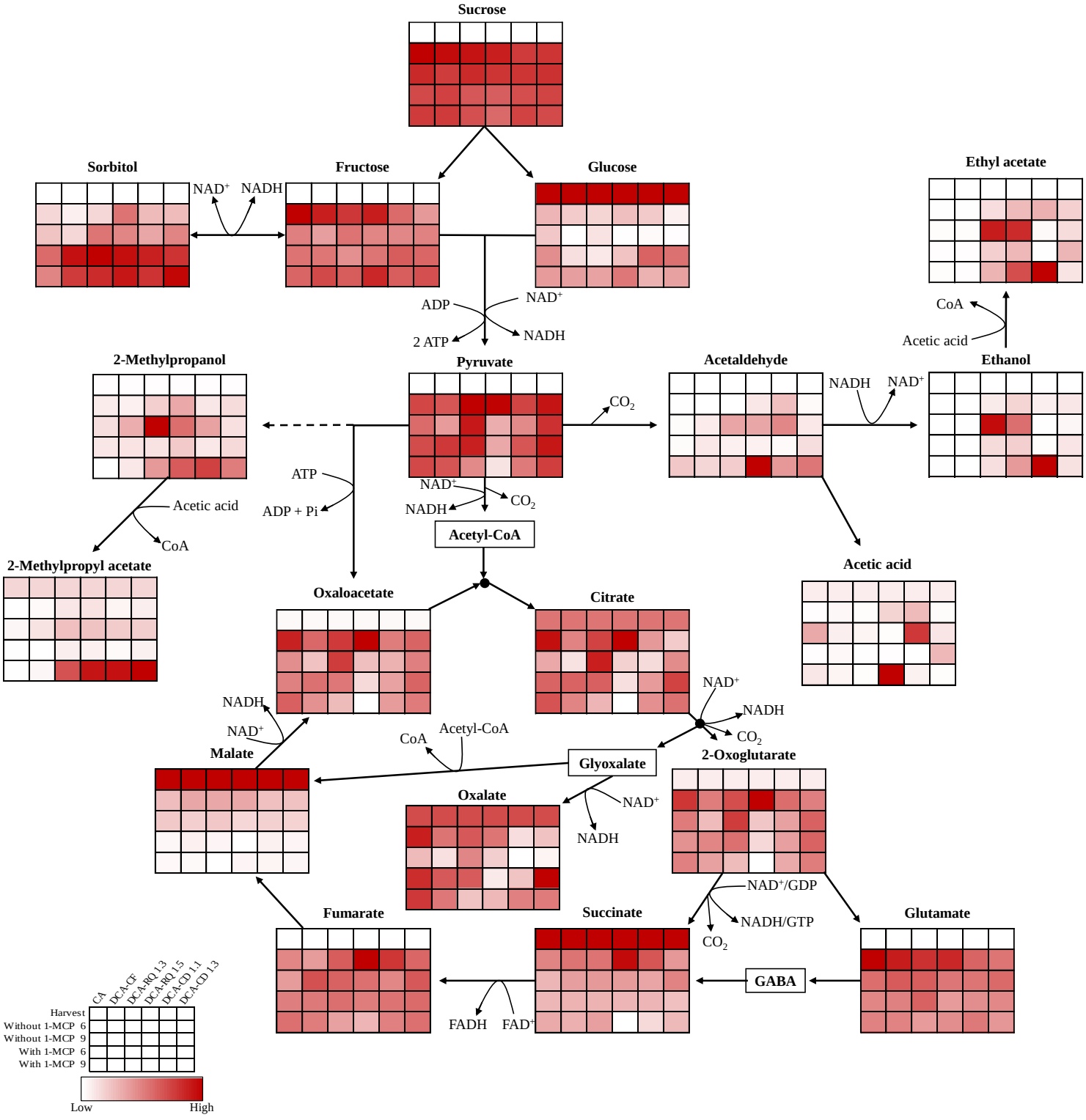

Figure 3. Heat map showing the relative change of sugars, organic acids and some volatile compounds of 'Elstar' apples after harvest, 6 and 9 months of storage under controlled atmosphere (CA), dynamic controlled atmosphere monitored by chlorophyll fluorescence (DCA – CF), by respiratory quotient (DCA – RQ) and by carbon dioxide (DCA – CD), either without or with 1-MCP treatment (0.650 µL L^{-1}), plus 7 d of shelf life at 20°C.

At harvest, the fructose content was low and its concentration was higher at the evaluation after 6 months of storage (Figure 3 and 4). These results are consistent with early studies that reported an increase of fructose over the storage period (Doerflinger et al., 2015; Lee et al., 2012; Thewes et al., 2019; Wang et al., 2010). For 'Elstar' apples without 1-MCP, fructose concentration was higher when fruit were stored over 6 months under CA, DCA – CF and DCA – RQ, differing from the one stored under DCA – CD (Figure 3). On the other hand, when fruit were treated with 1-MCP, the storage under DCA – RQ 1.5 resulted in the highest fructose concentration after 9 months (Figure 3). For 'Nicoter' apples stored without 1-MCP, CA, DCA – RQ 1.3 and DCA – CD 1.3 resulted in higher fructose concentration after 6 months (Figure 4). On the other hand, after 9 months of storage without 1-MCP, fruit under DCA – CF, DCA – RQ 1.5 and DCA – CD 1.1 had higher fructose concentration as compared the other atmosphere condition. When fruit were treated with 1-MCP, the storage under DCA – RQ 1.3 over 6 months resulted in the lowest fructose concentration (Figure 4). Probably, the 1-MCP treatment combined with DCA – RQ 1.3 delayed the accumulation of fructose from sucrose degradation in 'Nicoter' apples. Fructose is the most abundant sugar of apples (Aprea et al., 2017; Wang et al., 2010) and the sweetness one (Koehler and Kays, 1991), so fruit with higher fructose will be sweeter, but the apple sweetness is also a result of the interaction with other sugars, especially sorbitol and volatile compounds (Aprea et al., 2017).

Sorbitol is a sugar-alcohol present in apple fruit and its concentration decrease over the storage period in normal atmosphere conditions (Doerflinger et al., 2015; Wang et al., 2010). However, at the present study its concentration increased over the storage period, especially when fruit were stored under DCA – RQ and DCA – CD (Figure 3 and 4), showing a trend of its accumulation with the oxygen lowering as showed by the Pearson correlation (Figures 6 – 8). These results agree with (Thewes et al., 2019), which reported an increase of sorbitol by the storage under DCA – RQ. Additionally, sorbitol can protect the fruit cells from stress damages as demonstrated in yeast (Jain et al., 2012, 2011; Shen et al., 1999), probably, in our study the stress condition was the ethanol accumulation (Figures 3 and 4). A noteworthy fact is that the 1-MCP treatment increased the sorbitol accumulation in almost all storage conditions (Figure 3 and 4), with the exception for 'Nicoter' apples stored under CA over 6 months. In the literature is described the involvement of sorbitol in preventing chilling damage on 'Granny Smith' apples and *Arabidopsis thaliana*, being its concentration increased by the 1-MCP treatment (Busatto et al., 2018). The higher sorbitol accumulation of fruit stored under DCA – RQ and DCA – CD or 1-MCP treatment can contribute positively to fruit aroma, because it confers to the fruit a sweet-fruity aroma (Aprea et al., 2017).

The metabolism of glucose and/or fructose in the glycolysis will produce pyruvate, ATP and NADH (Bekele et al., 2016; Jain et al., 2012). Pyruvate is a key metabolite in fruit respiration process and, in normal atmosphere conditions (20.9 kPa O_2 + 0.04 kPa CO_2), the pyruvate is preferential metabolized via TCA, but if the oxygen partial pressure is below the ACP, pyruvate is directed to lactic and ethanoic fermentation (Ke et al., 1994; Saquet and Streif, 2008). It can also be converted to amino acids (Bekele et al., 2016, 2015), which are volatile compounds precursors. 'Elstar' apples stored under DCA – RQ and DCA – CD 1.3 had the highest pyruvate accumulation after 6 months of storage (Figure 3). When 'Elstar' fruit were stored under DCA – RQ 1.5, the oxygen lowering to extremely low partial pressures (Figure 1), resulted in an accumulation of TCA intermediaries, such as citrate, 2-oxoglutarate, succinate, fumarate and oxaloacetate (Figure 3), showing that the low oxygen suppressed the aerobic respiration, resulting in higher pyruvate accumulation after 6 months without 1-MCP. The pyruvate accumulation results in cytosol acidification, which actives the pyruvate decarboxylase (PDC) enzyme (Ke et al., 1994), which probably converted almost all pyruvate to acetaldehyde in 'Nicoter' apples (Figure 4), especially at the evaluation took place after 6 months of storage. The higher flow of pyruvate to acetaldehyde after 6 months of storage explains the reduction of TCA intermediaries under DCA – RQ 1.5 (Figure 4). If we look to the metabolites accumulation when fruit were stored under DCA – RQ 1.3 it is noteworthy that pyruvate concentration is high after 6 and 9 months of storage, but the TCA intermediates accumulated more after 9 months of storage for 'Elstar' apples (Figure 3). These results evidence that after 6 months storage, the oxygen partial pressure (Figure 1) did not suppress the electron transport chain sufficient to result in accumulation of anaerobic metabolism compounds when 'Elstar' fruit were stored under DCA – RQ 1.3, but after 9 months of storage, the oxygen set point was lower and, the TCA intermediaries accumulate starting anaerobic metabolism (Figure 3). In the literature is reported that the anaerobic metabolism started significantly generally by the storage under RQ higher than 1.3 (Both et al., 2017; de Oliveira Anese et al., 2019; Thewes et al., 2017b), but it depends on the temperature and the time that fruit remained under low oxygen (Boeckx et al., 2019).

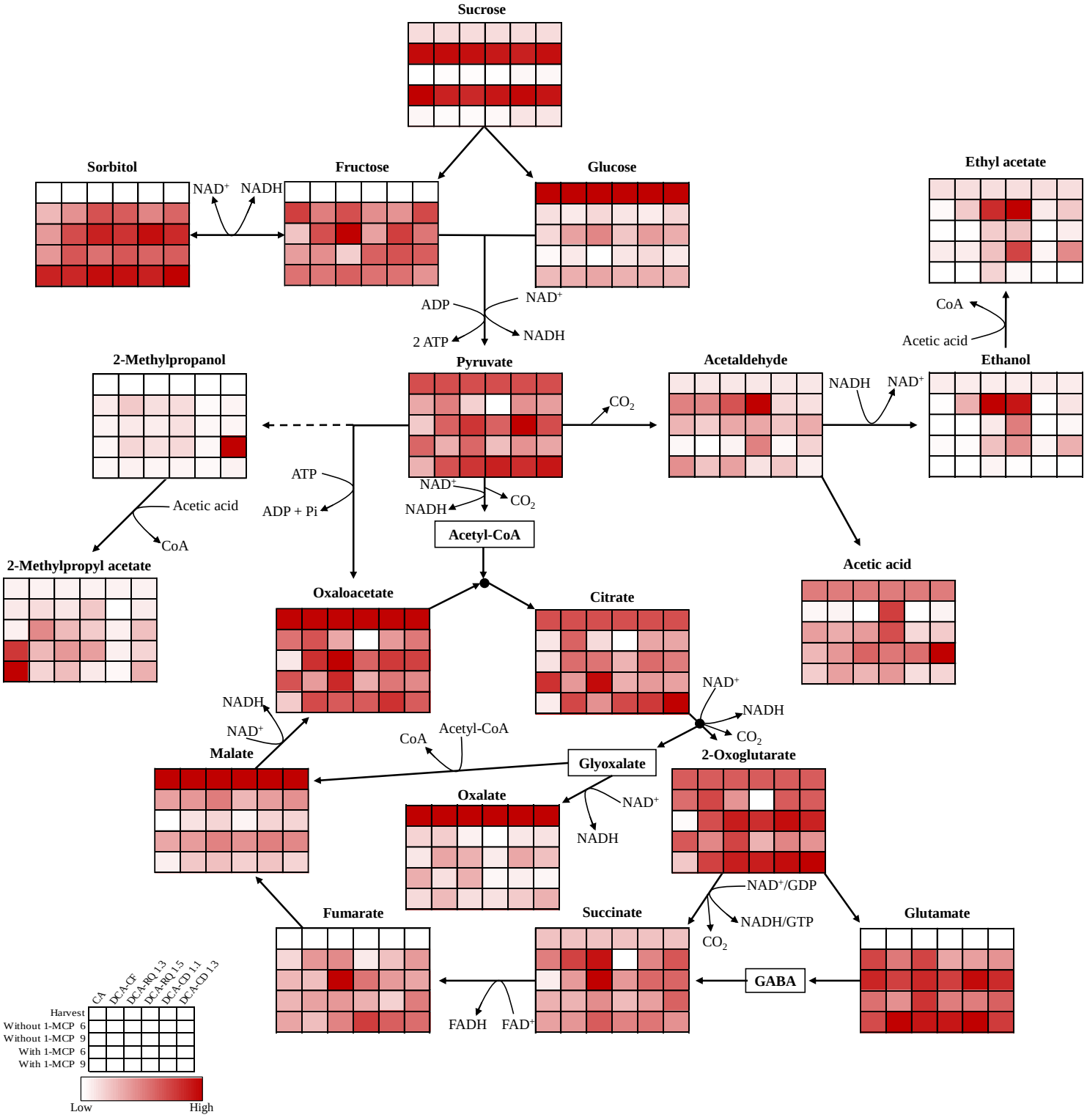

Figure 4. Heat map showing the relative change of sugars, organic acids and some volatile compounds of 'Nicoter' apples after harvest, 6 and 9 months of storage under controlled atmosphere (CA), dynamic controlled atmosphere monitored by chlorophyll fluorescence (DCA – CF), by respiratory quotient (DCA – RQ) and by carbon dioxide (DCA – CD), either without or with 1-MCP treatment (0.650 μL L^{-1}), plus 7 d of shelf life at 20°C.

An interesting result is that the storage of ‘Elstar’ apples under DCA – CD 1.1, which resulted in oxygen partial pressures lower than DCA – CF and higher than DCA – RQ 1.3 (Figure 1), had higher acetaldehyde concentration as compared to fruit under DCA – RQ 1.3 after 6 months storage without 1-MCP (Figure 3). The acetaldehyde is very toxic for the cells (Pesis, 2005), so it is actively being converted to other compounds, such as ethanol and acetic acid. The fruit stored under DCA – CD 1.1 without 1-MCP preferential converted acetaldehyde to acetic acid and fruit stored under DCA – RQ preferentially converted to ethanol (Figure 3). These results suggest that the oxygen partial pressure lowering to a level close to ACP results in an increment of acetaldehyde accumulation, which is converted to acetic acid, but not to ethanol. This is an important result, because acetic acid is a precursor of important esters, such as butyl acetate, 2-methylbutyl acetate and hexyl acetate (Brackmann et al., 1993; Echeverría et al., 2004), and ethanol is considered an off-flavour of apples (López et al., 2007; Wright et al., 2015), but it is also an important precursor of esters in ‘Fuji’ apples (Donadel et al., 2019; Niu et al., 2019; Thewes et al., 2017a). However, if we evaluate the anaerobic metabolism compounds accumulation when ‘Elstar’ apples were stored under DCA – CD 1.3, is verified higher pyruvate accumulation as compared to DCA – CD 1.1 after 6 and 9 months of storage (Figure 3). On the other hand, after 9 months of storage, DCA – CD 1.3 had higher ethyl acetate accumulation as compared to DCA – CD 1.1, which is a result of lower oxygen set points under DCA – CD 1.3 (Figure 3).

‘Elstar’ fruit stored under CA over 6 months had high accumulation of organic acids, such as citrate, 2-oxoglutarate, oxaloacetate, and oxalate and glutamate (Figure 3). Nevertheless, the accumulation of these acids is not due to O_2 shortage, but due to higher respiration rate (Figure 2), showing that these fruit had a higher metabolic rate. On the other hand, ‘Elstar’ apples stored under DCA – CF without 1-MCP over 9 months had low accumulation of TCA intermediaries and anaerobic metabolism compounds (Figure 3). These results show that the storage under DCA – CF, reduced aerobic respiration and did not start anaerobic metabolism by the oxygen lowering to ±0.4 kPa (Figure 1). The low metabolism rate, especially the anaerobic metabolism of fruit under DCA – CF may explain the lower volatile compounds emission (Both et al., 2017; Thewes et al., 2017a) because the TCA intermediaries, acetic acid and ethanol are important volatile compounds precursors.

When ‘Elstar’ apple were treated with 1-MCP, the highest effect on pyruvate and TCA intermediaries was observed under DCA – RQ 1.5 storage, at the evaluation after 9 months (Figure 3). This storage treatment (DCA – RQ 1.5 + 1-MCP) suppressed the pyruvate accumulation, which resulted in higher glucose and fructose concentration, especially after 9

months of storage (Figure 3). These results demonstrate that the combination of extremely low oxygen (Figure 1) with ethylene action blocking suppressed the glycolytic pathway, probably because of the low ethylene production (Figure 2) and action. Additionally, the low oxygen over the storage may result in more cytosol acidity (low pH), altering the phosphofructokinase enzyme activity (Gyulakhmedov et al., 2006). The lower pyruvate concentration in fruit stored under DCA – RQ 1.5 + 1-MCP is also a result of its conversion to acetaldehyde, which is converted to acetic acid in a higher proportion as compared to ethanol, contrarily to fruit stored under DCA – RQ 1.5 without 1-MCP (Figure 3). The TCA intermediaries are in very low concentrations by the storage under DCA – RQ 1.5 combined with 1-MCP (Figure 3), which is a result of low oxygen (Figure 1) and low pyruvate concentration because the pyruvate plays a central role on fruit respiration, linking glycolysis with TCA and anaerobic metabolism (Bekele et al., 2016).

Comparing the effect of 1-MCP on the anaerobic metabolism, when 'Elstar' apples were stored under DCA – CD 1.1 and DCA – RQ 1.5, is verified an inverse result. Fruit treated with 1-MCP and stored under DCA – CD 1.1 had higher formation of ethanol and ethyl acetate and low amount of acetic acid accumulation (Figure 3). However, under DCA – RQ 1.5, fruit without 1-MCP had higher ethanol and ethyl acetate accumulation, with low amount of acetic acid. Probably, this is a result of the modulation of ADH and aldehyde dehydrogenase (ALDH) enzymes activity as a function of the oxygen set point and 1-MCP treatment. These result explain in part why the 1-MCP application on 'Galaxy', 'Fuji' and 'Pink Lady' suppressed the ethanol and ethyl acetate accumulation by the storage under DCA – RQ 1.5 (Thewes et al., 2018), because it switches the acetaldehyde conversion to acetic acid (Figure 3). Nevertheless, the effect of 1-MCP in suppressing ethanol and ethyl acetate production is dependent on the oxygen set point that fruit remained over the storage period.

Analyzing the conversion of glucose and fructose to pyruvate for 'Nicoter' apples is verified a different response as compared to 'Elstar' apples (Figures 3 and 4). After 6 months of storage, the pyruvate concentration was lower in fruit under DCA – RQ 1.5 without 1-MCP (Figure 4). The TCA intermediaries also stay in low concentration when fruit were stored in this condition, showing that the TCA is suppressed by the extremely low oxygen storage (Figure 1). In this case, the pyruvate is metabolized via fermentative pathway (Figure 4), increasing the accumulation of acetaldehyde, ethanol, ethyl acetate and acetic acid. Analyzing these results, it is clearly demonstrated that the 'Nicoter' apples are much more sensitive to low oxygen over the storage period as compared to 'Elstar' one (Figure 1, 3 and 4). Probably, this is a result of the compact pulp of 'Nicoter' apples, which reduces the gas diffusion in flesh increasing the

LOL (Ho et al., 2013) or because the low skin porosity (Wang et al., 2020). On the other hand, after 9 months of storage under DCA – RQ 1.5 without 1-MCP, the oxygen setpoint was increased (Figure 1) reducing the anaerobic metabolism and the pyruvate flux was directed to the TCA cycle again, increasing the TCA intermediaries as compared to 6 months of storage. These results show that the low oxygen stress is reversible, corroborating the results obtained by (Wright et al., 2012). Pyruvate is a key molecule in fruit metabolism, linking glycolysis to TCA or anaerobic metabolism (Bekele et al., 2016) and the conversion of pyruvate to acetyl-CoA or acetaldehyde is dependent on the cytosol pH, which modulate the activity of PDC and PDH (Ke et al., 1994).

Evaluating the 'Nicoter' apples stored under CA without 1-MCP, it is verified a reduction of TCA intermediaries from harvest to 9 months of storage (Figure 4). This can be associated to the reduced fruit respiration (CO_2 release) over the storage period, when compared to harvest (Figure 2). These results evidence that fruit stored under CA without 1-MCP stay over the climacteric peak and close to senescence after 9 months of storage. This was not observed when fruit were stored under DCA without 1-MCP, were and increment of TCA intermediaries happen from 6 to 9 months of storage (Figure 4) also associated to an increased CO_2 release rate (Figure 2). This shows that probably the fruit stored under CA stay over the respiratory peak and fruit stored under DCA stay before it. These results corroborate the one reported in the literature, which suggests that apple fruit had an increment of ethylene production and respiration rate until a peak is reached, and thereafter it decreases (Saquet and Streif, 2017; Steffens et al., 2007), but this process is delayed by low oxygen storage, such as DCA (Brackmann et al., 2015; Thewes et al., 2015b).

Succinate accumulate generally when apple fruit are stored under CA with high CO_2 partial pressures due to the inhibition of succinate dehydrogenase enzyme activity (Bekele et al., 2016; Mathooko, 1996). At the present study is verified that the storage of 'Nicoter' apples under DCA – RQ 1.3 without 1-MCP also results in a higher accumulation of succinate as compared to the other storage conditions (Figure 4), fact that was not observed in 'Elstar' apples (Figure 3). After 9 months of storage under DCA – RQ 1.3 without 1-MCP, the higher succinate is due to a higher TCA intermediary's accumulation in 'Nicoter' apples stored under this condition, such as 2-oxoglutarate, fumarate, citrate and oxaloacetate (Figure 4). According to (Mathooko, 1996) this is a result of pH reduction, inhibiting the succinate dehydrogenase activity. Probably, the storage under DCA – RQ 1.3 reduced the cytosolic pH due to anaerobic metabolism, reducing the succinate dehydrogenase activity, especially after 6 months, where succinate was accumulated only.

The storage of ‘Nicoter’ apples under CA + 1-MCP and DCA – RQ 1.3 + 1-MCP over 6 months resulted in higher accumulation of pyruvate and TCA intermediaries, such as citrate, 2-oxoglutarate and oxaloacetate as compared to the other storage conditions (Figure 4). Additionally, in these two storage conditions (CA and DCA – RQ 1.3) 1-MCP application had an effect on these metabolite accumulation as compared to fruit stored under the same conditions without 1-MCP. On the other hand, after 9 months of storage with 1-MCP treatment, all DCA stored fruit had higher concentrations of pyruvate, citrate, 2-oxaloacetate when compared to CA stored fruit (Figure 4). However, if we compare fruit without and with 1-MCP at the same atmosphere conditions, only slight differences were observed (Figure 4). These results corroborate the one reported in the literature for ‘Jonagold’ (Bekele et al., 2015) and ‘Empire’ apples (Lee et al., 2012).

According to the literature, oxalate has some antioxidant activity (Zheng et al., 2007) and an anti-senescence effect of fruit (Wu et al., 2011). At the present study, its concentration decreased from harvest to 9 months of storage in ‘Elstar’ apples, regardless the atmosphere condition and 1-MCP treatment (Figures 3). However, for ‘Nicoter’ apples, there was a drastic reduction from harvest to 6 months of storage, and thereafter remained almost constant, regardless the storage condition (Figure 4). ‘Elstar’ apples stored under CA over 6 months had higher concentration of oxalate, and fruit under DCA – CD 1.1 the lowest concentration (Figure 3). After 9 months of storage without 1-MCP, DCA – RQ 1.3 stored fruit had higher oxalate concentration compared to the other storage conditions. In 1-MCP treated fruit, the storage under DCA – CD 1.3 resulted in the highest oxalate concentration. This response of oxalate concentration to the storage conditions is a result of citrate concentrations, which had a similar response to the treatments as compared to oxalate (Figure 3 and 5). In the literature is reported that the oxalate treatment reduced ethylene production in plums (Wu et al., 2011), but at the present study is verified that fruit with lower ethylene production had also lower oxalate accumulation, with some exception, such as DCA – CD 1.3 + 1-MCP (Figure 2 and 3).

From the pyruvate can be produced several amino acids, such as valine and alanine (Bekele et al., 2015). Valine is a precursor of 2-methylpropanol and 2-methylpropyl acetate (Kochevenko et al., 2012). ‘Elstar’ apples stored under DCA – RQ 1.3 (9 months) and ‘Nicoter’ apples stored under DCA – CD 1.3 + 1-MCP over 6 months had higher 2-methylpropanol concentration as compared to the other atmosphere conditions, but it did not result in higher 2-methylpropyl acetate concentrations (Figure 3 and 4). ‘Elstar’ apples stored over 9 months under DCA – RQ and DCA – CD with 1-MCP had higher concentration of 2-methylpropyl acetate as compared to CA and DCA – CF, probably because the higher precursor concentration

(2-methylpropanol). These results evidence that 2-methylpropyl acetate synthesis is not ethylene dependent, because 1-MCP inhibits ethylene action and the ethylene production in these conditions is very low (Figure 2). In the literature is reported that the branched-chain volatile compounds, such as 2-methylpropyl acetate, are less affected by 1-MCP as the straight-chain one (Thewes et al., 2017b, 2015a; Yang et al., 2016). For 'Nicoter' apples, the storage under CA + 1-MCP resulted in higher 2-methylpropyl acetate concentration as compared to fruit stored under DCA, regardless the storage period and DCA method.

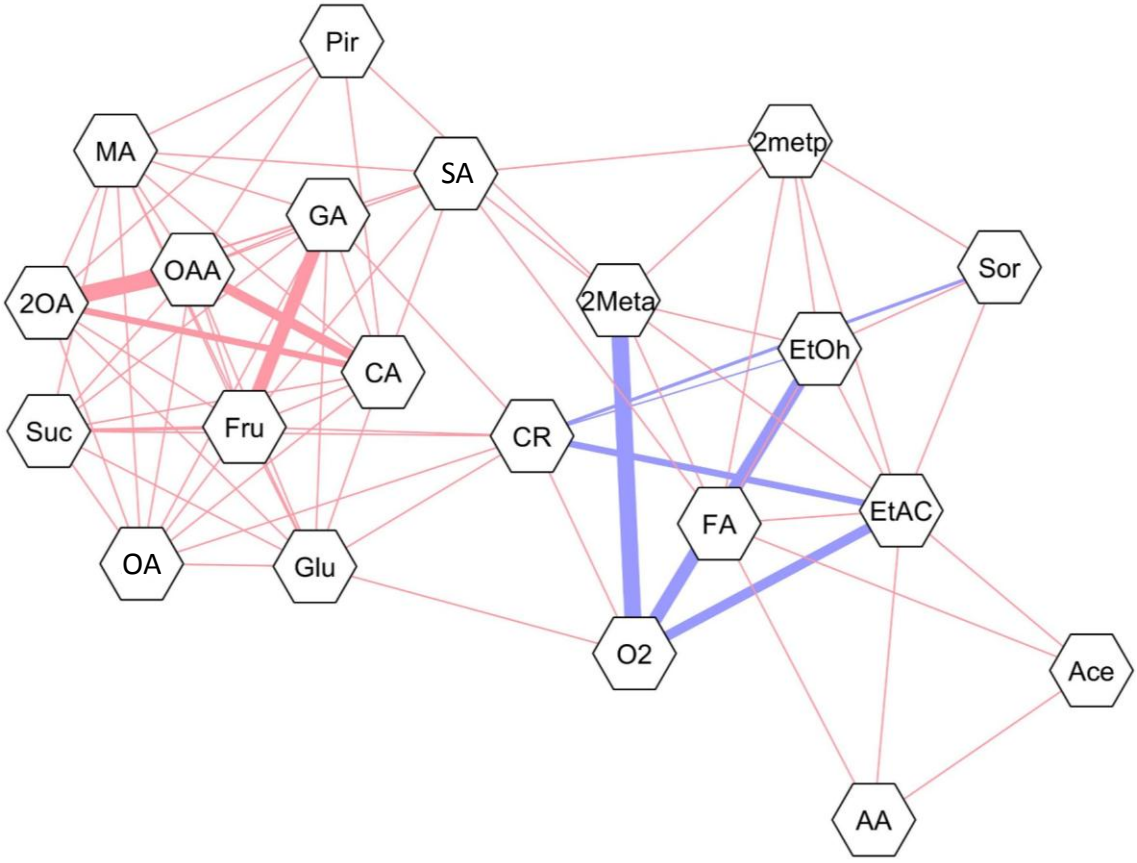

Figure 5. Correlation network for 'Elstar' apples after 6 months of storage under controlled atmosphere (CA), dynamic controlled atmosphere monitored by chlorophyll fluorescence (DCA – CF), respiratory quotient (DCA – RQ) and carbon dioxide (DCA – CD), either without or with 1-MCP treatment (0.650 µL L-1), plus 7 d of shelf life at 20°C. Blue and red lines show negative and positive correlation, respectively. The size of the line shows the correlation magnitude (all correlations showed are higher than 0.5). Suc: sucrose; Glu: glucose; Fru: fructose; Sor: sorbitol; Pir: pyruvate; Ace: acetaldehyde; EtOh: ethanol; EtAC: ethyl acetate; AA: acetic acid; CA: citrate; 2OA: 2-oxoglutarate; GA: glutamate; SA: succinate; FA: fumarate; MA: malate; OAA: oxaloacetate; OA: oxalate; 2metp: 2-methylpropanol; 2meta: 2-methylpropyl acetate; CR: carbon release.

7.1.3.4. Linear relationships between metabolites after storage

To better understand the relationships between the sugars, acids, volatile compounds, CO_2 release and the mean oxygen partial pressure over the storage, was undertaken a Pearson correlation ($p < 0.05$). The Pearson correlation matrix was used to build a correlation network for each outturn time evaluation (Figure 5 – 8). The oxygen set point was negatively correlated with sorbitol for both cultivars (Figures 6 – 8), showing a trend of its accumulation with oxygen lowering. These results corroborate the one of the literature, which reported sorbitol accumulation with oxygen lowering (Thewes et al., 2019). The oxygen set point was also negatively correlated to ethanol, ethyl acetate, 2-methylpropyl acetate for 'Elstar' apples after 6 months storage (Figures 5). This happen because the oxygen lowering results in anaerobic metabolism, resulting in fermentative compounds accumulation.

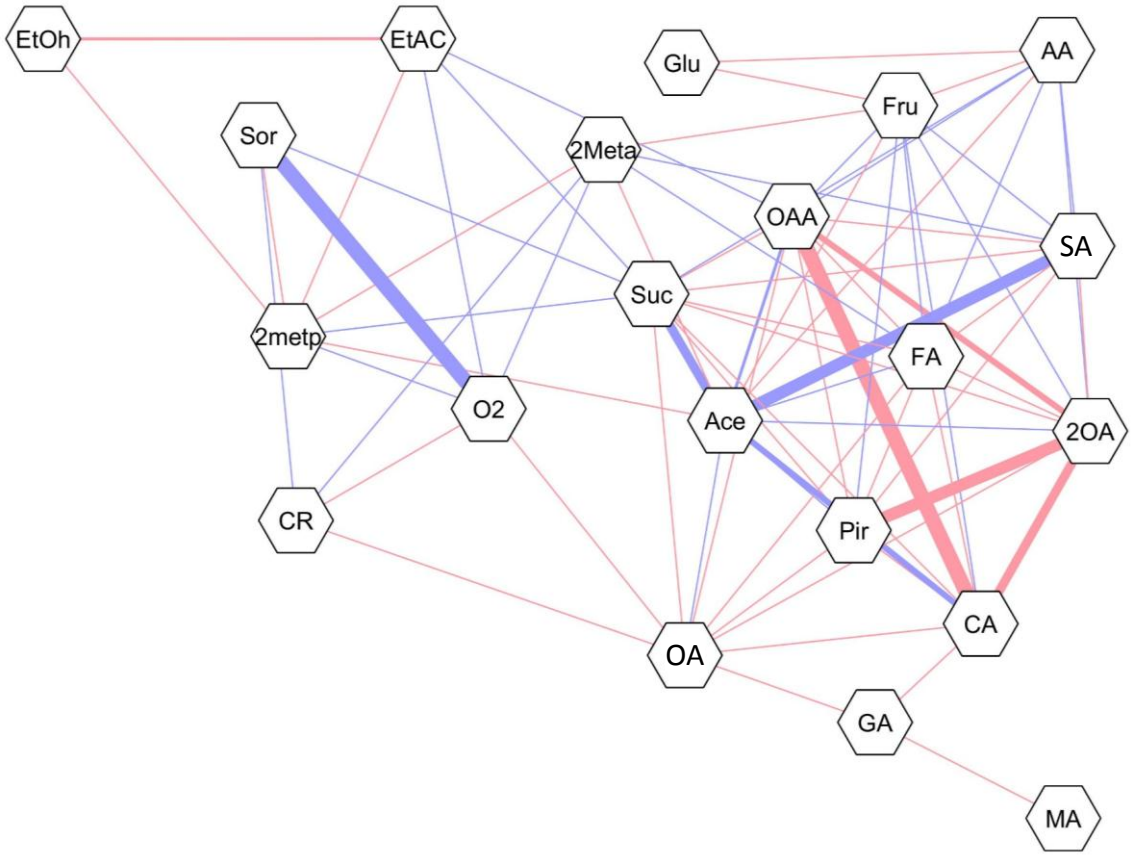

Figure 6. Correlation network for 'Elstar' apples after 9 months of storage under controlled atmosphere (CA), dynamic controlled atmosphere monitored by chlorophyll fluorescence (DCA – CF), respiratory quotient (DCA – RQ) and carbon dioxide (DCA – CD), either without or with 1-MCP treatment (0.650 µL L-1), plus 7 d of shelf life at 20°C. Blue and red

lines show negative and positive correlation, respectively. The size of the line shows the correlation magnitude (all correlations showed are higher than 0.5). Suc: sucrose; Glu: glucose; Fru: fructose; Sor: sorbitol; Pir: pyruvate; Ace: acetaldehyde; EtOH: ethanol; EtAC: ethyl acetate; AA: acetic acid; CA: citrate; 2OA: 2-oxoglutarate; GA: glutamate; SA: succinate; FA: fumarate; MA: malate; OAA: oxaloacetate; OA: oxalate; 2metp: 2-methylpropanol; 2meta: 2-methylpropyl acetate; CR: carbon release.

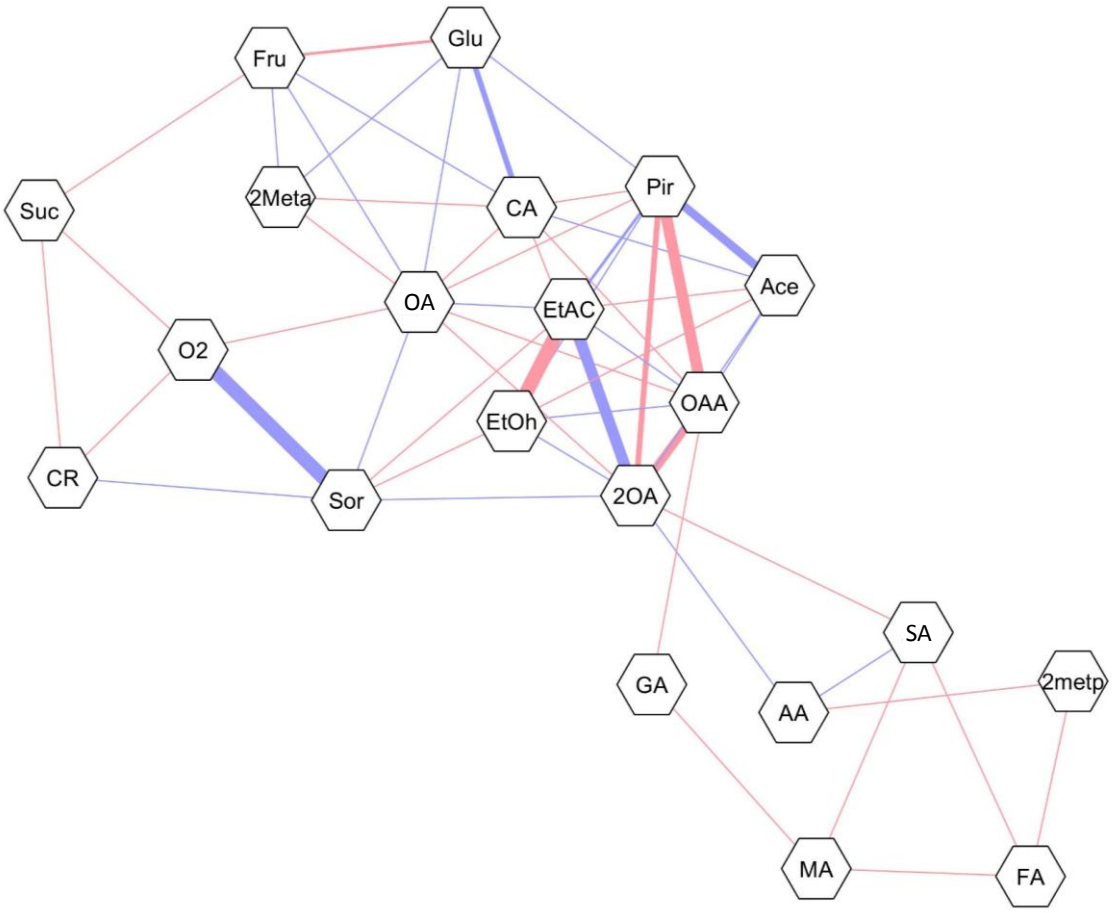

Figure 7. Correlation network for 'Nicoter' apples after 6 months of storage under controlled atmosphere (CA), dynamic controlled atmosphere monitored by chlorophyll fluorescence (DCA – CF), respiratory quotient (DCA – RQ) and carbon dioxide (DCA – CD), either without or with 1-MCP treatment (0.650 µL L-1), plus 7 d of shelf life at 20°C. Blue and red lines show negative and positive correlation, respectively. The size of the line shows the correlation magnitude (all correlations showed are higher than 0.5). Suc: sucrose; Glu: glucose; Fru: fructose; Sor: sorbitol; Pir: pyruvate; Ace: acetaldehyde; EtOH: ethanol; EtAC: ethyl acetate; AA: acetic acid; CA: citrate; 2OA: 2-oxoglutarate; GA: glutamate; SA: succinate; FA: fumarate; MA: malate; OAA: oxaloacetate; OA: oxalate; 2metp: 2-methylpropanol; 2meta: 2-methylpropyl acetate; CR: carbon release.

The correlation networks also evidence that the TCA intermediaries of ‘Elstar’ apples, such as citrate, 2-oxoglutarate and oxaloacetate, had a positive Pearson correlation among each other (Figures 5 and 6). These results evidence that citrate, 2-oxoglutarate and oxaloacetate had a similar response to the storage treatments, regardless the DCA method and 1-MCP application. On the other hand, for ‘Nicoter’ apples it is verified a strong and positive correlation between pyruvate and oxaloacetate after 6 and 9 months of storage (Figures 7 and 8). Probably the pyruvate is actively converted to citrate and/or oxaloacetate, by the enzymes pyruvate dehydrogenase and pyruvate carboxylase, respectively. The conversion of pyruvate to oxaloacetate uptakes CO_2, which in part can explain the apparent low RQ measurement in several studies (de Oliveira Anese et al., 2019; Stanger et al., 2018; Weber et al., 2017).

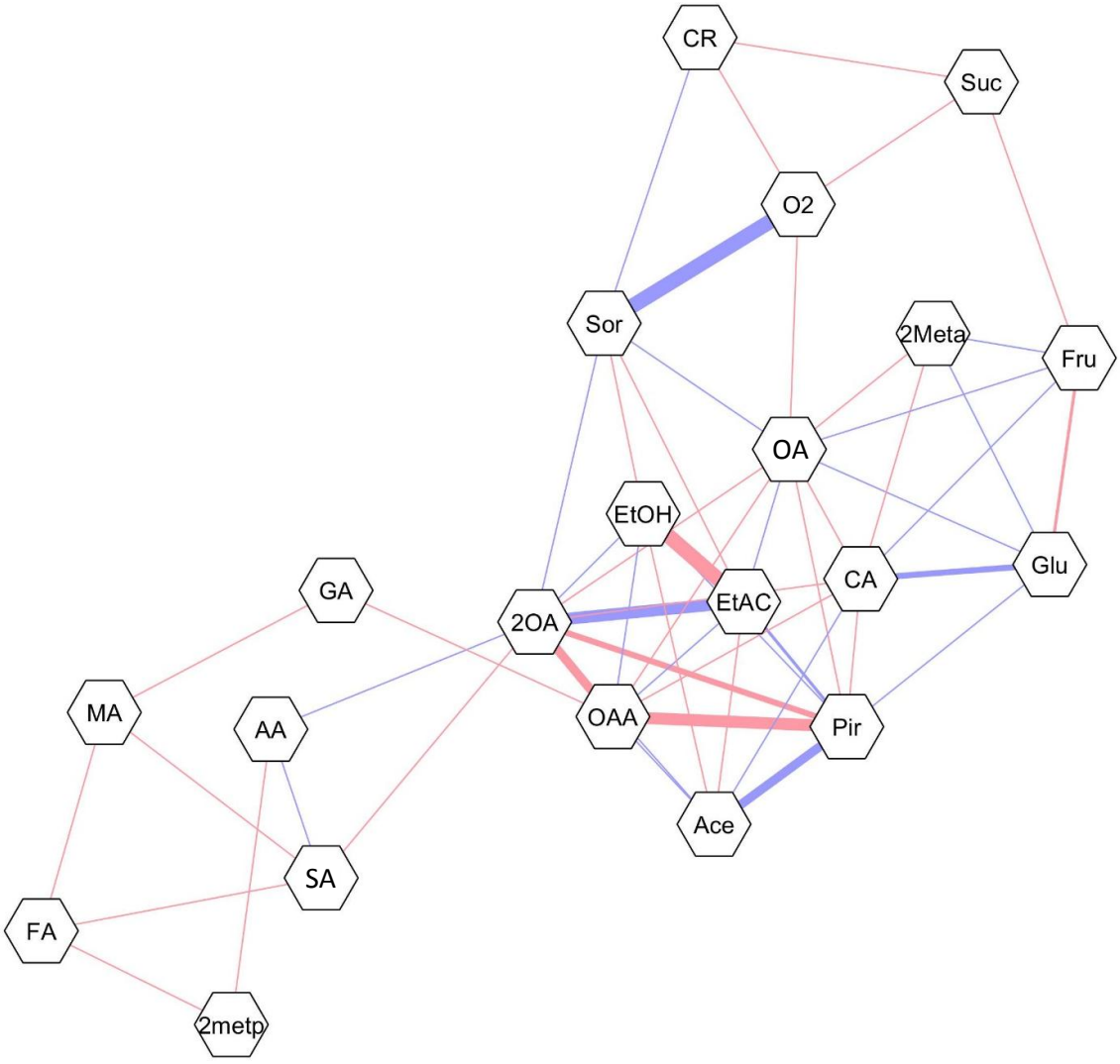

Figure 8. Correlation network for ‘Nicoter’ apples after 9 months of storage under controlled atmosphere (CA), dynamic controlled atmosphere monitored by chlorophyll fluorescence

(DCA – CF), respiratory quotient (DCA – RQ) and carbon dioxide (DCA – CD), either without or with 1-MCP treatment (0.650 μL L-1), plus 7 d of shelf life at 20°C. Blue and red lines show negative and positive correlation, respectively. The size of the line shows the correlation magnitude (all correlations showed are higher than 0.5). Suc: sucrose; Glu: glucose; Fru: fructose; Sor: sorbitol; Pir: pyruvate; Ace: acetaldehyde; EtOH: ethanol; EtAC: ethyl acetate; AA: acetic acid; CA: citrate; 2OA: 2-oxoglutarate; GA: glutamate; SA: succinate; FA: fumarate; MA: malate; OAA: oxaloacetate; OA: oxalate; 2metp: 2-methylpropanol; 2meta: 2-methylpropyl acetate; CR: carbon release.

7.1.4. Conclusions

In this paper, was demonstrated the effects of CA and three DCA methods, either with or without 1-MCP, on the metabolite variation at harvest, 6 and 9 months of storage, plus 7 days shelf life for ‘Elstar’ and ‘Nicoter’ apples. The metabolism of both cultivars differ between each other for the LOL variation over the storage period. LOL was higher for ‘Nicoter’ apples and the three DCA methods were able to detect this difference between cultivars, but LOL estimation was lower when fruit were stored under DCA – RQ and DCA – CD, because of the anaerobic metabolism induction.

The sugars metabolism was differently affected by the DCA methods and 1-MCP treatment, especially sorbitol, which had a trend of accumulation when fruit were stored under DCA – RQ and DCA – CD, especially in higher RQ and CD, showing a negative correlation with the oxygen partial pressure over the storage period. 1-MCP treatment induced sorbitol accumulation even when fruit were stored under CA (higher oxygen partial pressures), showing that sorbitol synthesis by fruit could be a response to stress, such as low oxygen and/or 1-MCP treatment.

Pyruvate flux to TCA is inhibit differently for both cultivars, being inhibit first for ‘Nicoter’ apples at higher oxygen partial pressures, evidencing higher tolerance to low oxygen for ‘Elstar’ apples. The TCA inhibition is reversible by the increase of oxygen set point resulting in higher TCA intermediaries after 9 months of ‘Nicoter’ apples stored under DCA – RQ 1.5. ‘Elstar’ apples stored under DCA – CD 1.1 result in higher accumulation of acetaldehyde that is converted to acetic acid in a higher amount, which is an important acetate esters precursor. 1-MCP application reduces the accumulation of ethanol and ethyl acetate when fruit were stored under DCA – RQ 1.5 by directing the conversion of acetaldehyde to acetic acid and not to ethanol for both cultivars studied.

TCA intermediaries, such as citrate, 2-oxoglutarate, succinate and oxaloacetate, are the most affected by the atmosphere conditions and 1-MCP treatment for both cultivars. These acids

generally have a positive Pearson correlation among each other. Malic acid is more affected by the storage time as the atmosphere conditions. Succinate and fumarate have an accumulation trend when fruit are stored under DCA – RQ, i.e., lowest oxygen partial pressures.

7.1.5. Acknowledgements

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8. DISCUSSÃO GERAL

O armazenamento e a distribuição de maçãs de qualidade durante o ano todo é um dos maiores desafios do setor de frutícola. Isso ocorre em função das significativas perdas que podem ocorrer no período de pós-colheita, especialmente, quando as condições de armazenamento não são as mais adequadas. Dentre as perdas de maior importância estão a ocorrência de podridões (BLUM et al., 2004; BRACKMANN et al., 2008), distúrbios fisiológicos e redução da firmeza e da emissão de compostos voláteis (AUBERT; MATHIEU-HURTIGER; VAYSSE, 2015; BOTH et al., 2017; JUNG; WATKINS, 2011; THEWES et al., 2017c; WEBER et al., 2020). Em função disso, é de fundamental importância o desenvolvimento de tecnologias de armazenamento que possibilitem a otimização das condições de armazenamento em tempo real, durante todo o período de armazenamento, em função das modificações metabólicas das frutas.

Na presente tese foram estudadas três tecnologias de monitoramento do metabolismo das maçãs a fim de permitir o armazenamento de forma interativa com a fruta. Com isso buscou-se preencher algumas lacunas do conhecimento para auxiliar os armazenadores a melhorar a manutenção da qualidade pós-colheita. No primeiro artigo foram avaliados o efeito do armazenamento em AC, ACD – FC e ACD – QR 1,5 com dois estresses múltiplos de baixo O_2 por semana e sua interação com a aplicação de 1-MCP sobre a expressão gênica de enzimas relacionadas à produção de etileno, atividade da lipoxigenase (LOX) e síntese de compostos voláteis. Esse trabalho comprovou que o armazenamento em ACD – QR 1,5, com um nível extremamente baixo de O_2 (0,18 kPa), incrementa a produção de compostos voláteis em maçãs ‘Galaxy’, pelo aumento da expressão da enzima álcool acetil transferase (*MdAAT1*) e aumento na concentração de precursores em função da indução do metabolismo anaeróbico. Também foi comprovado que em maçãs ‘Galaxy’ a indução da expressão da enzima ATT não é dependente de etileno quando em ACD – QR 1,5, uma vez que, mesmo quando as maçãs foram tratadas com 1-MCP, ocorre aumento na expressão do gene *MdAAT1*, resultando em maior acúmulo de ésteres.

Alguns trabalhos da literatura já relataram que a aplicação de 1-MCP não inibe completamente a expressão da *MdAAT1* em maçãs (BAN et al., 2010; YANG et al., 2016), o que ajuda a explicar o resultado encontrado no presente trabalho. Entretanto, o presente estudo demostrou que a combinação do armazenamento em ACD – QR 1,5, com O_2 extremamente baixo, combinado com a aplicação de 1-MCP aumenta a expressão gênica da enzima AAT em

comparação à AC + 1-MCP, evidenciando uma interação entre pressão parcial de O_2 e aplicação de 1-MCP sobre a expressão de *MdAAT1*.

No primeiro artigo também são levantadas evidências que explicam a menor emissão de compostos voláteis quando maçãs 'Galaxy' são armazenada em ACD – FC. Possivelmente, isso está associado à forte redução do metabolismo aeróbico sem induzir o metabolismo anaeróbico, reduzindo assim a formação de precursores de compostos voláteis a partir da oxidação de lipídeos e catabolismo de aminoácidos sem induzir a formação de precursores pelo metabolismo anaeróbico. Trabalhos recentes com maçãs mutantes de 'Fuji' já haviam levantado a hipótese de que a baixa emissão de ésteres por maçãs armazenadas em ACD – FC é função da menor produção de precursores (DONADEL et al., 2019; THEWES et al., 2017c). Entretanto, no presente estudo é comprovado que no armazenamento de maçãs 'Galaxy' em ACD – FC o menor acúmulo de precursores é função da baixa expressão da enzima lipoxigenase (*MdLOX1*), sendo a reduzida emissão de ésteres função da menor expressão da enzima AAT (*MdAAT1*) combinada ao menor acúmulo de precursores, como álcoois e ácidos.

Apesar da maior emissão de ésteres por maçãs armazenadas em ACD – QR, ocorre também maior emissão e acúmulo de acetaldeído e etanol, os quais em altas concentrações podem causar danos às células das frutas (DEUCHANDE et al., 2016; LIU et al., 2012; PESIS, 2005; WEBER et al., 2020). Porém, em maçãs armazenadas em ACD – QR as altas concentrações de etanol, que podem girar em torno de 100 a 400 mg L^{-1} (THEWES et al., 2017a; WEBER et al., 2019, 2020), não causam danos às membranas, mantendo baixa a permeabilidade da membrana celular (THEWES et al., 2017a).

Baseado nesses resultados, o segundo artigo buscou explicar a interação entre a dinâmica do metabolismo anaeróbico e o acúmulo de açúcares-álcoois, como glicerol e sorbitol, em maçãs armazenadas em AC e ACD – QR. A partir desse experimento foi possível associar o acúmulo de sorbitol ao armazenamento em ACD – QR, ou seja, em condições de O_2 extremamente baixas. De acordo com estudos realizados em leveduras, o sorbitol atenua o estresse salino (JAIN et al., 2011, 2012), no caso de maçãs em ACD o estresse é causado pelo baixo O_2. Nesse sentido, o sorbitol pode proteger as membranas celulares de maçãs do estresse causado pelo acúmulo de etanol e acetaldeído, reduzindo a permeabilidade da membrana celular em comparação à AC (THEWES; BRACKMANN; NEUWALD, 2019). Além disso, a síntese de sorbitol, a partir de frutose e glicose, pode ser uma forma alternativa de regeneração do NAD^+, composto chave para manutenção da síntese de ATP em nível de substrato, como observado em leveduras (JAIN et al., 2012; SHEN et al., 1999)

Com os resultados dos dois primeiros artigos da tese é possível verificar que o armazenamento em ACD – QR é promissor na redução das perdas pós-colheita de maçãs. Contudo, um dos maiores entraves do uso da ACD – QR em nível comercial é a dificuldade de determinação do QR em câmaras comerciais (VELTMAN, 2013; BRACKMANN, 2015). Buscando contornar esse problema do uso da ACD nessas câmaras, no terceiro artigo foi desenvolvido um método de ACD monitorado pela produção de CO_2 das frutas (ACD – DC). A grande vantagem da determinação da produção de CO_2 em comparação ao QR ($\Delta CO_2/\Delta O_2$) é a pouca influência do ambiente externo na medição da taxa de produção de CO_2 (BESSEMANS et al., 2016, 2018). Nesse artigo foi comprovado que é possível monitorar o LMO em maçãs de diversas cultivares apenas pela medição da produção de CO_2, resultando em *set points* de O_2 da câmara similares às maçãs armazenadas em ACD – QR. O método de ACD – DC também têm a vantagem de permitir a indução do metabolismo anaeróbico de maneira controlada, pelo emprego de fatores de metabolismo anaeróbico (FMA). Isso é de fundamental importância, pelo significativo efeito do etanol no metabolismo do etileno (ASODA et al., 2009; JIN et al., 2013; WEBER et al., 2016, 2020) e na síntese de compostos voláteis (DONADEL et al., 2019; LIU et al., 2012; THEWES et al., 2017b).

Depois de desenvolvido e calibrado para várias cultivares de maçã, o novo método ACD – DC foi testado e comparado aos métodos de ACD – FC, ACD – QR e à aplicação de 1-MCP sobre o metabolismo e qualidade de maçãs. No quarto artigo são apresentados dados do efeito da ACD – DC sobre o metabolismo, qualidade e perfil volátil de maçãs 'Elstar' em comparação a outras técnicas de ACD já consolidadas em nível mundial. De acordo com os resultados, é possível observar que o armazenamento de ACD – DC resultou em menor produção de etileno, incidência de podridões e distúrbios fisiológicos, e, consequentemente, em maior porcentagem de frutos sadios e firmeza de polpa em comparação à AC e ACD – FC após 9 meses de armazenamento mais 7 dias de vida de prateleira. O novo método de ACD apresentou resultados similares ao armazenamento em ACD – QR, especialmente, no que tange a distúrbios fisiológicos, firmeza de polpa e emissão de compostos voláteis, como acetato de butila, acetato de 2-metilbutila e hexila. Isso demonstra que com o método ACD – DC obtém-se resultados similares aos da ACD – QR, com a vantagem de apenas ser necessária a determinação da produção de CO_2 para estimação do LMO de maneira precisa. Esse resultado é de grande importância prática, uma vez que a medição da produção de CO_2 pode ser realizada de maneira precisa em câmara comerciais, sem a necessidade de equipamentos adicionais aos já presentes em uma câmara de AC normal.

A fim de entender como as condições de armazenamento em AC, ACD – FC, ACD – QR, ACD – DC e a aplicação de 1-MCP atuam no metabolismo de maçãs durante o armazenamento, foi desenvolvido o quinto artigo da tese. Nele são avaliados os perfis de açúcares, ácidos orgânicos e o metabolismo anaeróbico em duas cultivares de maçãs (Elstar e Nicoter) com tolerância distinta ao baixo O_2, Elstar maior tolerância e Nicoter menor tolerância (THEWES; BRACKMANN; NEUWALD, 2019). Essa diferença de tolerância ao baixo O_2 foi detectada de maneira precisa pelo método ACD – DC, mostrando que o mesmo é realmente eficaz na estimação do LMO. De maneira geral, o açúcar que sofreu maior influência das condições de ACD foi o sorbitol, apresentando maior acúmulo quando as maçãs foram submetidas às menores pressões parciais de O_2, o que corrobora com os resultados obtidos no segundo artigo. Entretanto, a aplicação de 1-MCP induziu o acúmulo de sorbitol mesmo em AC. Na literatura já é descrito que a aplicação de 1-MCP associada ao armazenamento refrigerado aumenta o acúmulo de sorbitol em maçãs 'Granny Smith' (BUSATTO et al., 2018), o que explica, em parte, o porquê do maior acúmulo de sorbitol também no armazenamento em ACD.

Com a redução da pressão parcial de O_2 próximo ou abaixo do PCA, ocorre o bloqueio parcial da respiração aeróbica, o que pode resultar no incremento da síntese de ATP à nível de substrato (KE et al., 1994), esperando-se acúmulo de piruvato. Analisando as duas cultivares, Elstar e Nicoter, percebe-se que o incremento no acúmulo de piruvato ocorre em diferentes momentos do armazenamento e em pressões parciais de O_2 diferenciadas, sendo que em maçãs 'Nicoter', aos seis meses de armazenamento em ACD – QR 1,5, praticamente todo piruvato foi convertido em acetaldeído, o que pode ser função da acidificação do citosol, ativando a enzima PDC como demonstrado nos artigos 2 e 4. Nesse sentido, foi possível observar que o nível de QR e do fator de metabolismo anaeróbico (FMA) induzem o metabolismo anaeróbico de maneira significativa e em diferentes pressões parciais de O_2 para as duas cultivares, ocorrendo já em pressões parciais maiores na maçã 'Nicoter'.

Avaliando os ácidos do ciclo de Krebs, foi possível verificar que o ácido majoritário de maçãs (malato) sofre pouca ou nenhuma influência das condições de armazenamento, porém reduz de maneira significativa ao longo do período de armazenamento. Esses resultados estão em concordância com a literatura, que relata pouca ou nenhuma influência das condições de armazenamento sobre a concentração de malato (BEKELE et al., 2015, 2016; LEE et al., 2012). Entretanto, os ácidos minoritários apresentam grande influência das condições de AC, ACD e da aplicação de 1-MCP. O armazenamento de maçãs 'Nicoter' em ACD – QR 1,5 por 6 meses, praticamente inibiu o acúmulo de citrato, 2-oxoglutarato, oxaloacetato, succinato e fumarato,

mostrando que as menores pressões parciais de O_2 bloqueiam o ciclo de Krebs, desviando o fluxo de piruvato para a rota anaeróbica, resultando em acúmulo de etanol e acetato de etila. Todavia, esse processo é reversível (WRIGHT et al., 2011), uma vez que, após 9 meses de armazenamento a pressão parcial de O_2 na condição de ACD – QR 1,5 era maior, ocorrendo um aumento no fluxo de piruvato para o ciclo de Krebs, acumulando mais citrato, 2-oxoglutarato, oxaloacetato, succinato e fumarato, e reduzindo o metabolismo anaeróbico.

Isso demonstra como o metabolismo da maçã é dinâmico e influenciado diretamente pela concentração de O_2 na câmara, uma vez que um aumento de 0,1 kPa na concentração de O_2 resulta em mudança do metabolismo anaeróbico para aeróbico. Esses resultados demonstram que maçãs armazenadas em ACD – QR e ACD – DC são submetidas a pressões parciais de O_2 extremamente baixas, induzindo a fruta se adaptar de maneira rápida e dinâmica entre metabolismo aeróbico e anaeróbico, a fim de manter o *pool* energético, não acumulando compostos do metabolismo anaeróbico em concentrações tóxicas, evitando danos aos tecidos do fruto.

A partir dos resultados apresentados na presente tese, é possível verificar o potencial das diferentes técnicas de ACD na manutenção da qualidade de maçãs após longos períodos de armazenamento. Dentre as três técnicas de ACD, a ACD – DC é a de mais fácil implementação em nível comercial, em função do baixo custo de sua implementação e segurança que oferece aos armazenadores. A grande vantagem das técnicas ACD – DC e ACD – QR é a detecção direta do LMO, seja pela medição da taxa de produção de CO_2 ou pelo QR, o que não ocorre na ACD – FC, onde um evento secundário da exposição das frutas ao baixo O_2 é monitorado, como a fluorescência de clorofilas. O armazenamento em ACD – DC e ACD – QR permite a indução, de maneira controlada, do metabolismo anaeróbico resultando na produção e acúmulo de etanol, que tem vários efeitos benéficos na manutenção da qualidade de maçãs na pós-colheita, especialmente pelos seus efeitos no metabolismo do etileno e síntese de compostos voláteis.

9. CONSIDERAÇÕES FINAIS

O armazenamento de maçãs em ACD – QR 1,5 com dois estresses múltiplos de baixo O_2 por semana resulta em frutas com menor produção de etileno, maior qualidade físico-química, especialmente maior emissão de ésteres. Isso é resultado da maior expressão de genes codificadores da enzima AAT (*MdAAT1*), mesmo em frutas tratadas com 1-MCP, evidenciando que a expressão dos genes que codificam a enzima AAT (*MdAAT1*) não são dependentes de etileno quando as frutas são armazenadas em ACD – QR 1,5.

A menor concentração de compostos voláteis em frutas armazenadas em ACD – FC é função do menor acúmulo de precursores e expressão de enzimas importantes para a síntese destes compostos (*MdAAT1*), pois reduz a níveis baixos o metabolismo aeróbico sem induzir o metabolismo anaeróbico.

A aplicação de 1-MCP em maçãs armazenadas em AC reduz significativamente a produção de compostos voláteis, em função da inibição da expressão de genes codificadores da enzima LOX (*MdLOX1*), AAT (*MdAAT1*) e síntese de precursores, como ácidos e álcoois voláteis.

O armazenamento de maçãs em ACD – QR, especialmente em QR 1,5, resulta na indução do metabolismo anaeróbico, acumulando acetaldeído e etanol, porém, também induz o acúmulo de sorbitol, o qual pode proteger as membranas celulares, reduzindo sua permeabilidade mesmo em condições de estresse por baixo O_2 com acúmulo de acetaldeído e etanol. Há uma correlação negativa entre pressão parcial de O_2 durante o armazenamento e acúmulo de sorbitol.

O tratamento das frutas com 1-MCP combinado com o armazenamento em baixo O_2, seja em AC ou ACD, incrementa o acúmulo de sorbitol em maçãs durante o armazenamento.

A determinação do LMO pode ser realizada de maneira precisa e em tempo real durante todo o período de armazenamento apenas pela medição da produção de CO_2 (ACD – DC) a fim de determinar o *set point* de O_2 de maneira dinâmica para várias cultivares de maçãs, locais de cultivo, com ou sem aplicação de fito-regulador, estádio de maturação e temperatura de armazenamento. Maçãs armazenadas em ACD – DC resultam em *set points* de O_2 similares à ACD – QR.

O armazenamento em ACD – DC mantém a qualidade de maçãs similar àquelas armazenadas em ACD – QR e superior àquelas armazenadas em AC, AC + 1-MCP e ACD –

FC, reduzindo a incidência de podridões e distúrbios fisiológicos, mantendo maior firmeza de polpa e proporção de frutos sadios.

O armazenamento em ACD – QR 1,5 e ACD – DC 1,3 aumenta a atividade da enzima PDC, porém não apresenta o mesmo efeito na enzima ADH, demonstrando que a enzima chave no metabolismo anaeróbico é a PDC. A conversão de aldeído em álcool é função direta da concentração do substrato (aldeído), com pouca ou nenhuma influência da atividade da enzima ADH.

Maçãs armazenadas em ACD – DC e ACD – QR apresentam maior acúmulo de ésteres importantes para o aroma, especialmente, quando comparados à AC + 1-MCP e ACD – FC. Isso é resultado da indução do metabolismo anaeróbico em frutas armazenadas nessas duas técnicas de ACD.

As condições de AC, ACD e aplicação e 1-MCP não apresentam efeito no acúmulo/degradação de malato, sendo a sua concentração mais influenciada pelo tempo de armazenamento.

O armazenamento em AC e ACD tem maior influência nos ácidos minoritários do ciclo de Krebs, citrato, 2-oxoglutarato, fumarato, succinato e oxaloacetato. A concentração desses ácidos é reduzida quando as maçãs são armazenadas na menores pressões parciais de O_2 (ACD – QR 1,5 e ACD – DC 1,3).

A aplicação de 1-MCP tem pouca influência no acúmulo/degradação dos ácidos minoritários do ciclo de Krebs, demonstrando que o etileno não desempenha regulação direta do ciclo de Krebs de maçãs armazenadas.

A partir dos resultados obtidos no presente trabalho, levando em consideração a qualidade físico-química, produção de compostos voláteis e expressão genica, as melhores condições de armazenamento de maçãs por longos períodos segue a seguinte ordem: ACD – DC = ACD – QR > ACD – FC = AC + 1-MCP > AC.

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