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Faculdade de Agronomia Eliseu Maciel
Departamento de Ciência e Tecnologia Agroindustrial
Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos

Tese

Efeito do déficit hídrico e hipóxia pós-colheita no sistema antioxidante enzimático e não enzimático de frutos de tomate 'Micro-Tom' com diferentes expressões de MT-sHSP23.6

Gabriela Niemeyer Reissig

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Gabriela Niemeyer Reissig
Bacharela em Química de Alimentos - UFPel
Mestra em Ciência e Tecnologia de Alimentos - UFPel

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Orientador: Prof. Dr. Marcos Antonio Baccarin

Coorientador: Prof. Dr. Cesar Valmor Rombaldi

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Banca examinadora:

_____

Prof. Dr. Marcos Antonio Baccarin
Doutor em Ciências Agrárias (Fisiologia Vegetal) pela Universidade Federal de Viçosa (UFV).

_____

Prof. Dr. Cesar Valmor Rombaldi
Doutor em Biologie Moléculaire Végétale pela Ecole Nationale Superieure Agronomique de Toulouse.

_____

Prof. Dra. Joseana Severo
Doutora em Ciência e Tecnologia de Alimentos pela Universidade Federal de Pelotas (UFPel).

_____

Prof. Dr. Sidnei Deuner
Doutor em Agronomia (Fisiologia Vegetal) pela Universidade Federal de Lavras (UFLA).

_____

Prof. Dra. Vanessa Galli
Doutora em Biologia Celular e Molecular pela Universidade Federal do Rio Grande do Sul (UFRGS).

Dedicatória

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“Não existe meio de verificar qual é a decisão acertada, pois não existe termo de comparação. Tudo é vivido pela primeira vez e sem preparação. Como se um ator entrasse em cena sem nunca ter ensaiado. Mas o que pode valer a vida, se o primeiro ensaio da vida já é a própria vida?”

A Insustentável Leveza do Ser
Milan Kundera

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"*Diante da vastidão do tempo e da imensidão do universo, é um imenso prazer para mim dividir um planeta e uma época com você.*"

Carl Sagan

RESUMO

REISSIG, Gabriela Niemeyer. **Efeito do déficit hídrico e hipóxia pós-colheita no sistema antioxidante enzimático e não enzimático em frutos de tomate 'Micro-Tom' com diferentes expressões de MT-sHSP23.6**. 2018. 101f. Tese (Doutorado em Ciência e Tecnologia de Alimentos) – Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos, Faculdade de Agronomia Eliseu Maciel. Universidade Federal de Pelotas, Pelotas, 2018.

O tomate (*Solanum lycopersicum* L.) é uma hortaliça de grande importância para a alimentação e para a pesquisa. É extremamente versátil, nutritivo e rico em compostos bioativos. Outrossim, é uma excelente planta modelo para estudos que envolvem o processo de amadurecimento, em especial a cv. 'Micro-Tom', que apresenta tamanho pequeno, ciclo de vida curto e fácil transformação. Sabe-se que as pequenas HSPs (*small heat shock proteins*) desempenham papel importante na tolerância a estresses de origem abiótica, que por sua vez podem levar ao dano oxidativo. A utilização de tomate cv. 'Micro-Tom' modificado quanto a expressão do gene que condifica a proteína MT-sHSP23.6 é uma excelente ferramenta de pesquisa para entender o seu papel como chaperona nos processos celulares em condições potencialmente estressantes. Isto posto, o presente estudo teve por objetivo investigar a resposta do sistema antioxidante enzimático e não enzimático em genótipos de tomate cv. 'Micro-Tom' com diferentes níveis de expressão de MT-sHSP23.6 submetidos ao déficit hídrico pré-colheita e hipóxia pós-colheita. Foram utilizados frutos de genótipos de tomate c.v 'Micro-Tom' tipo selvagem (WT) e com maior expressão do gene que codifica a proteína MT-sHSP23.6 (Sense). Para o experimento do primeiro capítulo, foram colhidos frutos no estádio *breaker* que foram submetidos às condições de normoxia (23 ºC, escuro) e hipóxia (fluxo de nitrogênio diário de 0,0098 MPa/10 minutos, 23 ºC, escuro) por 5 e 8 dias, respectivamente. Para o experimento do segundo capítulo, foram utilizados frutos no estádio *breaker,* oriundos plantas que foram submetidas a tratamentos sob irrigação normal e sob déficit hidrico (8 dias de suspensão). Após a colheita, os frutos foram submetidos a condição de hipóxia similar a do primeiro experimento. Em ambos os estudos foi avaliada a tonalidade de cor dos frutos (Hueº), bem como as enzimas do sistema antioxidante enzimático, espécies reativas de oxigênio, sistema antioxidante não-enzimático e a atividade antioxidante. No primeiro experimento, constatou-se que as enzimas antioxidantes apresentaram maior atividade no período de hipóxia para os frutos do genótipo Sense em comparação aos WT. Também apresentaram menor concentração de ânion superóxido, tanto sob condições de normoxia quanto hipóxia. Quanto aos antioxidantes não enzimáticos analisados, destaque para o maior acúmulo de compostos fenólicos no período pós-hipóxia e ácido L-ascórbico no período de hipóxia para o genótipo Sense. Durante o período de hipóxia, o genótipo Sense apresentou a maior atividade antioxidante. Já no segundo experimento, observou-se que o déficit hídrico promoveu um maior acúmulo de compostos do sistema antioxidante não enzimático, como os compostos fenólicos totais e ácido ascórbico. Também se observou que algumas vezes, em especial no período de hipóxia, a influência de uma maior expressão de MT-sHSP23.6 superou a do déficit hídrico. Os resultados obtidos nos dois experimentos demonstraram que uma maior expressão do gene que codifica a proteína MT-sHSP23.6 influencia positivamente o sistema antioxidante de frutos de tomate submetidos ao déficit hídrico pré-colheita e hipóxia pós-colheita, através do aumento da atividade de enzimas antioxidantes e

acúmulo de compostos antioxidantes, evidenciando que estas proteínas podem estar relacionadas aos mecanismos de tolêrancia das plantas à diferentes fatores abióticos de estresse.

Palavras-chave: fisiologia pós-colheita; sHSP; déficit hídrico; armazenamento sob baixas concentrações de oxigênio; enzimas antioxidantes; metabólitos secundários antioxidantes; espécies reativas de oxigênio

ABSTRACT

REISSIG, Gabriela Niemeyer. **Effect of water deficit and post-harvest hypoxia on the enzymatic and non-enzymatic antioxidant system in 'Micro-Tom' tomato fruits with different MT-sHSP23.6 expression**. 2018. 101f. Tese (Doutorado em Ciência e Tecnologia de Alimentos) – Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos, Faculdade de Agronomia Eliseu Maciel. Universidade Federal de Pelotas, Pelotas, 2018.

Tomato (*Solanum lycopersicum* L.) is a horticultural crop of pivotal importance for human nutrition and research. Tomato fruits are extremely versatile, nutritious and rich in bioactive compounds. It is also an excellent plant model for studies involving ripening, especially cv. 'Micro-Tom', which features small size, short life cycle, and easy transformation. It is well known that sHSPs (small heat shock proteins) play an important role in tolerance to abiotic stresses which can lead to oxidative damage. Genetically transformed 'Micro-Tom' plants with different expression levels of MT-sHSP23.6 are an excellent research tool to understand its role as a chaperone in cellular processes under potentially stressful conditions. Therefore, the present study aimed to investigate the role of the MT-sHSP23.6 protein in the enzymatic and non-enzymatic antioxidant system of 'Micro-Tom' tomato fruits subjected to pre-harvest water deficit and post-harvest hypoxia. Fruits of 'Micro-Tom' tomato wild type (WT) and with higher expression of the gene coding for the protein MT-sHSP23.6 (Sense) genotypes were used. For the experiment of the first chapter, fruits were harvested at the breaker stage and subjected to normoxia (23 °C, dark) and hypoxia (daily nitrogen flow of 0.0098 Mpa/10 minutes, 23 °C, dark) storage for 5 and 8 days, respectively. For the experiment of the second chapter, fruits were used in the breaker stage, from plants that underwent treatments under normal irrigation and under water deficit (8 days of suspension). After harvest, the fruits were subjected to hypoxia conditions similar to those of the first experiment. In both studies the color tone of the fruits (Hue°) was evaluated, as well as the components of the enzymatic antioxidant system, reactive oxygen species, non-enzymatic antioxidant system and antioxidant activity. In the first experiment, it was found that the antioxidant enzymes presented higher activity in the period of hypoxia for the fruits of the Sense genotype in comparison to the WT. They also presented lower concentration of superoxide anion, both under normoxia and hypoxia storage. Regarding non-enzymatic antioxidants, the highest accumulation of phenolic compounds in the post-hypoxia period and L-ascorbic acid in the period of hypoxia for the Sense genotype was the most significant. During hypoxia period, the Sense genotype had the highest antioxidant activity. In the second experiment, it was observed that the water deficit promoted a greater accumulation of compounds of the non-enzymatic antioxidant system, such as total phenolic compounds and L-ascorbic acid. It has also been observed that sometimes, especially in the period of hypoxia, the influence of a higher expression of MT-sHSP23.6 surpassed that of the water deficit. The results obtained in the two experiments demonstrated that a higher expression of the gene coding for the MT-sHSP23.6 protein positively influences the antioxidant system of tomato fruits subjected to pre-harvest water deficit and post-harvest hypoxia, by increasing the activity of antioxidant enzymes and accumulation of antioxidant compounds, evidencing that these proteins may be related to the mechanisms of plant tolerance to different abiotic stress factors.

Keywords: post-harvest physiology; sHSP; water deficit; low oxygen storage; antioxidant enzymes; antioxidant secondary metabolites; reactive oxygen species

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ABTS	Ácido 2,2'-azino-bis (3-etilbenzotiazolina-6-sulfónico)
APX	Ascorbato peroxidase
AsA	Ácido ascórbico
CAT	Catalase
DPPH	2,2-Difenil-1-picrilhidrazil
EROS	Espécies Reativas de Oxigênio
GPOD	Guaiacol peroxidase
HSP	Heat shock protein
H_2O_2	Peróxido de Hidrogênio
$O_2^{\bullet-}$	Ânion superóxido
ROS	Reactive Oxygen Species
sHSP	Small heat shock protein
SOD	Superóxido dismutase
ºHue	Ângulo Hue

Sumário

1 Introdução

O tomate (*Solanum lycopersicum* L.) é uma hortaliça de grande importância mundial, estando presente na alimentação diária de muitas pessoas, tanto na forma *in natura* quanto processada (SLIMESTAD e VERHEUL, 2009). Durante o cultivo e o armazenamento pós-colheita, o tomate pode enfrentar diversos fatores estressantes, bióticos e abióticos, que podem levar a perda de produtividade e/ou qualidade, contudo, se usados de forma intencional, moderada e controlada, podem levar a um acúmulo de compostos que incrementam a qualidade do fruto, aumentando a tolerância da planta e preparando a mesma para um estresse subsequente (CAPANOGLU, 2010; PEDRESCHI e LURIE, 2015; RIPOLL et al., 2014).

Assim como nos demais organismos vivos, nos mais diversos níveis de complexidade, o tomate apresenta um grupo de proteínas que desempenham papel fundamental para a manutenção da sua homeostase celular protéica, atuando como chaperonas moleculares, em condições normais ou quando submetido a algum fator estressante. Estas proteínas são conhecidas como HSPs ("*heat shock protein*s" ou proteínas de choque térmico) e fazem parte de uma grande família que é classificada pelo seu peso molecular. Integrando esta família temos as pequenas HSPs (sHSPs), que podem degradar proteínas anormalmente configuradas, assim como se ligar a proteínas desnaturadas ou parcialmente dobradas, prevenindo a agregação ou o dobramento não funcional (FU, 2014; RODZIEWICZ et al., 2014; SUN, VAN MOTANGU e VERBRUGGEN, 2002).

Apesar de terem sido originalmente descobertas nas glândulas salivares de moscas da fruta (*Drosophila*) em resposta ao choque térmico (ASHBURNER e BONNER, 1979, PEGORARO et al., 2011), atualmente sabe-se que as HSPs são induzidas nos mais diferentes estresses, como no caso do déficit hídrico (SATO e YOKOYA, 2008, TIMPERIO, EGIDI e ZOLLA, 2008) e hipóxia pós-colheita (PEGORARO et al., 2012).

O déficit hídrico e hipóxia podem levar a uma resposta ao estresse conhecido como dano oxidativo, que pode danificar os componentes da célula e causar sua disfunção. O estresse oxidativo é causado por uma superprodução e acúmulo de espécies reativas de oxigênio (EROs), levando a um desbalanço entre a produção e detoxificação de EROs, devido ao distúrbio na fisiologia normal da célula (DEMIDCHIK, 2015; MORALES e MUNNÉ-BOSCH, 2016).

A limitação de água na planta pode levar a um aumento da produção de espécies reativas de oxigênio, devido à interrupção do sistema de transporte de elétrons na fase fotoquímica da fotossíntese e dos processos metabólicos oxidantes que ocorrem no cloroplasto, mitocôndria e microcorpos (AHMAD et al., 2010; DEMIDCHIK, 2015; MURSHED, LOPEZ-LAURI e SALLANOM, 2013).

Em condições de baixa concentração de oxigênio, mesmo com o decréscimo do substrato essencial (O_2) para o metabolismo oxidativo, é possível observar um maior acúmulo de espécies reativas de oxigênio quando plantas são submetidas a condições de hipóxia. Mesmo em baixas concentrações de oxigênio, o escape de elétrons da cadeia transportadora de elétrons da respiração e da fotossíntese possibilita a formação de EROs (CHOUDHURY et al., 2017; MITTLER, 2017; PUCCIARIELLO, BANTI e PERATA, 2012).

As espécies reativas de oxigênio apresentam duplo papel nas células, podendo agir como marcadores e como moléculas sinalizadoras para indução de respostas ao estresse oxidativo. Para poderem desempenhar esta dupla função, a sua produção e varredura são fortemente reguladas por uma ampla rede de genes que é ativada sob condições de estresse (PUCCIARIELLO, BANTI e PERATA, 2012). O sistema de defesa antioxidante é um dos principais responsáveis por elevar a tolerância de plantas expostas as mais diversas condições ambientais que causam dano oxidativo (AHMAD, 2010).

Ao longo da evolução, as plantas desenvolveram estratégias para lidar com o estresse oxidativo, como a ativação ou a síntese “de novo” de enzimas antioxidantes que removem as espécies reativas de oxigênio. São exemplos de enzimas antioxidantes a catalase (CAT), a superóxido dismutase (SOD), a ascorbato peroxidase (APX) e a guaiacol peroxidase (GPOD) (CHOUDHURY et al., 2017; MITTLER, 2017; DEMIDCHIK, 2015). Além do sistema antioxidante enzimático, as plantas produzem metabólitos especializados, que desempenham diversas funções nas células e podem apresentar capacidade antioxidante. São exemplos destes compostos os tocoferóis, o ácido L-ascórbico, os compostos fenólicos e os carotenoides (GILL e TUTEJA, 2010; JITHESH et al., 2006; THATOI, PATRA e DAS, 2014).

As proteínas HSPs e o sistema antioxidante se complementam para aumentar a tolerância de plantas em situações em que ocorra um desbalanço redox nas células. Enquanto os antioxidantes (enzimáticos e não enzimáticos) promovem a detoxificação das espécies reativas de oxigênio na célula, as proteínas HSPs atuam impedindo que ocorram danos estruturais nas proteínas celulares (TIMPERIO, EGIDI e ZOLLA, 2008). As proteínas de choque térmico podem ter sua expressão aumentada devido ao papel sinalizador das EROs, demonstrando o duplo papel destas moléculas na célula e sua atuação na regulação redox (PUCCIARIELLO, BANTI e PERATA, 2012; TIMPERIO, EGIDI e ZOLLA, 2008). É possível que plantas que superexpressem sHSPs apresentem uma melhor resposta do sistema antioxidante quando submetidas a diferentes estresses, já que o mesmo é constituído por enzimas (enzimas antioxidantes e enzimas para biossíntese de antioxidantes não enzimáticos) que também são proteínas suscetíveis aos danos oxidativos.

A utilização de plantas transformadas geneticamente, com diferentes expressões de MT-sHSP23.6 é uma excelente alternativa para estudar o papel desta proteína no sistema antioxidante de plantas. O tomate cv. ‘Micro-Tom’ é um cultivar anão que vem sendo muito utilizado para transformação, tendo sido inicialmente criado para cultivo doméstico. Por ser uma planta de tamanho pequeno, rápdio crescimento, ciclo de vida curto para a colheita dos frutos após a semeadura e fácil transformação, o cv. ‘Micro-Tom’ se tornou um modelo conveniente para os mais diversos campos de pesquisa (GONZALEZ et al., 2015; MALACRIDA et al., 2006; SUN et al., 2006). O tomate também é um modelo preeminente para o estudo do amadurecimento de frutos e controle do etileno nos processos de desenvolvimento, pelo fato de ser um fruto climatérico dependente do etileno para o amadurecimento (KLEE e GIOVANNONI, 2011).

O grupo de pesquisa do laboratório de Metabolismo Vegetal da Universidade Federal de Pelotas tem estudado o papel da proteína mitocondrial sHSP23.6 frente a diferentes fatores ambientais de estresse. Para isso, são utilizados genótipos de tomate cv. ‘Micro-Tom’ geneticamente modificados quanto ao nível de expressão do gene que codifica a proteína MT-sHSP23.6, através do silenciamento e da superexpressão do gene.

Para validar o modelo experimental, foi verificado o acúmulo relativo de transcritos e produto da tradução, onde confirmou-se que a transformação para uma

maior expressão de MT-sHSP23.6 foi efetiva (HUTHER et al., 2013). Além disso, dissertações, teses e artigos já foram divulgados com os resultados obtidos, demonstrando que uma maior expressão de MT-sHSP23.6 resultou em menores danos à atividade fotossintética quando as plantas de tomate foram submetidas a altas temperaturas (HUTHER et al., 2013), bem como uma maior tolerância ao alagamento (HUTHER et al., 2017).

Com o intuito de estudar o papel da proteína MT-sHSP23.6 em plantas de tomate, frente a fatores abióticos de estresse, este trabalho tem como objetivo verificar se genótipos com com diferentes níveis de expressão da proteína MT-sHSP23.6, submetidos ao déficit hídrico pré-colheita e hipóxia pós-colheita, apresentam diferenças no sistema antioxidante enzimático e não enzimático.

1.1 Hipótese

Considerando que as proteínas HSPs, em especial a MT-sHSP23.6, estão envolvidas na tolerância das plantas aos mais diversos fatores ambientais de estresse, uma maior expressão do gene que codifica esta proteína promove uma maior atividade de enzimas antioxidantes e maior acúmulo de compostos antioxidantes não enzimáticos em tomates ‘Micro-Tom’ quando submetidos ao déficit hídrico pré-colheita e/ou hipóxia pós-colheita.

1.2 Objetivo Geral

Estudar se as respostas do sistema antioxidante enzimático e não enzimático são afetadas pelo déficit hídrico e hipóxia em frutos de tomate ‘Micro-Tom’ do genótipo tipo selvagem e do genótipo com maior expressão do gene que codifica a proteína a MT-sHSP23.6.

1.3 Objetivos Específicos

- Avaliar a atividade de enzimas do sistema antioxidante (catalase, ascorbato peroxiase, guaiacol peroxiase e superóxido dismutase) em frutos de tomate submetidos ao déficit hídrico pré-colheita e hipóxia pós-colheita;
- Avaliar as espécies reativas de oxigênio (peróxido de hidrogênio e ânion superóxido) em frutos de tomate submetidos ao déficit hídrico pré-colheita e hipóxia pós-colheita;
- Avaliar o acúmulo de metabólitos (compostos fenólicos totais, β-caroteno, licopeno e ácido L-ascórbico) com potencial antioxidante em frutos de tomates submetidos ao déficit hídrico pré-colheita e hipóxia pós-colheita;
- Avaliar a ativade antioxidante de compostos não enzimáticos, pelos métodos de captura dos radicais DPPH (2,2-difenil-1-picril-hidrazila) e ABTS (2,2-azinobis-[3-etil-benzotiazolin-6-ácido sulfônico]), em frutos de tomate submetidos ao déficit hídrico pré-colheita e hipóxia pós-colheita.

1.4 Metas

Demonstração de que o aumento da expressão do gene que codifica a proteína MT-sHSP23.6 melhora a eficiência do sistema antioxidante enzimático e não-enzimático na varredura de espécies reativas de oxigênio e radicais livres em frutos de tomate submetidos ao déficit hídrico pré-colheita e hipóxia pós-colheita.

2 Revisão de Literatura

2.1 Tomate (*Solanum lycopersicum* L.): importância na alimentação e na pesquisa

O tomate (*Solanum lycopersicum* L.) é um fruto de grande importância mundial, estando presente na alimentação diária de grande parte das pessoas, tanto na forma *in natura* como processada. No ano de 2017, o Brasil produziu 4,5 milhões de toneladas de tomate, e o estado de Goiás foi o que teve a produção mais significativa (1,3 milhões de toneladas) no mesmo período (IBGE, 2017). Segundo dados da FAO (2017), a China continua sendo a maior produtora de tomates.

É uma espécie originária da América do Sul, mais especificamente das regiões andinas do Peru, Bolívia e Equador. Botanicamente, o fruto do tomateiro é do tipo baga, mas é cultivado e consumido como um vegetal (SLIMESTAD, 2009). Os frutos de tomate são fontes de compostos bioativos, como os carotenoides (em especial o licopeno), ácido ascórbico, compostos fenólicos e vitamina E. Estudos epidemiológicos têm demonstrado que o consumo de tomate diminui o risco de alguns tipos de câncer, como o de próstata, e problemas cardiovasculares (GEORGE et al., 2004; GÜMÜSAY et al., 2015).

O tomate é um fruto carnoso climatérico, dependente do etileno para seu processo de amadurecimento. Frutos deste tipo apresentam um incremento (pico) na taxa respiratória e na síntese do etileno durante o amadurecimento. Também apresentam a capacidade de amadurecer depois de colhidos e respondem à aplicação de etileno (ALEXANDER e GRIERSON, 2002; KLEE e GIOVANNONI, 2011).

No processo de amadurecimento do tomate, que coincide com a maturação total das sementes, uma das transformações mais marcantes é a conversão do cloroplasto em cromoplasto. Com esta conversão a capacidade fotossintética dos cloroplastos é perdida, devido à degradação das clorofilas e "desmontagem" das estruturas do tilacóide. Com isso, começa a ocorrer um acúmulo de plastoglóbulos dentro dos cromoplastos formados, que são partículas lipoprotéicas que acumulam grandes quantidades de carotenoides, principalmente licopeno e β-caroteno, levando a uma mudança irreversível no fruto e sinalizando visualmente que o fruto está maduro. Além desta transformação, ocorre o amolecimento do fruto, devido a

alterações que ocorrem na parede celular, bem como formação de açúcares, ácidos e compostos voláteis responsáveis pelo sabor único e atrativo do tomate maduro (GIOVANNONI, 2004; SEYMOUR et al., 2002).

Por ser climatérico, dependendo do etileno para o seu amadurecimento, o tomate se tornou um modelo muito utilizado em estudos de amadurecimento e sobre o controle de etileno nos processos de desenvolvimento (KLEE e GIOVANNONI, 2011). Cultivares como a cv. 'Micro-Tom', são convenientes para a pesquisa, pois apresentam tamanho pequeno, rápido crescimento, ciclo de vida curto para a colheita dos frutos após semeadura e fácil transformação (GONZALEZ et al., 2015; MALACRIDA et al., 2006; SUN et al., 2006). A utilização de tomates cv 'Micro-Tom' transformados, com diferentes expressões do gene MT-sHSP23.6, possibilita o estudo do papel que as proteínas sHSP podem desempenhar em plantas de tomates submetidas a diferentes condições estressantes. Huther et al. (2013) observaram que sob altas temperaturas, tomateiros com alta expressão do gene sHSP23.6 mitocondrial mostraram menos danos à atividade fotossintética do que o tipo selvagem e o com baixa expressão, o que de alguma forma pode indicar o papel destas proteínas em uma maior tolerância a estresses abióticos.

2.2 Fatores abióticos de estresse: déficit hídrico e hipóxia pós-colheita

De acordo com Larcher (1987), estresse é a desordem no aparato estrutural e na coordenação de vários processos em nível molecular, celular e ao nível de organismo como um todo. No entanto, segundo Lichtenthaler (1988), o conceito de estresse em plantas pode ser divido em eu-estresse e di-estresse, sendo o primeiro caracterizado como estimulante para o desenvolvimento da planta, e o segundo como um estresse real e severo que causa injúria e afeta negativamente o desenvolvimento da planta. Diversos fatores podem causar perturbações nas plantas, podendo ser de ordem biológica (estresses bióticos) ou não (estresses abióticos). Entre estes podem ser citados o déficit hídrico e hipóxia.

2.2.1 Déficit hídrico

A seca, condição ambiental que ocorre devido à limitação de recursos hídricos, é um dos maiores limitantes de produção na agricultura. De acordo com Pedro-Monzonís et al. (2015), a seca, quando definida como um risco natural (*natural hazard*, em inglês) pode ser divida em três tipos: meteorológica (escassez continuada de precipitação, geralmente origina os outros tipos), agricultural (déficit de umidade na região da raiz) e hidrológica (período de baixo fluxo em cursos de água, lagos e níveis de água subterrânea abaixo do normal).

Durante a exposição à seca, ocorrem diversas alterações bioquímicas, fisiológicas e metabólicas na planta, afetando o metabolismo, rendimento e desempenho de diversas culturas (CAPANOGLU, 2010; CHAVES e OLIVEIRA, 2004; SHAFI et al., 2009). As respostas celulares a este tipo de estresse incluem ajuste osmótico, regulação da circulação de água, proteção e degradação de proteínas e proteção contra o estresse oxidativo (TIMPERIO, EGIDI e ZOLLA, 2008).

A fotossíntese é um dos processos celulares mais afetados pelo déficit hídrico. Com a finalidade de manter o balanço de água, as plantas fecham os estômatos, diminuindo a perda de água por transpiração. No entanto, este mecanismo que as plantas desenvolveram tem um preço, que é a redução na captação do CO_2, que acaba levando a uma redução na taxa fotossintética (GRASSMANN, HIPPELI e ELSTNER, 2002; RIPOLL et al., 2014). Isso leva a uma diminuição na regeneração do $NADP^+$ através do ciclo de Calvin-Benson. Com a falta de aceptor final de elétrons, ocorre uma interrupção do sistema de transporte de elétrons, acarretando em um extravasamento de elétrons em direção ao O_2. Ocorre então, um aumento na produção de espécies reativas de oxigênio, que além de serem formadas pelos processos oxidantes no cloroplasto, também são formadas na mitocôndria e micro corpos em condições de déficit hídrico (AHMAD et al., 2010; ASADA, 1999; MURSHED, LOPEZ-LAURI e SALLANOM, 2013; SHARMA et al., 2012). Nestas condições também há um aumento na fotorrespiração, principalmente quando a oxigenação da RUBP (Ribulose-1,5-bisfosfato) é máxima, devido à limitação da fixação do CO_2 (NOCTOR et al., 2002).

O estresse oxidativo, que é desencadeado por diferentes estresses abióticos, como o déficit hídrico, leva a produção de compostos antioxidantes, na tentativa de diminuir a quantidade de espécies reativas de oxigênio na célula. Em condições de déficit hídrico, a produção de espécies reativas de oxigênio excede a capacidade de o sistema antioxidante removê-los (NOCTOR e FOYER, 1998). O sistema antioxidante enzimático das plantas é constituído por enzimas como a catalase, ascorbato peroxidase, guaiacol peroxidase, superóxido dismutase, entre outras. Com relação ao sistema não enzimático, podem ser citados a glutationa, betaínas, fenóis, carotenoides, α-tocoferol, ácido ascórbico, etc (SHARMA et al., 2012).

Quando aplicado moderadamente e de forma controlada, o déficit hídrico pode melhorar a qualidade de frutas e vegetais, através de um maior acúmulo de compostos antioxidantes não enzimáticos que são importantes na dieta humana, devido ao potencial bioativo que podem apresentar. Entre estes compostos temos os fenóis, carotenoides e ácido ascórbico (NORA et al., 2012; RIPOLL et al., 2014). Quando se visa melhorar a qualidade de produtos vegetais através de fatores abióticos que promovam um estresse moderado, vários fatores devem ser levados em consideração, como: estádio de desenvolvimento, tipo e severidade do estresse, susceptibilidade do produto de origem vegetal, entre outros. Estudo realizado com frutos de tomate demonstrou que os efeitos do déficit hídrico dependem do genótipo e do estágio de desenvolvimento do fruto (RIPOLL et al., 2016). Veit-Köhler, Krumbein e Kosegarten (1999) observaram que a aplicação de déficit hídrico quando o tomate estava vermelho aumentou mais a qualidade do fruto do que quando foi aplicado no fruto verde e laranja.

O déficit hídrico também pode ser utilizado como um pré-tratamento/pré-aclimatação para aumentar a tolerância de plantas a um estresse futuro. Os impactos do estresse subsequente podem ser marcadamente reduzidos, quando comparados com plantas que não sofreram um pré-tratamento com estresse. Como diferentes tipos de estresses abióticos dividem as mesmas respostas, é possivel realizar a pré-aclimatação de plantas utilizando diferentes estresses, através de tolerância cruzada (CAPANOGLU, 2010; RIPOLL et al, 2014). Seguindo este pensamento, é possível esperar que a aplicação de déficit hídrico em plantas de tomate seja capaz de aumentar a tolerância dos frutos quando submetidos à hipóxia pós-colheita.

2.2.2 Hipóxia pós-colheita

O termo hipóxia designa a condição em que a concentração de oxigênio do ambiente está menor que as condições de oxigênio atmosférico normais (21%, v/v), e pode ser utilizado para baixos níveis de oxigênio dentro do tecido, mesmo em ambientes com concentrações normais de oxigênio (VAN DONGEN e LICAUSI, 2015). No pós-colheita, quando a hipóxia é utilizada com a finalidade de conservação, para aumentar a vida de prateleira dos produtos de origem vegetal, ela não é vista como uma condição adversa. A utilização de atmosferas modificadas e controladas, embalagens plásticas, aplicação de coberturas (cera, amido, xantana, etc) são exemplos de técnicas de conservação que propiciam uma menor concentração de oxigênio ou restrição de trocas gasosas, aumentando a vida de prateleira de alimentos altamente perecíveis (CORTELLINO et al., 2015; PEDRESCHI e LURIE, 2015).

As repostas às baixas concentrações de O_2 dependem das características do produto, da duração e da intensidade do estresse (PEDRESCHI e LURIE, 2015). De modo geral, condições de hipóxia levam a redução do ciclo dos ácidos tricarboxílicos (respiração aeróbia), ocorrendo acúmulo de intermediários deste ciclo, redução significativa na produção de ATP e ativação do metabolismo anaeróbio. Em vez da fosforilzação oxidativa mitocondrial, a obtenção de energia passa a ser pela via glicolítica. Para manter a glicólise sob baixas concentrações de oxigênio, o NAD^+ precisa ser continuamente regenerado, e este processo ocorre pelas vias fermentativas, levando a formação de produtos como etanol e lactato (HORCHANI et al., 2009; SOUZA e SODEK, 2002; ZABALBA et al., 2009).

O retardamento do amadurecimento também é uma resposta a condições de hipóxia. O amadurecimento é um processo altamente coordenado, onde o fitormônio etileno desempenha um papel fundamental. A rota biossintética do etileno é relativamente simples, com participação de apenas duas enzimas, onde S-adenosilmetionina (AdoMet) é convertida em 1-aminociclopropano-1-carboxilato (ACC) pela ACC sintase. Logo, o ACC (Ácido 1-aminociclopropano-1-carboxílico) é convertido em etileno pela ACC oxidase. Em baixas concentrações de O_2 a conversão do ACC em etileno é inibida, o que contribui para o atraso no amadurecimento (KLEE e GIOVANNONI, 2011).

A hipóxia pós-colheita também leva a indução de metabólitos e proteínas responsivas ao estresse, como compostos do ciclo ascorbato-glutationa e proteínas HSP (PEDRESCHI e LURIE, 2015). Pegoraro et al. (2012) submeteram frutos de tomate a condições de hipóxia e observaram uma maior indução de genes referentes as proteínas HSP, demonstrando que estas proteínas estão envolvidas na proteção da homeostase celular em condições de baixas concentração de oxigênio.

Quando hortaliças são expostas a períodos prolongados de hipóxia, em vez de aumentar a vida de prateleira do produto, poderá ocorrer o oposto, estando os mesmos sujeitos a desordens fisiológicas, associadas ao desbalanço entre os processos oxidativos e redutivos nas células (PEDRESCHI et al., 2009; PEDRESCHI e LURIE, 2015).

2.3 Proteínas HSP e sua importância na homeostase celular protéica

As HSPs (*heat shock proteins*) pertencem a um amplo grupo de proteínas que atuam mantendo a homeostase celular, tanto sobre ótimas condições de crescimento quanto sobre condições de estresse (AL-WHAIBI, 2011; FU, 2014; RODZIEWICZ, 2014). Estas proteínas existem ubiquamente em todas as formas de vida e a maioria delas são conhecidas como chaperonas moleculares, ou seja, proteínas que auxiliam outras proteínas a adquirirem estruturas ativas e funcionais. São responsáveis pelo enovelamento adequado, translocação e degradação de proteínas malformadas (FU, 2014; LASKEY et al., 1978).

Condições adversas, como os estresses abióticos, induzem uma cascata de eventos altamente regulada, levando a um aumento transcricional dos genes referentes às proteínas HSPs. A expressão das HSPs é regulada por HSFs (heat shock factors) e a ativação dos HSFs sob condições de estresse ocorre via processo multi-etapas que envolvem a formação de homotrímeros e aquisição de competência transcricional para indução do gene alvo (DRIEDONKS, 2015; KOTAK et al., 2007; LIU et al., 2013)

As proteínas HSP são classificadas com base no seu peso molecular em cinco diferentes famílias: Família HSP100, Família HSP90, Famíla HSP70, Família HSP60 e Família sHSP (RODZIEWICZ et al. 2014). A família das sHSPs (*small heat-shock proteins*) apresentam peso molecular entre 15 e 40 kDa e atuam na supressão da agregação proteica independente de ATP, bem como na estabilização de membranas celulares danificadas por estresse (FU, 2014; HORWITZ, 1992; JAKOB et al., 1993; TÖRÖK et al., 2001; TSVETKOVA et al., 2002).

As sHSPs podem ser divididas em seis classes, que são classificadas com base na sequencia de aminoácidos, funções e localização intracelular (GUPTA et al., 2010; WATERS et al., 1994). Três classes estão localizadas no citosol e no núcleo, e as demais estão localizadas nos plastídeos, retículo endoplasmático e mitocôndria (HELM et al., 1995; LAFAYETTE et al., 1996; SUN et al., 2010). A quantidade e os tipos de sHSPs variam para cada espécie vegetal, havendo mais de 30 sHSPs em plantas, estando presentes no citosol e em diferentes organelas, sob diversas isoformas (HASLBECK, 2002).

Foi observado que proteínas sHSP originadas do material genético presente no cloroplasto e na mitocôndria desempenham papel importante na tolerância de plantas submetidas a altas temperaturas (HECKATHORN et al., 1998; SANMIYA et al., 2004; SARKAR, KIM e GROVER, 2009). A responsividade de diferentes tipos de proteínas de choque térmico, em uma ampla variedade de respostas ao estresse, indica que a estabilização e a correta formação de proteínas mitocondriais são de alta prioridade para as células de plantas sujeitas a condições de estresse (VANDENBROUCKE et al., 2008). A utilização de plantas com diferentes expressões de genes que codificam estas proteínas, como, por exemplo, MT-sHSP23.6, são excelentes ferramentas para estudar o papel destas pequenas proteínas na tolerância a estresses abióticos, os quais podem levar ao dano oxidativo, danificando as estruturas celulares devido ao acúmulo de espécies reativas de oxigênio.

2.4 Espécies reativas de oxigênio e o sistema antioxidante enzimático e não enzimático

Condições adversas para o crescimento e desenvolvimento de plantas podem levar a uma superprodução e acúmulo de moléculas que contém oxigênio reativo, levando ao estresse oxidativo, que pode danificar as estruturas presentes na célula e causar sua disfunção (DEMIDCHIK, 2015). Estas moléculas, produzidas em excesso durante o estresse oxidativo, são conhecidas como espécies reativas de oxigênio (EROs, em inglês ROS). Estes compostos são conhecidos por apresentarem um ou mais átomos de oxigênio reativo, como exemplos tem-se o oxigênio singlete ($^1O_2$), ânion superóxido ($O_2^{\bullet -}$), radicais hidroxila ($OH^{\bullet}$) e o peróxido de hidrogênio (H_2O_2) (AHMAD et al., 2010). Estas moléculas danificam estruturas celulares e macromoléculas, podendo levar a morte celular (GILL e TUTEJA, 2010; MURSHED, LOPEZ-LAURI e SALLANON, 2013).

As espécies reativas de oxigênio podem ser formadas em diferentes compartimentos celulares, mas é na mitocôndria e no cloroplasto que ocorre a maior produção destes compostos, principalmente devido à fuga de elétrons que ocorre na cadeia transportadora de elétrons. Além destas duas organelas, os peroxissomos também produzem grandes quantidades de EROs quando a planta está sob condições de estresse (ASADA, 2006; MURPHY, 2009). Apesar de todo os efeitos prejudiciais que as EROs podem provocar, é importante ressaltar que elas também desempenham papel importante como moléculas sinalizadoras e reguladoras de genes em plantas submetidas a estresses abióticos (DRIEDONKS et al., 2015; MOLASSIOTIS e FOTOPOULOS, 2011).

Para lidar com os efeitos deletérios das EROs, ao longo de milhares de anos de evolução as plantas desenvolveram mecanismos antioxidantes para se proteger do estresse oxidativo. Entre estes mecanismos estão as enzimas antioxidantes, que são capazes de diminuir ou suprimir o efeito deletério das espécies reativas de oxigênio nas células. A superóxido dismutase (SOD), catalase (CAT), ascorbato peroxidase (APX), guaiacol peroxidase (GPOD), entre outras, são exemplos de enzimas antioxidantes (GILL e TUTEJA, 2010; RODZIEWICZ et al., 2014).

A superóxido dismutase (EC 1.15.1.1) é uma metaloenzima que catalisa a reação de dismutação do ânion superóxido ($O_2^{\bullet-}$) em peróxido de hidrogênio (H_2O_2) e oxigênio molecular (O_2). Estão presentes em diversas partes da célula, como na mitocôndria, peroxissomos, cloroplasto e citosol. São classificadas de acordo com o cofator metálico requerido: ferro (Fe-SOD), cobre/zinco (Cu/Zn-SOD) e manganês (Mn-SOD) (MITTLER, 2002; RODZIEWICZ et al., 2014).

A catalase (EC 1.11.1.6) é uma enzima tetramérica, que possui um grupo heme em cada uma de suas subunidades e é capaz de catalizar a reação de conversão de 2 H_2O_2 em O_2 e $2H_2O$. São encontradas nos peroxissomos e podem ser divididas em três classes: (1) estão mais presentes nos tecidos fotossintéticos e estão envolvidas na remoção do peróxido de hidrôgenio produzido na fotorrespiração; (2) são produzidas nos tecidos vasculares e desempenham papel na lignificação; (3) abundante em plantas jovens e sementes e sua atividade está relacionada com a remoção do excesso de peróxido de hidrogênio formado no ciclo do glioxilato (AHMAD et al., 2010; WILLEKENS et al., 1994).

A ascorbato peroxidase (EC 1.11.1.11) é uma heme peroxidase que possibilita a conversão do H_2O_2 em H_2O pelo ciclo ascorbato-glutationa e utiliza o ácido ascórbico como doador de elétrons. É encontrada nos cloroplastos e no citosol de células não fotossintetizantes, sendo muito importante na proteção contra o estresse fotoxidativo. A família ascorbato peroxidase é constituída de pelo menos cinco isoformas, incluindo as formas ligadas ao tilacóide e membranas microssomais, e as solúveis no citosol, apoplasto e estroma (AHMAD et al., 2010; ASADA, 1992; NOCTOR e FOYER, 1998).

A guaiacol peroxidase (EC 1.11.1.7) é uma proteína que possui o grupo prostético heme e preferencialmente oxida compostos aromáticos doadores de elétrons, como o guaicol e o pirogalol, as custas do peróxido de hidrogênio. Diversas isoformas de GPOD são encontradas em plantas, estando localizadas no citosol, vacúolo e parede celular. Além da defesa contra estresses bióticos e abióticos, a GPOD está associada a importantes processos biossintéticos, como a lignificação da parede celular, biossíntese do etileno e degradação do ácido indolacético (ASADA, 1992; SHARMA et al., 2012).

Outro mecanismo desenvolvido pelas plantas para lidar com o acúmulo de espécies reativas de oxigênio e radicais livres é a utilização de compostos do metabolismo primário e secundário que possuem estruturas que proporcionam atividade antioxidante. Estes compostos, aliados ao sistema antioxidante enzimático, ajudam na varredura de radicais livres formados normalmente nas células e durante a exposição da planta a condições estressantes. Como exemplos destes compostos temos o ácido ascórbico, os carotenoides, os compostos fenólicos e os tocoferóis (DEMIDCHIK, 2015; GILL e TUTEJA, 2010).

O ácido ascórbico, além de agir como antioxidante está envolvido na modulação de algumas enzimas e regulação da transcrição de alguns genes, participa da fotossíntese e também atua como precursor na síntese do oxalato e do tartarato em plantas (HOREMANS et al., 2000; NOCTOR e FOYER, 1998). No cloroplasto, o AsA está relacionado com a fotoproteção e é capaz de regenerar o α-tocoferol a partir de radicais α-tocoferoxil. Ademais, atua como um cofator para a formação da zeaxantina, carotenoide essencial para a dissipação de energia na forma de calor nos cloroplastos (DEMMIG-ADAMS et al., 2012; MUNNÉ-BOSH e ALEGRE, 2002). Na mitocôndria, o ácido ascórbico é sintetizado na membrana interna e reage quimicamente com $^{1}O_2$, $O_{2}^{\cdot -}$, HO^{-} (NOCTOR e FOYER, 1998). O AsA atua como sequestrante de espécies reativas de oxigênio, formando compostos com uma reatividade menor, como o radical ascorbil e o ácido dehidroascórbico (DAVEY et al., 2000).

Os carotenoides são pigmentos lipossolúveis que estão amplamente distribuídos na natureza, sendo produzidos por todos os organismos fotossintetizantes. Na fotossíntese, atuam como compostos coletores de luz e como moléculas fotoprotetoras (DOMONKOS et al., 2013; DURCHAN et al., 2014; ESTEBAN et al., 2015). Basicamente, os carotenoides são tetraterpenos formados por uma cadeia poliênica de 40 carbonos, podendo apresentar de 3-13 duplas ligações conjugadas (MELÉNDEZ-MARTÍNEZ et al., 2007). É esta estrutura altamente insaturada dos carotenoides que possibilita a atuação destes compostos como antioxidantes, sequestrando espécies reativas de oxigênio como o radical peroxil e o oxigênio singlete (STAHL e SIES, 2003). Em altas pressões de oxigênio, os carotenóides podem apresentar ação pró-oxidante (BURTON e INGOLD, 1984).

Os fenóis são compostos formados por anéis aromáticos, com um ou mais substituintes hidroxil. Os compostos fenólicos são moléculas que vão desde simples fenóis até compostos altamente polimerizados (BRAVO, 1998). Os fenóis são considerados antioxidantes multifuncionais, atuando como agentes redutores, quelantes de metais e extintor de oxigênio singlete (KANCHEVA, 2009). A estrutura destes compostos é o fator determinante da sua capacidade de varredura de radicais livres e quelação de metais. Tomando como exemplo os fenóis ácidos, sua atividade antioxidante é devida ao número e as posições dos grupos hidroxil em relação ao grupo funcional carboxil (BALASUNDRAM, SUNDRAM e SAMMAN, 2006; ROBARDS et al., 1999). Assim como os carotenoides, os fenóis podem exibir atividade pró-oxidante na presença de metais de transição (PISOSCHI e POP, 2015). Além de desempenharem papel importante no sistema antioxidante, os compostos fenólicos conferem as plantas resistência a predadores e pesticidas. Também tornam as folhas, flores e frutos mais atraentes, devido à coloração proporcionada por suas moléculas de natureza fenólica (CHANG, ALASALVAR e SHAHIDI, 2016; da SILVA, BARREIRA e OLIVEIRA, 2016).

Além dos compostos citados anteriormente, outros compostos, como a glutationa, a prolina, as poliaminas e as betaínas também desempenham papel importante na defesa das plantas contra o estresse oxidativo (DEMIDCHIK, 2015).

3 Capítulo 1 – High MT-sHSP23.6 expression increases antioxidant system in 'Micro-Tom' tomato fruits during post-harvest hypoxia

Capítulo referente ao artigo publicado na revista Scientia Horticulturae (2018), como requisito para a defesa de Tese.

Abstract: The effect of hypoxia on the antioxidant system during the storage of tomato fruits was investigated using plants with different expression levels of the encoding gene for the MT-sHSP23.6 protein. Fruits from two 'Micro-Tom' tomato plant genotypes (wild type and high expression of MT-sHSP23.6) were harvested at the breaker stage and subjected to normoxia (23 ºC, dark) and hypoxia (daily nitrogen flow of 0.098 Mpa/10 min, 23 °C, darkness) treatments for 5 and 8 days, respectively. We evaluated the color tone (Hueº) of the fruits, as well as the components of the enzymatic antioxidant system, reactive oxygen species, non-enzymatic antioxidant system components and radical scavenging activity. Fruits from plants with high MT-sHSP23.6 expression had the highest activity for all the antioxidant enzymes under hypoxia conditions (25, 20, 76, and 48% higher than wild type for SOD, CAT, APX, and GPOD, respectively). They also had the lowest concentrations of superoxide anion under both normoxia and hypoxia storage. At the end of the normoxia storage period, the genotype with high MT-sHSP23.6 expression had the highest accumulation of lycopene (13%), whereas at the end of hypoxia storage, the wild type genotype had the highest lycopene accumulation (39%). Higher accumulation of phenolic compounds was observed in the post-hypoxia period for plants subjected to the hypoxia treatment in high MT-sHSP23.6 expression genotype (12% on the fourth day and 9% on the eighth day). In addition, ascorbic acid concentration was considerably higher on the third and fourth storage days (47% and 8%, respectively). During the hypoxia period, the genotype with high MT-sHSP23.6 expression exhibited the highest radical scavenging activity. High MT-sHSP23.6 expression stimulated the enzymatic antioxidant system during the hypoxia period and led to higher accumulation of phenolic compounds and ascorbic acid, evidence that this protein may be related to tolerance mechanisms in plants subjected to post-harvest abiotic stresses.

Keywords: post-harvest physiology, low oxygen storage, sHSP, antioxidant secondary metabolites, antioxidant enzymes, reactive oxygen species

3.1 Introduction

Tomato (*Solanum lycopersicum* L.) is a horticultural crop of pivotal importance for human nutrition and research. Regarding human nutrition, the tomato is extremely versatile, nutritious and rich in bioactive compounds (George et al., 2004; Gümüsay et al., 2015). It is a climacteric fruit, entirely dependent on ethylene for ripening, and is an excellent plant model for studies involving ripening (Klee and Giovannoni, 2011). The 'Micro-Tom' tomato cultivar is suitable for research owing to its small size, rapid growth, short life cycle for fruit harvest after sowing, and easy transformation (Gonzalez et al., 2015; Malacrida et al., 2006; Sun et al., 2006).

Using genetically transformed 'Micro-Tom' plants with different expression levels of MT-sHSP23.6 provides a good means to understand how the translated protein acts as a chaperone in cellular processes under stressful conditions. It is well known that small heat shock proteins (sHSPs) play an important role in tolerance to abiotic stresses which can lead to oxidative damage, and this damage, in turn, can damage cellular structures owing to the accumulation and action of reactive oxygen species (Fu, 2014; Rodziewicz et al. 2014; Huther et al., 2013; Huther et al., 2017; Huther et al., 2018).

Fruit storage in low-oxygen conditions, from a physiological perspective, can be considered a stressful post-harvest condition. When used moderately for preservation, to increase the shelf life of fruits and vegetables, it is not seen as an adverse condition. The use of modified and controlled atmospheres, plastic packaging, and the application of coatings (wax, starch, xanthan, etc.) are examples of conservation techniques that allow a lower concentration of oxygen or restriction of gas exchange, increasing the shelf life of highly perishable products (Cortellino et al., 2015; Pedreschi and Lurie, 2015).

Hypoxic conditions lead to a break in the cycle of tricarboxylic acids (aerobic respiration), with an accumulation of intermediates in this cycle, a significant reduction in ATP production, and an activation of anaerobic metabolism (Horchani et al., 2009; van Dongen and Licausi, 2015; Pegoraro et al., 2012; Zabalza et al., 2009). Delayed ripening is also a response to hypoxia. Ripening is a highly coordinated process in which the phytohormone ethylene plays a key role. At low O_2 concentrations the conversion of ACC (1-aminocyclopropane-1-carboxylic acid) to ethylene is inhibited, which contributes to the observed delay in ripening (Klee and

Giovannoni, 2011). Under hypoxic conditions, even with the decrease of O_2 for oxidative metabolism, higher accumulation of reactive oxygen species (ROS) is sometimes observed (Pucciariello et al., 2012). Even at low concentrations of oxygen, the electron escape from both photosynthetic and mitochondrial electron transport chains allows the formation of ROS (Mittler, 2017). Studies using Arabidopsis seedlings have presented evidence of mitochondria-associated ROS production in response to low-oxygen conditions (Chang et al., 2012).

The mitochondria and chloroplasts are the major producers of ROS, mainly due to the leakage of electrons from the electron transport chain (Asada, 2006; Murphy, 2009). During ripening, chloroplasts are converted into chromoplasts and their photosynthetic capacity is lost owing to the degradation of chlorophyll and disassembly of the thylakoid structures (Giovannoni, 2004; Seymour et al., 2002). Thus, the mitochondria become the main producers of ROS. To cope with the excessive accumulation of ROS, through evolution, plants have developed antioxidant mechanisms to deal with the harmful effects of these molecules and maintain the cellular redox balance, including the production of the antioxidant enzymes catalase (CAT), superoxide dismutase (SOD), ascorbate peroxidase (APX), and guaiacol peroxidase (GPOD), and primary and secondary metabolism compounds with structures that provide antioxidant activity, such as ascorbic acid, carotenoids and phenols (Demidchik, 2015; Rodziewicz et al., 2014).

Assuming the above background, based on a comparison of 'Micro-Tom' tomato fruits with high expression (Sense) of the MT-sHSP23.6 protein vs. wild type (WT), the objective of this work was to evaluate the role of MT-sHSP23.6 in the enzymatic and non-enzymatic antioxidant system during normoxia and hypoxia storage conditions.

3.2 Material and Methods

3.2.1 Plant material and treatments

Seeds of two tomato genotypes of cultivar 'Micro-Tom' - the wild type (WT) and those with high expression of MT-sHSP23.6 protein (Sense) - were used. The transformation stability and physiological behavior of tomato plants under normal cultivation conditions has been described previously (Huther et al., 2013; Huther et al., 2018).

Seeds of each genotype (WT and Sense) were germinated in Gerbox® with blotting paper moistened with distilled water, where they remained for a period of 10 days in a germinating chamber (25 ºC, photoperiod of 12 hours). After this period, the seedlings were transplanted to 0.5 L plastic pots filled with commercial organic substrate. Tomato plants were grown in a greenhouse located at Federal University of Pelotas, Capão do Leão campus (geographical coordinates: 31º 52’ 32” S and 52º 21’ 24” W, altitude 13 m). The average temperature in the greenhouse during the experimental period was 25 ± 5 ºC and the irradiance, from natural light, was 800 µmol photons m^{-2} s^{-1}.

After transplantation, the plants were watered on alternate days (50 mL) and supplied with 15 mL of nutrient solution (Hoagland and Arnon, 1938) three times a week. When the fruit reached the breaker stage (90 days after germination), they were harvested and subjected to normoxia and hypoxia post-harvest storage (Fig. 1). The sampling times differed between storage conditions because hypoxia delayed ripening, as observed in earlier preliminary studies conducted to establish the experimental conditions (hypoxia condition, time, and ripening evolution).

For normoxia treatment, 12 fruits of the two genotypes were harvested and stored in opened transparent plastic pots (500 mL) at 23 ºC and kept in the dark. Three fruits were collected immediately before storage (0 time), 1, 3, and 5 days after the beginning of the treatment (post-harvest storage; Fig. 1).

In the hypoxic treatment, 12 fruits of the two genotypes were collected and stored in transparent plastic pots (500 mL), sealed with silicone glue to reinforce their own packing at 23 °C, and kept in the dark. The plastic pots had an air flow control system, consisting of an inlet and an outlet for the flow of gases. The hypoxia condition was generated by the introduction of nitrogen gas and a gas scan was

performed daily (0.098 MPa / 10 min) on all 3 days of hypoxia. After 3 days of hypoxia, the samples were subjected to the normoxia condition for 5 days, similar to the normoxia treatment. Three fruits were collected immediately before storage (time 0) and after hypoxia treatment, corresponding to a 3-day period (Fig. 1). After being returned to the normoxia condition, three fruits were collected on the first and fifth days, corresponding to 4 and 8 days after the beginning of the post-harvest treatment (Fig. 1).

At each sampling point color analysis was performed on the fruit epicarp, which was immediately stored at -86 ºC for further biochemical analysis. Color parameters, such as Hue angle, are efficient for following the ripening evolution in the 'Micro-Tom' tomato.

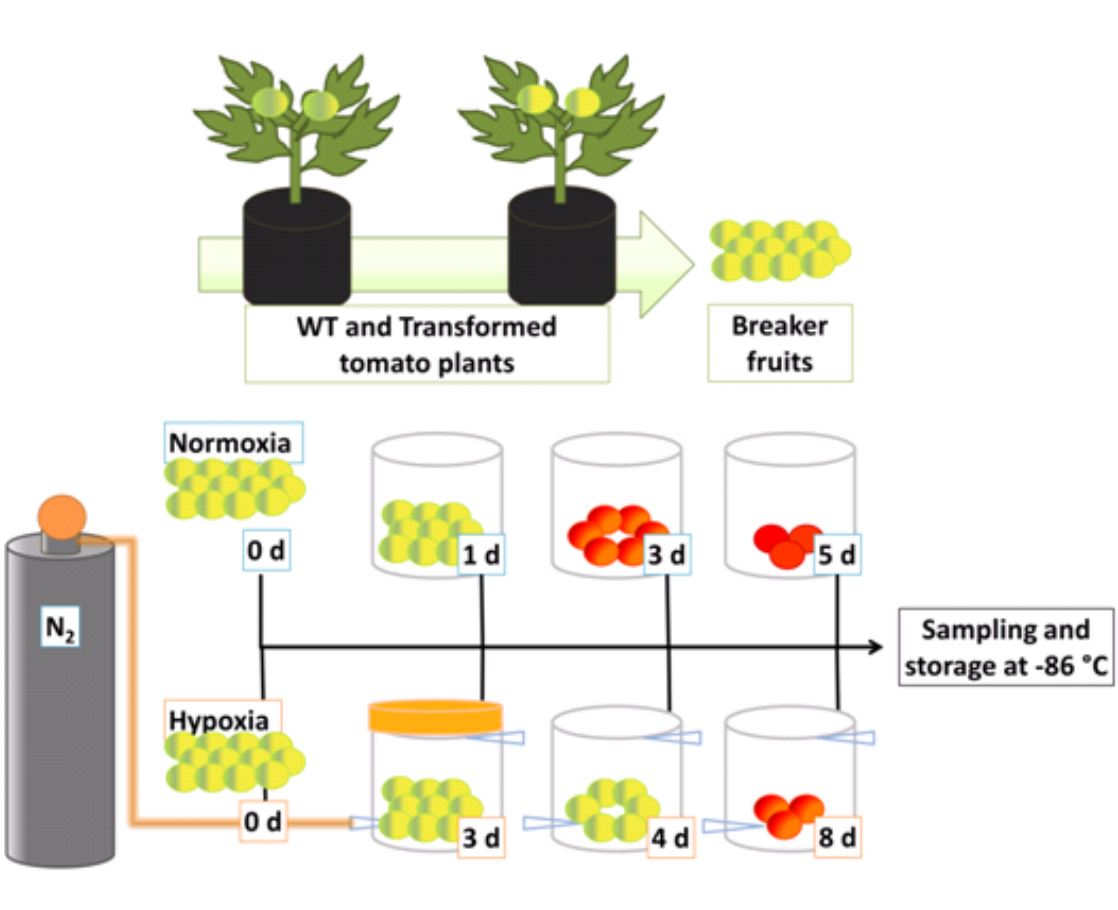

Figure 1. Scheme of tomato fruits sampling and normoxia/hypoxia post-harvest treatments.

3.2.2 Color tone analysis

Color tone changes during fruit storage under conditions of normoxia and hypoxia were evaluated using the Minolta CR-300 colorimeter, with the parameters L* (luminosity), a* and b*. Three fruits from each biological replicate were randomly chosen, and from each fruit, three readings were taken from different parts of the fruit to better represent the coloration. The results were expressed in hue angle (hº), with the equation hº = [arc tangent (b*/ a*)].

3.2.3 Initial sample processing

Tomato fruits (three from each biological repetition) were macerated in a ball mill with liquid nitrogen and stored in 50 mL falcon tubes in an ultrafreezer (-86 ºC) until further weighing to perform the following biochemical analysis.

3.2.4 Enzymatic antioxidant system

For the measurement of enzyme activities, fruit samples (± 0.2 g) were homogenized with 5 % (w:v) polyvinylpolypyrrolidone (PVPP) and 1.5 mL of 100 mM potassium phosphate buffer, pH 6.0, containing 0.1 mM ethylenediaminetetraacetic acid (EDTA) and 20 mM sodium ascorbate. The homogenate was centrifuged at 12 000 *g* for 20 min at 4 ºC and the supernatant obtained was used as a crude enzyme extract (Azevedo et al., 2006). From the same extract, the total soluble proteins content (Bradford et al., 1976) was determined for expression of enzymatic activity.

Superoxide dismutase (SOD - EC 1.15.1.1) activity was assayed by monitoring the inhibition of the nitro blue-tetrazolium (NBT) coloration at 560 nm in a 2 mL reaction medium containing 50 mM potassium phosphate buffer (pH 7.8), 14 mM methionine, 0.1 µM EDTA, 75 µM NBT and 2 µM riboflavin (Giannopolitis and Ries, 1977). The results were expressed as U mg^{-1} protein.

Catalase (CAT - EC 1.11.1.6) activity was determined based on the oxidation rate of hydrogen peroxide (Beers and Sizer, 1952), which was monitored by the decrease in absorbance at 240 nm (ε_{240nm} = 39.4 x 10^3 M^{-1} cm^{-1}) for 2 minutes at reading intervals of 10 s. A 4 mL reaction medium containing enzymatic extract, 100 mM potassium phosphate buffer (pH 7.0), and 12.5 mM hydrogen peroxide was used. The results were expressed as µmol H_2O_2 min^{-1} mg^{-1} protein.

Ascorbate peroxidase (APX - EC 1.11.1.11) activity was analyzed as described by Nakano and Asada (1981), using sodium ascorbate as substrate. The reaction medium (final volume 4 mL) was composed of 100 mM potassium phosphate buffer (pH 7.0), 0.5 mM sodium ascorbate, 0.1 mM hydrogen peroxide, and enzyme extract. The activity was monitored using ascorbate oxidation rate for 2 min with absorbance readings every 10 seconds at 290 nm (ε_{290nm} = 2.80 x 10^3 M^{-1} cm^{-1}). The results were expressed in µmol AsA min^{-1} mg^{-1} protein.

Guaiacol peroxidase (GPOD 1.11.1.7) activity was assayed as described by Azevedo et al. (2006), by monitoring the production rate of tetraguaiacol at 470 nm (ε_{470nm} = 26.6 x 10^3 M^{-1} cm^{-1}) in a spectrophotometer for 2 min at 10 second intervals. The reaction medium (final volume 4 mL) consisted of 100 mM potassium phosphate buffer (pH 7.0), 0.1 µM EDTA, 5 mM guaiacol, 15 mM hydrogen peroxide, and enzyme extract. The results were expressed as µmol H_2O_2 min^{-1} mg^{-1} protein.

3.2.5 Phytochemical compounds

Total phenols were determined spectrophotometrically according to the methodology proposed by Singleton and Rossi (1965). Approximately 0.25 g of tomato fruit sample was weighed and diluted in methanol. The extract was homogenized in an Ultra Turrax for 1 min and centrifuged at 5488 *g* for 15 min at 4 °C. In a 15 mL falcon tube, ultrapure water and 0.25N Folin-Ciocalteau solution were added to the supernatant obtained. After 3 min of reaction, 1N sodium carbonate was added. After 2 hours of reaction, the absorbance of the sample was read at 725 nm. Total phenols were quantified using a calibration curve obtained by reading the absorbances of gallic acid standards. The results were expressed in milligram equivalents of gallic acid in 100 g of fresh weight.

β-carotene and lycopene content were determined by spectrophotometry according to the methodology proposed by Nagata and Yamashita (1992). Pigments were extracted from approximately 0.25 g of tomato fruit in 10 mL of acetone/hexane (2:3, v/v). The mixture was homogenized in an Ultra Turrax for 1 min and the supernatant was read at the absorbances of 453, 505, 645 and 663 nm, respectively. The following equations were used to calculate the concentration of β-carotene and lycopene: β-carotene (mg/100 mL) = $0.216A_{663}$ - $1.22A_{645}$ - $0.304A_{505}$ + $0.452A_{453}$; lycopene (mg/100 mL) = $-0.0458A_{663}$ + $0.204A_{645}$ + $0.372A_{505}$ - $0.0806A_{453}$. The results were expressed in mg/100 g fresh weight.

Ascorbic acid was determined according to the method of Oliveira (2010). Samples (± 0.5 g) were vortexed in oxalic acid (0.4%) for 1 min and then centrifuged (5488 g / 15 min / 4ºC). The supernatant was collected and mixed with 2,6-dichlorophenolindophenol, and ascorbic acid was quantified spectrophotometrically at 520 nm using a calibration curve obtained from ascorbic acid standards. Results were expressed as milligrams of ascorbic acid per 100 g of fresh weight.

3.2.6 Hydrogen peroxide and superoxide content

The determination of hydrogen peroxide was performed using a methodology described by Velikova et al. (2000). Tomato fruits (± 0.2 g) were mixed with 2 mL of 0.1 % trichloroacetic acid (TCA) in a vortex for 2 min. The homogenate was centrifuged at 12 000 *g* at 4 ºC for 20 min and the supernatant obtained was added to the reaction medium, which was composed of 10 mM potassium phosphate buffer (pH 7.0) and 1 M potassium iodide, with a final volume of 2 mL. The absorbance was read at 390 nm. The H_2O_2 content was determined by a standard curve prepared with known concentrations of H_2O_2 and the results expressed in µmol H_2O_2 g^{-1} of fresh weight.

The determination of superoxide anion content was carried out using the methodology of Elstner and Heupel (1976). Approximately 0.2 g of tomato fruit was mixed with 1.8 mL of 65 mM phosphate buffer (pH 7.8) in a vortex for 2 min and centrifuged at 5000 *g* at 4 °C for 10 min. The supernatant obtained was mixed with 65 mM phosphate buffer (pH 7.8) and 10 mM hydroxylamine. Then the reaction medium was immediately placed in a water bath (Grant® Y28) at 25 °C for 20 min.

Thereafter, 17 mM sulfanilamide and 7 mM α-naphthylamine were added, and again the reaction medium was incubated at 25 °C for 20 min. The absorbance of the solution was measured at 530 nm. A standard curve of sodium nitrite ($NaNO_2$) was used to calculate the superoxide anion content. The results were expressed in μmol $O_2^{\bullet-}$ g^{-1} of fresh weight.

3.2.7 Radical scavenging activity

The radical scavenging activity from the capture of the DPPH radical (2,2-Diphenyl-1-picrylhydrazyl) was performed by spectrophotometer according to the methodology proposed by Brand-Williams et al. (1995), using the same extract obtained for determination of total phenolic compounds. In a falcon tube (15 mL), the previously obtained extract and DPPH solution in methanol (adjusted for absorbance of 1.10 ± 0.02) were added. The solution was homogenized on a tube shaker and held in the dark for 24 hours. After that, the absorbance was read at 517 nm.

Radical scavenging activity by the capture of the ABTS radical (2,2′-Azino-bis [3-ethylbenzothiazoline-6-sulfonic acid]) was also performed using a spectrophotometer employing methodology proposed by Rufino et al. (2007), and using the same extract obtained for determination of total phenolic compounds. The ABTS radical was obtained from the reaction of 140 mM potassium persulfate solution and 5 mL of 7 mM ABTS solution kept in the dark (room temperature, 16 h). Then, 1 mL of the mixture was diluted in absolute ethyl alcohol to an absorbance of 0.700 ± 0.05, at 734 nm. The reaction was carried out with the extract and the solution of the adjusted ABTS radical. The mixture was homogenized on a tube shaker and the reading performed after a 6 min reaction at 734 nm. Both results were expressed as percentage (%) DPPH and ABTS radical remaining, according to the equation: % inhibition = $[(A_{control} - A_{sample})/A_{control}]x\ 100$.

3.2.8 Experimental design and statistical analysis

Each treatment consisted of four biological replicates (three fruits per repetition) for each genotype of ‘Micro-Tom’ tomato, in a fully randomized design. All variables analyzed were evaluated in triplicate. The data were analyzed by a one-way analysis of variance (ANOVA). When *F* was significant, the treatment means for each genotype were compared with a Student t-test ($p \leq 0.05$), and each storage period was compared by a Tukey test ($p \leq 0.05$). We also subjected the data for the antioxidant system, reactive oxygen species and radical scavenging activity to a Pearson’s correlation coefficient analysis. Statistical analyses were performed using the SAS 8.0 statistical software program (SAS Institute Inc. Cary, NC, USA).

3.3 Results

Ripening evolution was monitored using the color tone (Hueº) parameter in both normoxia and hypoxia storage (Fig. 2). The closer to the 0º Hue, the greater the tendency for reddish color. In normoxia conditions, the red tone begins to be pronounced from the first day after the harvest, reaching the reddest color tone on the third day, which did not differ significantly from that of the fifth day (Fig. 2A). The genotype with the high MT-sHSP23.6 expression was the one with the most reddish color at the end of the treatment (Fig. 2C). Under hypoxia conditions, the fruits of the transformed genotype (Sense) only started to have a reddish color tone on the fourth day (Fig. 2B), and the wild type genotype (WT) showed the reddish color only at the end of storage (Fig. 2D).

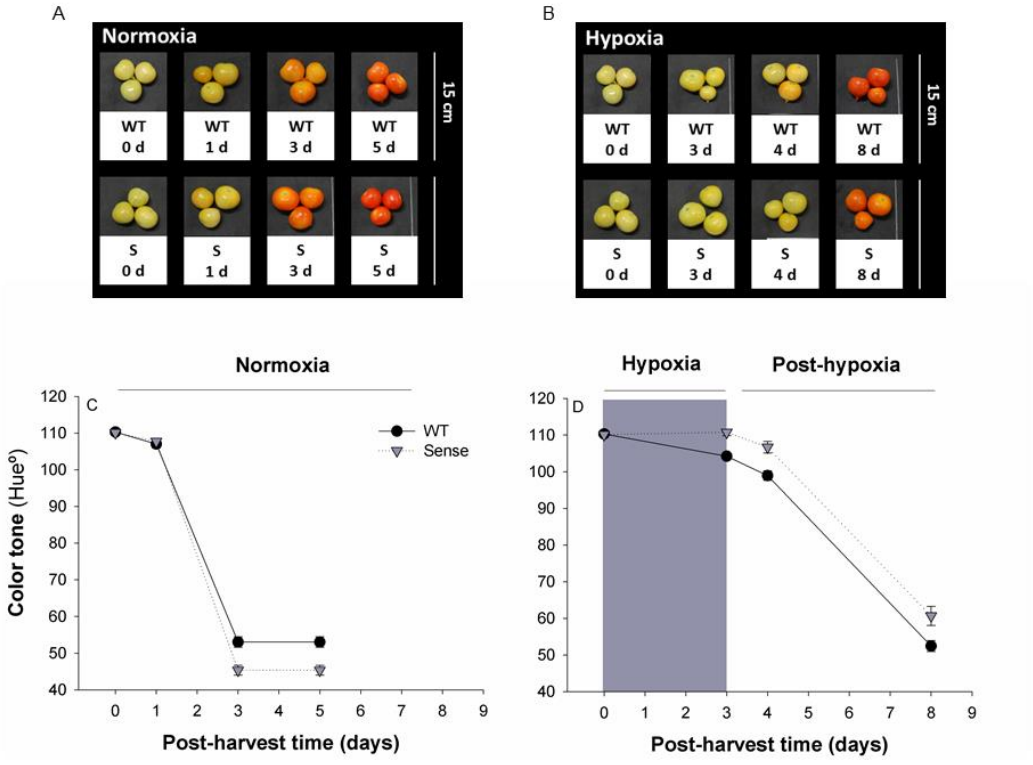

Figure 2. Color tone (ºHue) of 'Micro-Tom' tomato during post-harvest normoxia (A and C) and hypoxia (B and D) storage. Values represent the mean ± SD (n = 4). WT: wild type; Sense: high MT-sHSP23.6 expression.

Under normoxia conditions, the wild type genotype (WT) showed the highest superoxide dismutase (SOD) activity on the first and third days of storage (Fig. 3A). The transformed genotype (Sense) showed the highest SOD activity on the fifth and harvest days. For the hypoxia treatment, the genotype that presented the highest antioxidant activity in the period with low oxygen concentration was that with high MT-sHSP23.6 expression (Fig. 3B). On the first day post-hypoxia, there was no significant difference ($p \leq 0.05$) in SOD activity between the genotypes, but on the last day of storage, the WT genotype showed the lowest activity.

Regarding catalase activity under normoxia storage, the transformed genotype (Sense) presented lower activity on the harvest day and on the first day of storage under normal conditions (Fig. 3C). On the last day of storage there was no significant difference ($p \leq 0.05$) between the genotypes. In the treatment with hypoxia, there was an increase in CAT activity in the period at low oxygen concentrations in the transformed genotype (Fig. 3D). Higher CAT activity was observed on the third and fourth days, varying among genotypes. At the end of storage, the transformed genotype showed the highest activity.

Under normoxia storage, the transformed genotype (Sense) started with the lowest activity of the enzyme ascorbate peroxidase (APX) on harvest day and had the highest activity on the first day (Fig. 3E). APX activity was highest on the last day in hypoxia storage (Fig. 3F), 310% higher than that in the wild type. In the post-hypoxia period, the activity of the transformed genotype decreased significantly ($p \leq 0.05$), but even so it had a higher activity compared to that of the wild type genotype (WT).

Regarding guaiacol peroxidase (GPOD) activity in normoxia storage, only on the last day was there a significant difference ($p \leq 0.05$) between the genotypes, with the transformed genotype (Sense) presenting the highest activity (Fig. 3G). Under hypoxia storage, the transformed genotype showed significantly ($p \leq 0.05$) higher GPOD activity than the wild type (WT). However, on the eighth day there was no significant difference ($p \leq 0.05$) between treatments (Fig. 3H).

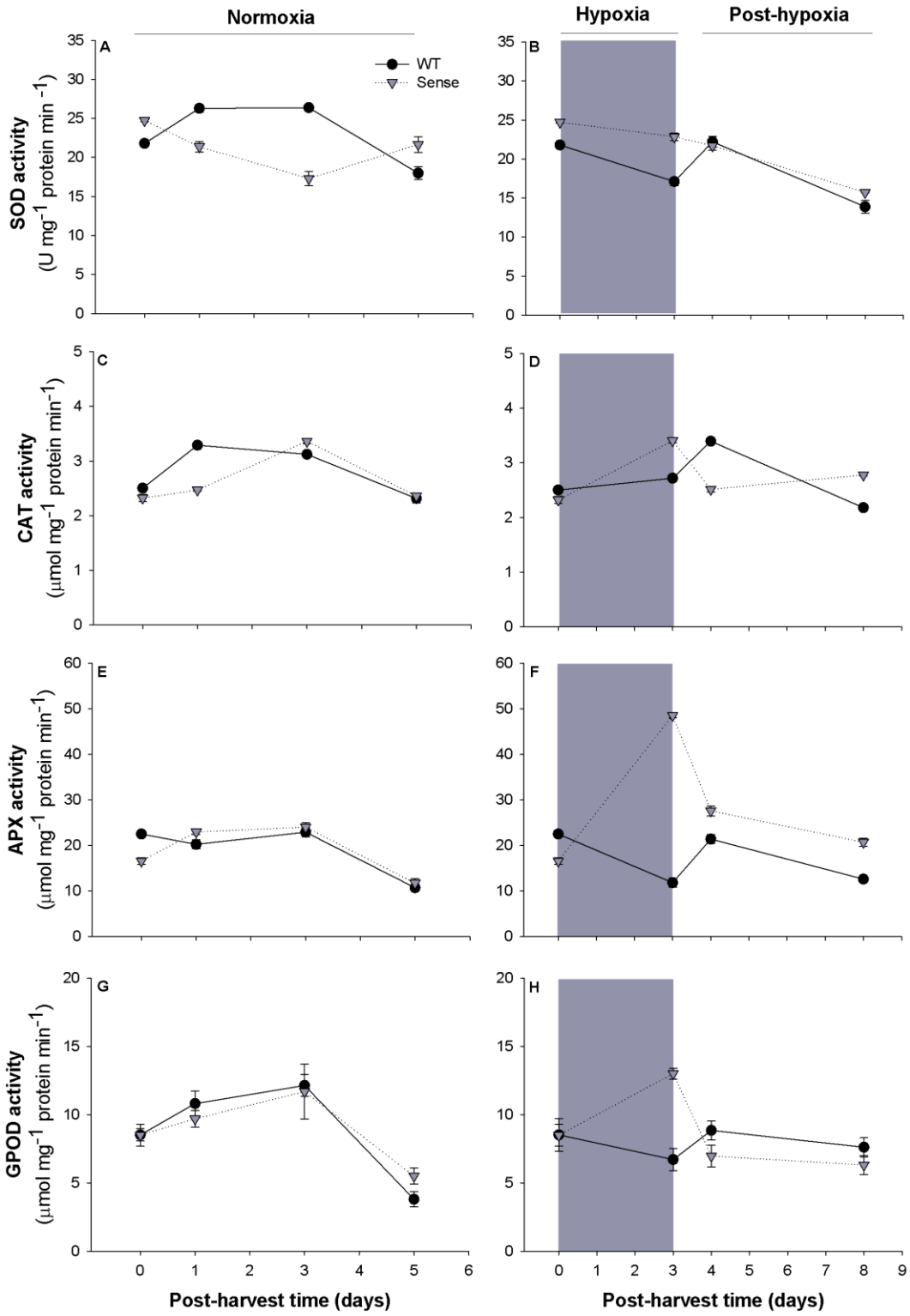

Figure 3. Antioxidant enzymes activity of 'Micro-Tom' tomato during post-harvest normoxia and hypoxia storage. Superoxide dismutase (SOD) activity (A and B); Catalase (CAT) activity (C and D); Ascorbate peroxidase (APX) activity (E and F); Guaiacol peroxidase (GPOD) activity (G and H). Values represent the mean ± SD (n = 4). WT: wild type; Sense: high MT-sHSP23.6 expression.

Under normoxia storage, superoxide anion content did not show significant difference ($p \leq 0.05$) between genotypes on harvest day (Fig. 4A). At the end of storage, the transformed genotype (Sense) had 56% less superoxide anion content than WT. Under hypoxia storage, the WT genotype showed the highest concentration in the period of low oxygen concentration and on the fourth day of storage (Fig. 4B). The Sense genotype showed a decrease in superoxide anion concentration in the same period. At the end of storage period, there was no significant difference ($p \leq 0.05$) between the two genotypes.

Regarding hydrogen peroxide under normoxia conditions, the WT genotype presented the lowest concentrations on the day of harvest and after one day of storage (Fig. 4C). On the fifth day, the Sense genotype had the highest concentration. In hypoxia storage, the Sense genotype had a decrease in peroxide content in the period at low oxygen concentrations, which continued to decline, even when normal oxygen levels were reestablished (Fig. 4D). The WT genotype showed an increase in peroxide content during hypoxia, and at the end of the storage it had the highest concentration.

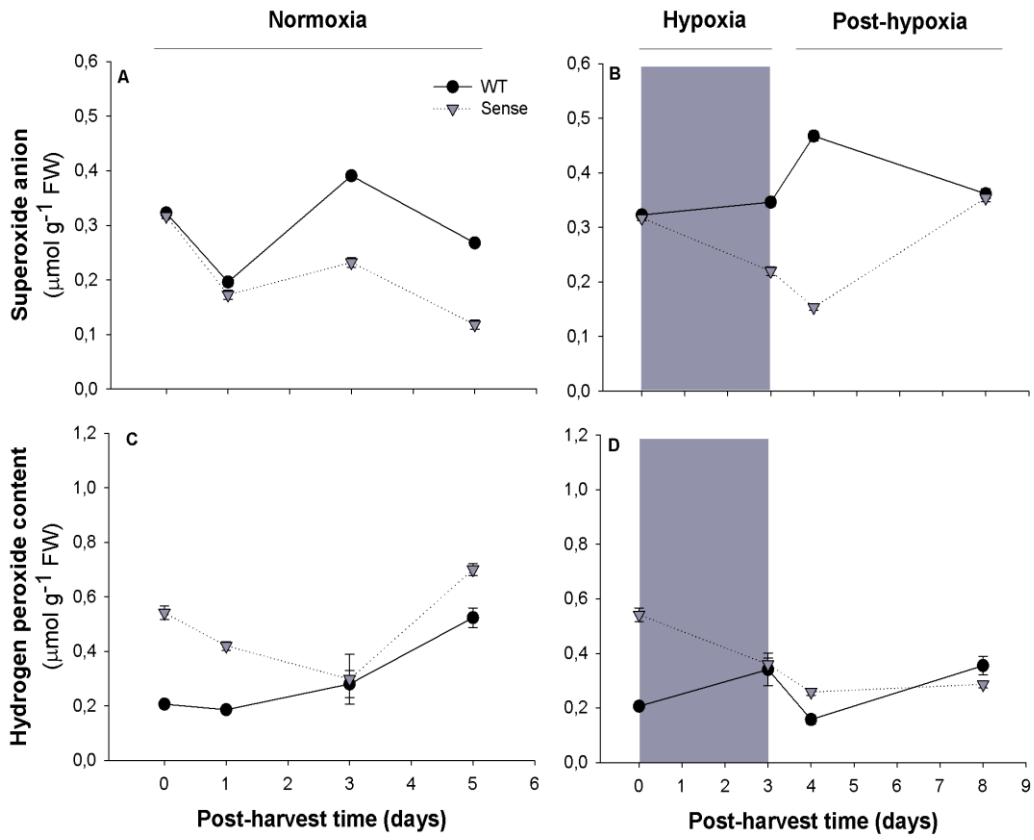

Figure 4. Superoxide anion and hydrogen peroxide content of 'Micro-Tom' tomato during normoxia and hypoxia storage. Superoxide anion (A and B); Hydrogen peroxide content (C and D). Values represent the mean ± SD (n = 4). WT: wild type; Sense: high MT-sHSP23.6 expression.

β-carotene content in normoxia storage was higher in the Sense genotype. Only in the third day was there no significant difference ($p \leq 0.05$) between the two genotypes (Fig. 5A). The highest accumulation of β-carotene was observed in the last two days of storage. Under hypoxia storage, there was no significant difference ($p \leq 0.05$) between genotypes during the hypoxia and post-hypoxia periods (Fig. 5B). The highest accumulation of β-carotene for both genotypes was observed on the last storage day.

Under normoxia conditions, the Sense genotype had the highest content of lycopene on the last day of storage (Fig. 5C). Under hypoxia storage, during three days at low oxygen concentrations, there was no significant increase ($p \leq 0.05$) in lycopene accumulation (Fig. 5D). Only after the fourth day, under normal oxygen conditions, did lycopene began to increase in concentration. At the end of storage, the WT genotype had the highest concentration of this carotenoid.

In normoxia storage, there was a significant increase in total phenol concentration on the third day, but there was no significant difference ($p \leq 0.05$) between genotypes in any sampling period (Fig. 5E). Under hypoxia storage there was no significant difference ($p \leq 0.05$) between genotypes on the third day (Fig. 5F). Under post-hypoxia storage, Sense genotype showed a higher concentration of total phenols until the eighth day of storage.

In both normoxia and hypoxia storage, the genotype with the high expression of MT-sHSP23.6 (Sense) had a higher concentration of ascorbic acid (AsA), except for the eighth day of hypoxia storage, where the WT genotype had a higher concentration (Figs. 5G and 5H). During low oxygen concentration periods, the WT genotype showed no significant difference ($p \leq 0.05$) in the AsA accumulation, whereas the Sense genotype showed a significant increase ($p \leq 0.05$) (Fig. 5H).

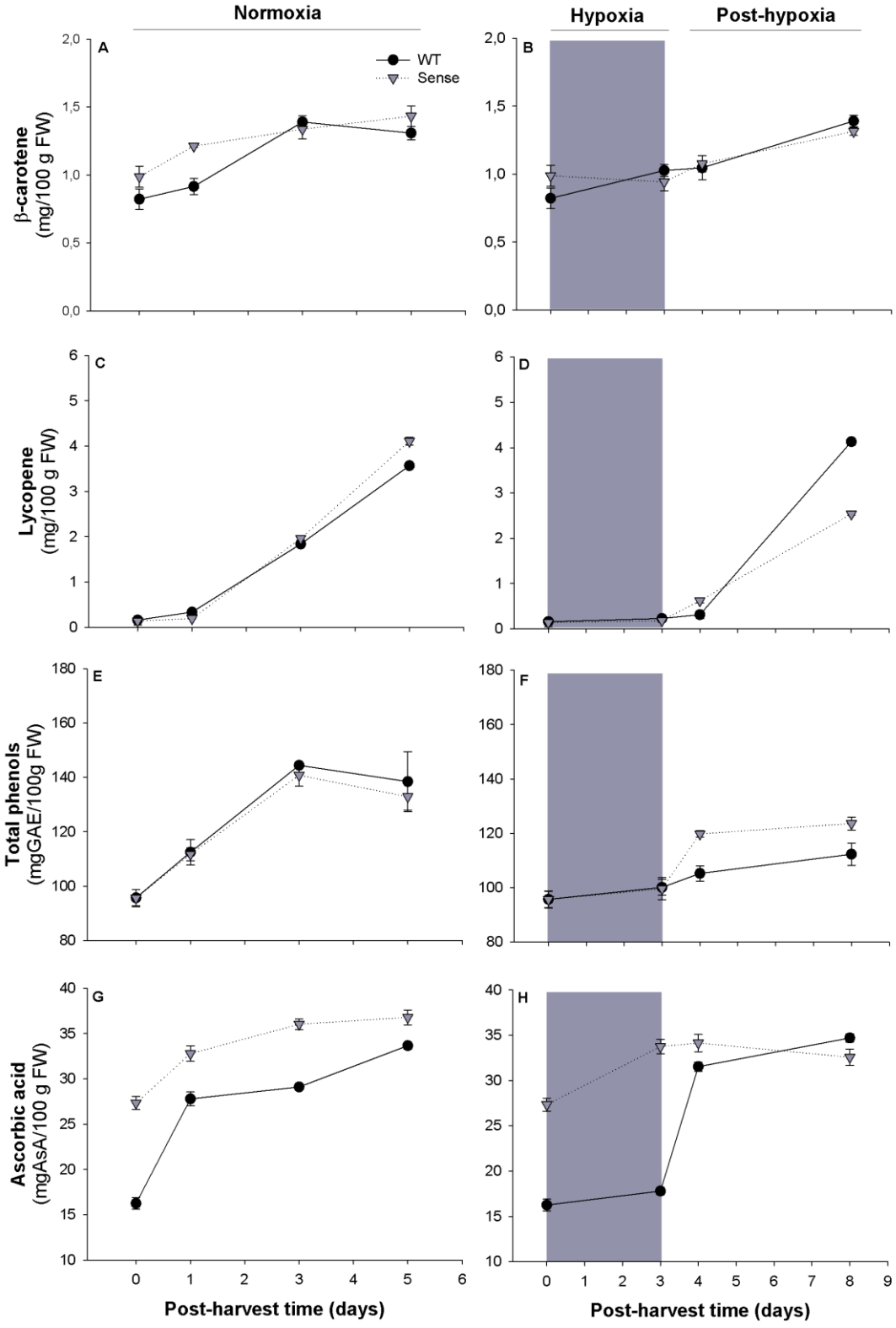

Figure 5. Non-enzymatic antioxidant system of 'Micro-Tom' tomato during normoxia and hypoxia storage. β-carotene (A and B); Lycopene (C and D); Total phenols (E and F); Ascorbic acid (G and H). Values represent the mean ± SD (n = 4). WT: wild type; Sense: high MT-sHSP23.6 expression

In relation to the antioxidant activity on the harvest day, there was no significant difference ($p \leq 0.05$) between the genotypes in radical scavenging activity (Fig. 6). Regarding the DPPH method, under normoxia storage there was no significant difference ($p \leq 0.05$) between genotypes during the entire storage period (Fig. 6A). However, under hypoxia conditions, Sense genotype showed the highest antioxidant activity in the period at low oxygen concentrations, and post-hypoxia as well (Fig. 6B). In normoxia storage, the highest activity for the ABTS radical was observed on the third day of storage (Fig. 6C). In the treatment with hypoxia, the Sense genotype showed increased activity during the period of low oxygen concentrations and did not show significant increase ($p \leq 0.05$) in the antioxidant activity from the fourth day until the end of storage (Fig. 6D). However, the WT genotype increased its activity on the fourth day and continued to increase until the end of the storage period.

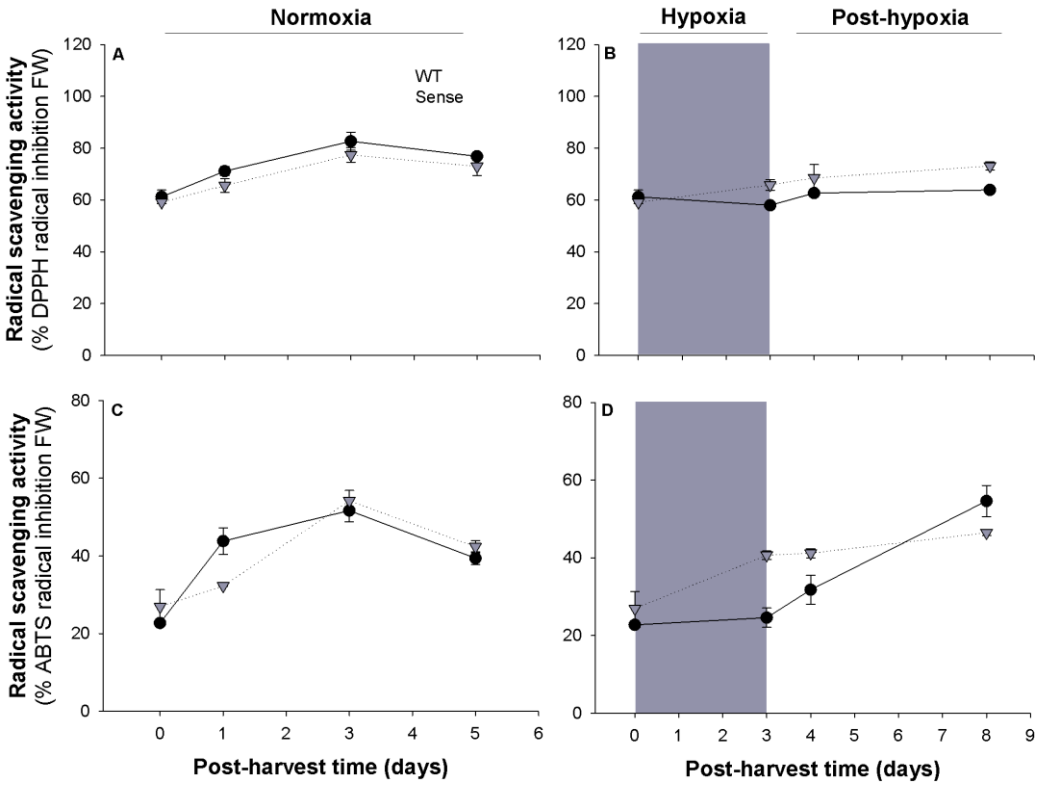

Figure 6. Antioxidant activity of 'Micro-Tom' tomato during normoxia and hypoxia storage. ABTS radical inhibition (A and B); DPPH radical inhibition (C and D). Values represent the mean ± SD (n = 4). WT: wild type; Sense: high MT-sHSP23.6 expression.

3.4 Discussion

As shown previously, tomato plants with high MT-sHSP23.6 expression showed an increased hypoxia tolerance during cultivation (Huther et al., 2017), and it was believed that this behavior could also occur in fruit. To test this hypothesis, the tomato genotypes with high expression levels of this gene were harvested in the breaker stage and maintained in conditions of normoxia and hypoxia. From this study, we observed that generally hypoxia delayed maturation, as evidenced by the lower color evolution (Fig. 2).

On the third day of normoxia storage the transformed genotype (Sense) began to differentiate, acquiring significantly redder color tone than the WT. Differently, under hypoxic conditions, from the third day (end of hypoxia), the genotype that presented more reddish color tone was the WT. This fact can be explained by the observed differences between the genotypes in the accumulation of lycopene at the end of the storage period. Rather than participating in the synthesis of carotenoids, it is likely that MT-sHSP23.6 indirectly affected its synthesis in a different manner when the fruit was subjected to low oxygen storage.

During tomato ripening, variations in the activities of the main enzymes involved with the enzymatic antioxidant system are expected (Murshed et al., 2013, Ripoll et al., 2016) due to the acceleration of metabolism and ROS accumulation (Pedreschi and Lurie, 2015, Pucciariello et al., 2012). This variation of enzyme activity occurs with all the enzymatic activities studied (Fig. 3). However, no uniform response was observed for the antioxidant enzymes between the studied genotypes. For each enzymatic activity analyzed, the results were different, which makes it difficult to explain the role of the MT-sHSP23.6 protein in the enzymatic antioxidant system in general. However, under the conditions of hypoxia, the Sense genotype showed greater activity in comparison to the WT in all the enzymes analyzed, demonstrating that the MT-sHSP23.6 protein influenced the enzymatic antioxidant system of tomato fruits stored under conditions of low oxygen concentration.

Moreover, at the end of the evaluations the activity of antioxidant enzymes reached values similar to the initial levels or even lower, demonstrating that as ripening evolves, the activity decreases, probably compensated for the accumulation of phytochemicals with antioxidant activity, such as ascorbic acid, phenols, and carotenoids (Fig. 5).

In the present work, we found a poor linear correlation between anion superoxide and SOD activity, which was the highest found for the genotype with high expression of sHSP23.6 under hypoxia (r=-0.38). Regarding hydrogen peroxide, the highest correlations were found with APX (r=-0.98) and GPOD (r=-0.99) in the genotype with a high expression of sHSP23.6 under normoxia storage. Overall the non-enzymatic antioxidant system studied showed a very poor correlation with the ROS analyzed. Establishing linear causation between ROS detoxification, antioxidant enzymes and antioxidant phytochemicals does not seem to be the most appropriate, considering that the plants have different mechanisms and compounds that act in the removal of ROS. Even small HSPs such as those studied in this work, may have antioxidant activity (Aghdam et al., 2015), as do components of mitochondria (alternative oxidase and mitochondrial uncoupling protein), and compounds such as L-ascorbic acid, carotenoids, phenols, and proline (Rodziewicz et al. al., 2014).

As the fruits were ripening, β-carotene synthesis decelerated, with lycopene overtaking it in both storage conditions. Under conditions of low oxygen concentration, there was no difference in the accumulation of the two carotenoids studied between the genotypes. At the end of storage it was possible to observe differences in the accumulation of these compounds between genotypes. Lycopene is found in large quantities in ripe tomato fruits and it has antioxidant ability to sweep singlet oxygen and free radicals, reducing oxidative stress and preventing the generation of reactive oxygen species (Bacanli et al., 2017; Tapiero et al., 2004). β-carotene, like lycopene, also acts to inhibit singlet oxygen, in addition to interacting synergistically with vitamin E, inhibiting lipid peroxidation (Schwenke, 1998).

In normoxia storage, the genotype with the highest expression of MT-sHSP23.6 showed the highest accumulation of lycopene at the end of the treatment. In the treatment with a period of hypoxia, the transformed genotype showed the lowest accumulation of lycopene at the end of the treatment, a fact that was also observed in the color analysis. In this way, the MT-sHSP23.6 protein has a different influence on the synthesis of lycopene under different storage conditions. A study carried out with tomato fruits has shown that Orange (OR) and ClpB3 chaperones protect enzymes from the carotenoid biosynthetic pathway deoxyxylulose 5-phosphate synthase and phytoene synthase, and promote the differentiation of chromoplasts, preventing carotenoid degradation (D'Andrea et al., 2018).

Regarding the accumulation of total phenolic compounds in hypoxia storage, after three days at low oxygen concentrations, the Sense genotype had a higher accumulation of these compounds. The MT-sHSP23.6 protein may play some role in the biosynthetic pathway of the phenolic compounds by preventing aggregation or malformation of important route enzymes under abiotic stress conditions, or by acting putatively as an antioxidant and preserving these compounds throughout storage. Phenolic compounds are considered to be multifunctional antioxidants, acting as reducing agents, metal chelators, and singlet oxygen extinguishers (Kancheva, 2009; Bravo, 1998).

Transgenic tomato overexpressed a transcription factor of Arabidopsis HSTF AtHSFA1b, which resulted in a higher production of HSPs, showing a higher expression of sHSPs and an increase in APX enzyme activity when submitted to cold (Aghdam et al., 2015). In the present study, higher APX activity was observed on the last day of hypoxia of tomato with high MT-sHSP23.6 expression (Sense) than in WT. The Sense genotype was also the one with the highest accumulation of ascorbic acid during the two storage conditions, except for the eighth day of hypoxia treatment, when the wild type genotype presented the highest concentration.

Among the antioxidant phytochemicals studied, ascorbic acid was the most influenced by the transformation for a higher expression of MT-sHSP23.6. APX is a peroxidase found in several cellular compartments, such as mitochondria and chloroplasts, and it requires ascorbic acid as a reducing molecule to act as a cofactor for regeneration (Sharma et al., 2012). In mitochondria, ascorbic acid is synthesized on the inner membrane and chemically reacts with H_2O_2, and HO^- (Noctor and Foyer, 1998). The ascorbic acid acts as a sequestrant of reactive oxygen species, forming compounds with a lower reactivity, such as ascorbyl radical and dehydroascorbic acid (Davey et al., 2000).

Regarding the radical scavenging activity of DPPH and ABTS radical capture, there was no uniform response between the genotypes. In the period of low oxygen concentration, the Sense genotype presented higher antioxidant activity than the WT genotype, evidenced by the high expression of MT-sHSP23.6 influencing the antioxidant activity of tomato fruits under conditions of post-harvest abiotic stress. Under normoxia conditions, generally the third day of storage was when the highest radical scavenging activity was observed among the genotypes, both by the DPPH method and ABTS.

As in the activity of antioxidant enzymes, the third day seems to be the period where the antioxidant system is most active, both for ROS scavenging and for free radicals. The occurrence of a peak in the cellular oxidative processes in this period probably should be at the moment of ripening, with a greater increase in respiration, and consequently operation of the electron transport chain of the mitochondria. Overall we found a good positive correlation between radical scavenging activity and the phytochemicals analyzed, with the highest values found for the genotype with a high expression of MT-sHSP23.6 under normoxia storage (r=0.99, total phenols and DPPH) and for the wild type genotype under hypoxia storage (r=0.97, lycopene and ABTS).

3.5 Conclusions

The overexpression of the MT-sHSP23.6 protein promotes positive responses in the enzymatic and non-enzymatic antioxidant systems of tomato fruits, especially during periods of low oxygen concentrations, highlighting ascorbate peroxidase, ascorbic acid and total phenols. Further studies are needed to understand the specific mechanism by which the MT-sHSP23.6 protein acts on the tolerance to post-harvest abiotic stress in fruit.

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4 Capítulo 2 – High MT-sHSP23.6 expression and moderate water deficit influences antioxidante system in ‘Micro-Tom’ tomato fruits under hypoxia

Capítulo referente ao manuscrito que será submetido à revista Journal of the Science of Food and Agriculture no ano de 2018.

Running title: Antioxidant system is influenced by water deficit and expression level of MT-sHSP23.6

ABSTRACT

BACKGROUND: Pre-harvest abiotic factors, as water deficit, can influence post-harvest responses in the antioxidant system of crops subject to low oxygen storage conditions. We investigated the influence of moderate water deficit in the ripening evolution, enzymatic and non-enzymatic antioxidant system, reactive oxygen species, and radical scavenging activity in ‘Micro-Tom’ tomato fruits with different MT-sHSP23.6 expression levels under hypoxia storage.

RESULTS: Sense genotype under water deficit presented a redder color tone at the end of storage period compared to other treatments. Regarding antioxidant enzymes, the transformed genotype (Sense) showed the highest activity under hypoxia period for all enzymes analysed, both for irrigated and water deficit treatments. On the last storage day, Sense genotype under water deficit showed the lowest superoxide anion content. Regarding hydrogen peroxide, water deficit treatments showed the highest content on the harvest day, however on the last day they presented the lowest content. Sense genotype under water deficit had the highest β-carotene content on the fourth storage day, and in post-hypoxia period this treatment showed the highest lycopene content. Total phenols content was higher at the end of storage and the water deficit treatments had the higher content. Treatments under water deficit had higher contents of ascorbic acid on the harvest day, and at the end of storage irrigated treatments showed the highest content. We found that tomato under water deficit treatment had higher radical scavenging activity, together with high total phenols and lycopene content at the end of storage period.

CONCLUSION: Water deficit and expression level of MT-sHSP23.6 influenced differently the components of antioxidant system studied. Different genotypes showed different responses to water deficit pre-harvest treatment. We highlight the antioxidant enzymes during hypoxia for genotype with high MT-sHSP23.6 expression for both irrigated and with moderate water deficit treatments. As well as an increment in total phenols, radical scavenging and SOD activity on the eighth storage day in both wild type and high MT-sHSP23.6 expression genotypes with pre-harvest water deficit treatment.

KEYWORDS: post-harvest physiology, water deficit, low oxygen storage, sHSP, antioxidant metabolites, antioxidant enzymes

4.1 Introduction

Tomato (*Solanum lycopersicum* L.) is a horticultural crop of great importance worldwide, being present in the daily diet of many people, both *in natura* and processed.[1] During cultivation and post-harvest storage, tomato plants may face several stress factors, biotic and abiotic, which may lead to loss of productivity and/or quality, but if used intentionally, moderately and controlled can lead to an accumulation of compounds that increase the quality of the fruit, increasing the tolerance and preparing the plant for subsequent stress.[2,3,4]

As in other living organisms, in the most diverse levels of complexity, tomato plants present a group of proteins that play a fundamental role for the maintenance of its protein cellular homeostasis, acting as molecular chaperones under normal conditions or when subjected to some stressors. These proteins are known as heat shock proteins (HSPs).[5,6]

Stress factors such as water deficit and hypoxia can lead to a stress response known as oxidative damage, which can damage the components of the cell and cause its dysfunction. Oxidative stress is caused by an overproduction and accumulation of reactive oxygen species (ROS), leading to an imbalance between the production and detoxification of ROS, due to a disorder in normal cell physiology.[7,8] However, water deficit can also be used as a pre-treatment to increase plant tolerance to future stress. Impacts of subsequent stress can be markedly reduced when compared to plants that have not been pre-treated with stress. As different types of abiotic stresses share the same responses, it is possible to perform pre-acclimatization of plants using different stresses, through cross-tolerance.[2,4]

Throughout evolution, plants have developed strategies to deal with oxidative stress, such as the activation or "de novo" synthesis of antioxidant enzymes that remove reactive oxygen species. Examples of antioxidant enzymes are catalase (CAT), superoxide dismutase (SOD), ascorbate peroxidase (APX) and guaiacol peroxidase (GPOD). In addition to the enzymatic antioxidant system, plants produce specialized metabolites that perform various functions in cells and may have antioxidant capacity. Examples of these compounds are tocopherols, L-ascorbic acid, phenolic compounds, and carotenoids.[7,9]

HSPs proteins and the antioxidant system complement each other to increase plant tolerance in situations where redox unbalance occurs in cells. While antioxidants (enzymatic and non-enzymatic) promote the detoxification of reactive oxygen species in the cell, HSPs act to prevent structural damage to cellular proteins.[10] Previous studies with tomato plants overexpressing a mitochondrial small HSP (MT-sHSP23.6) showed an increment in tomato plants plasticity[11] and demonstrated that these plants are able to restore the photosynthetic parameters after heat stress.[12] However, any evaluation was carried out in fruit during ripening and with an abiotic pre-harvest treatment. Thus, it is possible that fruits could change the biochemical and physiological behavior when submitted to a mild water deficit before storage under hypoxia.

Therefore, the aim of this study was to investigate the influence of a pre-harvest moderate water deficit in the antioxidant system of two tomato plants genotypes with different MT-sHSP23.6 expression levels subjected to post-harvest hypoxia storage.

4.2 Materials and methods

4.2.1 Plant growth and treatments

Seeds of two tomato genotypes of cultivar 'Micro-Tom' - the wild type (WT) and those with high expression of MT-sHSP23.6 protein (Sense) - were used. The transformation stability and physiological behavior of tomato plants under normal cultivation conditions has been described previously.[11,12]

Seeds of each genotype were germinated in Gerbox® with blotting paper moistened with distilled water, where they remained for a period of 10 days in germinating chamber (25 ºC, photoperiod of 12 hours). After this period, the seedlings were transplanted to 0.5 L plastic pots filled with commercial organic substrate. Tomato plants were grown in a greenhouse located at Federal University of Pelotas, Capão do Leão Campus (geographical coordinates: 31º 52’ 32” S and 52º 21’ 24” W, altitude 13 m).

After transplantation, the plants were irrigated on alternate days (50 mL) and 15 mL of nutrient solution[13] were applied three times a week. When the fruits were green (84 days after germination), irrigation and nutrient solution were suspended for eight days (induction of water deficit). The irrigated plants received water normally, but did not receive nutrient solution while the treatment of the plants under water deficit lasted. The average temperature in the greenhouse during the experimental period was 25 ± 5ºC and the irradiance, from natural light, was of 800 µmol photons m^{-2} s^{-1}. During the irrigation suspension, stomatal conductance and soil moisture were monitored.

When the soil moisture was around 3% and the conductance was approximately 50% lower than the control (which corresponded to 8 days of treatment), the water and nutrient solution were restored. After 2 days of irrigation reestablishment, when the fruits reached the breaker stage (approximately 90 days after sowing), they were harvested and then subjected to hypoxia conditions.

In the hypoxic treatment, 12 fruits of the two genotypes were collected and stored in transparent plastic pots (500 mL) at 23 °C and kept in the dark. The plastic pots had an air flow control system, consisting of an inlet and an outlet for the flow of gases. The hypoxia condition was generated by the introduction of nitrogen gas and a gas scan was performed daily (0.098 MPa / 10 min). This procedure was performed during the three days of hypoxia. After three days of hypoxia, the samples were submitted to normal atmospheric condition for five days. Three fruits were collected immediately before storage (time 0) and after hypoxia treatment, corresponding to a 3-day period. After being returned to normal atmospheric condition, three fruits were collected on the first and fifth days, corresponding to the times 4 and 8 days after the beginning of the post-harvest treatment. At each sampling point color analysis was performed on the fruit epicarp, which was immediately stored at -86 ºC for further biochemical analysis. Color parameters, such as Hue angle, are efficient for following the ripening evolution in the ‘Micro-Tom’ tomato.

4.2.2 Color tone analysis

Color tone changes during fruit storage were evaluated using the Minolta CR-300 colorimeter, with the parameters L* (luminosity), a* and b*. Three fruits from each biological repetition were randomly chosen, and from each, fruit three readings were taken from different parts of the fruit to better represent the coloration. The results were expressed in angle hue (h^o), by the equation h^o = [arc tangent (b*/ a*)].

4.2.3 Enzymatic antioxidant system

For the measurement of enzyme activities, fruit samples (± 0.2 g) were homogenized with 5 % (w:v) polyvinylpolypyrrolidone (PVPP) and 100 mM potassium phosphate buffer, pH 6.0, containing 0.1 mM ethylenediaminetetraacetic acid (EDTA) and 20 mM sodium ascorbate. The homogenate was centrifuged at 12 000 *g* for 20 min at 4 ºC and the supernatant obtained was used as crude enzyme extract.[14] From the same extract was determined the content of total soluble proteins[15], to express the enzymatic activity as a specific activity.

Superoxide dismutase (SOD - EC 1.15.1.1) activity was assayed by monitoring the inhibition of the nitro blue-tetrazolium (NBT) coloration at 560 nm in 2 mL reaction medium containing 50 mM potassium phosphate buffer (pH 7.8), 14 mM methionine, 0.1 µM EDTA, 75 µM NBT and 2 µM riboflavin.[16] The results were expressed as U mg^{-1} protein.

Catalase (CAT - EC 1.11.1.6) activity was determined as described by Beers and Sizer[17], which is based on the oxidation rate of hydrogen peroxide, the activity being monitored by the decrease in absorbance at 240 nm (ε_{240nm} = 39.4 x 10^3 M^{-1} cm^{-1}) for 2 minutes at a reading interval of 10 s. A 4 mL reaction medium containing enzymatic extract, 100 mM potassium phosphate buffer (pH 7.0) and 12.5 mM hydrogen peroxide was used. The results were expressed as µmol H_2O_2 min^{-1} mg^{-1} protein.

Ascorbate peroxidase (APX - EC 1.11.1.11) activity was analyzed according to Nakano and Asada[18], using sodium ascorbate as substrate. The reaction medium (final volume 4 mL) was composed of 100 mM potassium phosphate buffer (pH 7.0), 0.5 mM sodium ascorbate, 0.1 mM hydrogen peroxide and enzyme extract. The activity was monitored by the ascorbate oxidation rate for 2 minutes with absorbance reading in the range of 10 seconds at 290 nm (ε_{290nm} = 2.80 x 10^3 M^{-1} cm^{-1}). The results were expressed in µmol AsA min^{-1} mg^{-1} protein.

Guaiacol peroxidase (GPOD 1.11.1.7) activity was assayed as described by Azevedo et al.[14], by monitoring the production rate of tetraguaiacol at 470 nm (ε_{470nm} = 26.6 x 10^3 M^{-1} cm^{-1}) in a spectrophotometer for 2 minutes in a 10 s interval. The reaction medium (final volume 4 mL) consisted of 100 mM potassium phosphate buffer (pH 7.0), 0.1 µM EDTA, 5 mM guaiacol, 15 mM hydrogen peroxide and enzyme extract. The results were expressed as µmol H_2O_2 min^{-1} mg^{-1} protein.

4.2.4 Hydrogen peroxide and superoxide content

The determination of hydrogen peroxide was performed using a methodology described by Velikova et al.[19]. Tomato fruits (± 0.2 g) were mixed with 0.1 % trichloroacetic acid (TCA) in a vortex for 2 min. The homogenate was centrifuged at 12000 *g* at 4ºC for 20 min and the supernatant obtained was added to the reaction medium, which was composed of 10 mM potassium phosphate buffer (pH 7.0) and 1 M potassium iodide, with a final volume of 2 mL. The absorbance was read at 390 nm. The H_2O_2 content was determined by a standard curve prepared with known concentrations of hydrogen peroxide and the results expressed in µmol H_2O_2 g^{-1} of fresh weight.

The determination of the superoxide anion was carried out using a methodology of Elstner and Heupel[20]. Approximately 0.2 g of tomato fruit was mixed with 65 mM phosphate buffer (pH 7.8) in a vortex for 2 minutes and centrifuged at 5000 *g* at 4 °C for 10 min. To the supernatant obtained was added 65 mM phosphate buffer (pH 7.8) and 10 mM hydroxylamine, then the reaction medium was immediately placed in a water bath (Grant® Y28) at 25 °C for 20 min. Thereafter, 17 mM sulfanilamide and 7 mM α-naphthylamine were added and again the reaction medium was incubated at 25 °C for 20 min. The absorbance of the solution was measured at 530 nm. A standard curve of sodium nitrite (NaNO2) was used to calculate the superoxide anion content. The results were expressed in μmol $O_2^{\bullet-}$ $g^{-1}$of fresh weight.

4.2.5 Non-enzymatic antioxidants

Total phenols were determined spectrophotometrically according to the methodology proposed by Singleton and Rossi[21]. Approximately 0.25 g of tomato fruit sample was weighed and diluted in methanol. The extract was homogenized in an Ultra Turrax for 1 min and centrifuged at 5488 *g* for 15 min at 4 °C. In a 15 mL falcon tube, ultrapure water and 0.25 N Folin-Ciocalteau solution were added into the supernatant obtained. After 3 min of reaction 1N sodium carbonate was added. After two hours of reaction, the absorbance of the sample was read at 725 nm. The quantification of the total phenols was performed through a calibration curve obtained by reading the absorbances of gallic acid standards. The results were expressed in milligram equivalent of gallic acid in 100 g of fresh weight.

Carotenoids β-carotene and lycopene were determined by spectrophotometry according to methodology proposed by Nagata and Yamashita[22]. Approximately 0.25 g of tomato fruit was used to extract the pigments in 10 mL of acetone/hexane (2:3, v/v). The mixture was homogenized in Ultra Turrax for 1 min and the supernatant was read at the absorbances of 453, 505, 645 and 663 nm. The following equations were used to calculate the concentration of β-carotene and lycopene: β-carotene (mg/100 mL) = $0.216A_{663}$ - $1.22A_{645}$ - $0.304A_{505}$ + $0.452A_{453}$; lycopene (mg/100 mL) = - $0.0458A_{663}$ + $0.204A_{645}$ + $0.372A_{505}$ - $0.0806A_{453}$. The results were expressed in mg/100 g fresh weight.

Ascorbic acid was determined according to the method of Oliveira[23]. Samples (± 0.5 g) were vortexed in oxalic acid (0.4%) for 1 min and then centrifuged (5488 g / 15 min / 4ºC). The supernatant was collected and mixed with 2,6-dichlorophenolindophenol and ascorbic acid was quantified spectrophotometrically at 520 nm using a calibration curve obtained from ascorbic acid standards. Results were expressed as milligrams of ascorbic acid per 100 g of fresh weight.

4.2.6 Radical scavenging activity

The radical scavenging activity by the capture of the DPPH radical (2,2-Diphenyl-1-picrylhydrazyl) was performed by spectrophotometer, according to the methodology proposed by Brand-Williams et al.[24]. It was used the same extract obtained for determination of total phenolic compounds. In a falcon tube (15 mL), the previously obtained extract and DPPH solution in methanol (adjusted for absorbance of 1.10 ± 0.02) was added. The solution was homogenized on a tube shaker and held in the dark for 24 h. After that, the absorbance was read at 517nm.

Radical scavenging activity by the capture of the ABTS radical (2,2′-Azino-bis[3-ethylbenzothiazoline-6-sulfonic acid]) was also performed by spectrophotometer, through a methodology proposed by Rufino et al.[25]. It was used the same extract obtained for determination of total phenolic compounds. The ABTS radical was obtained from the reaction of 140 mM potassium persulfate solution and 5 mL of 7 mM ABTS solution kept in the dark (room temperature, 16 h). Then, 1 mL of the mixture was diluted in absolute ethyl alcohol to the absorbance of 0.700 ± 0.05, at 734 nm. The reaction was carried out with the extract and the solution of the adjusted ABTS radical. The mixture was homogenized on a tube shaker and the reading performed after 6 min of reaction at 734 nm. Both results were expressed as radical scavenging activity (%) of DPPH and ABTS.

4.2.7 Experimental design and statistical analysis

The experiment was analyzed as a completely randomized design in a factorial scheme, where one factor was "Genotype" (with 2 levels) and the other was "Water deficit" (2 levels) with 4 biological replicates each treatment. The experiment was evaluated at 0, 3, 4 and 8 days of hypoxia storage. Each date was analyzed separately. Variance analysis (ANOVA) was performed, and when the interaction was significant the interaction deployment was performed, comparing the means by the Tukey test, comparing genotypes within each level of water deficit and comparing each level of water deficit within each level of genotypes. A *p* value less than or equal to 0.05 was considered significant. Also, data regarding the antioxidant system, reacitive oxygen species and radical scavenging activity was subjected to Pearson’s correlation coeficiente analysis. The analyzes were performed in software R 3.5.1 (2018).

4.3 Results

Significant interaction occurred for all evaluated variables, for which the interaction unfolding was made. It can not be said whether the effect of the water deficit on the antioxidant system is higher or lower than in the treatment with irrigation without considering the genotype. Nor can it be said whether the enzymatic activity, phytochemicals content or antioxidant activity is greater in a given genotype, as it will depend on the treatment.

On the harvest day (0 day) the responses were different depending on the enzyme analyzed. SOD activity was higher in the irrigated treatments, being the genotype Sense the highest (Tab. 1). CAT activity was not influenced by water deficit in Sense genotype, as well for WT genotype, who presented the highest activity. APX and GPOD activity presented similar influences, being Sense genotype influenced by water deficit and presentig the highest activity and WT genotype not influenced.

During low oxygen concentration period (3 day), Sense genotype always presented higher activity for all enzymes analized, compared to WT genotype (Tab. 1). The water deficit treatment did not promote higher enzyme activity in this period for Sense genotype. However, for WT genotype, we observed higher activity for water deficit treatment in CAT enzyme.

On the first day post-hypoxia (4 day), GPOD activity was influenced by water deficit for both genotypes, with WT genotype presenting the highest (Tab. 1). CAT activity was also influenced, but only for Sense genotype. SOD and APX were not influenced by water deficit, with the highest activity for irrigated WT genotype and irrigated Sense genotype, respectively.

On the last storage day (8 day), the response was different among enzymes. For SOD, the water deficit treatment influenced the response, presenting higher activity for both Sense and WT genotype, being WT the one with the highest (Tab. 1). For CAT, APX, and GPOD, water deficit influenced differently, depending on the genotype. WT genotypes under water deficit showed higher activity and Sense genotypes presented the lowest.

Related to H_2O_2, on the harvest day, the highest content was observed in water deficit treatment, for both genotypes (Tab. 1), with no significant difference between genotypes. During hypoxia period and the first day after hypoxia, pre-harvest water deficit treatment did not influence the response. As well on the last storage day, being the irrigated WT genotype the one with the highest content.

Regarding to superoxide anion (O_2^-), on the harvest day, WT genotype presented the highest content, being influenced by water deficit. However, Sense genotype was not, presenting the highest content in the irrigated treament. During hypoxia period, Sense genotype was influenced by water deficit, and WT genotype was not. On the first post-hypoxia day, irrigated Sense genotype presented the lowest content and on the last day, Sense genotype subjected to pre-harvest water deficit presented the lowest.

Table 1 – Averages of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), guaiacol peroxidase (GPOD), hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-), from genotypes with different MT-sHSP23.6 expression levels (WT and Sense), with or without irrigation (WD and Irrig.), evaluated on different hypoxia storage days (0, 3, 4 and 8 days). Multiple comparisons between genotypes within each level of irrigation and Irrigantion within each level of genotype.

		SOD		CAT		APX		GPOD		H_2O_2		O_2^-	
Days	Gen.	DH	Irrig.	DH	Irrig.	DH	Irrig.	DH	Irrig.	DH	Irrig.	DH	Irrig.
0	Sense	16,8 bB	24,7 aA	2,2 aA	2,2 bA	25,5 aA	16,6 bB	13,0 aA	8,5 aB	0,71 aA	0,54 aB	0,24 bB	0,32 aA
	WT	18,6 aB	21,8 bA	1,6 bB	2,5 aA	15,4 bB	23,2 aA	7,1 bB	9,4 aA	0,63 aA	0,21 bB	0,46 aA	0,32 aB
3	Sense	19,2 aB	22,9 aA	3,2 aB	3,4 aA	19,9 aB	48,6 aA	13,6 aA	13,0 aA	0,31 aB	0,36 aA	0,25 aA	0,22 bB
	WT	12,6 bB	17,1 bA	3,1 bA	2,7 bB	14,1 bA	11,8 bA	5,7 bB	7,0 bA	0,21 aB	0,34 aA	0,13 bB	0,35 aA
4	Sense	17,0 bB	21,7 aA	2,6 aA	2,5 bB	15,2 bB	28,2 aA	10,4 bA	7,0 bB	0,22 aA	0,26 aA	0,47 aA	0,15 bB
	WT	14,8 aB	22,2 bA	2,4 bB	3,4 aA	19,7 aB	21,4 bA	13,1 aA	8,9 aB	0,21 aA	0,16 bB	0,47 aA	0,47 aA
8	Sense	18,0 bA	15,7 aB	2,3 bB	2,8 aA	11,9 bB	21,2 aA	5,9 bA	6,3 bA	0,22 aB	0,29 bA	0,17 bB	0,35 aA
	WT	21,8 aA	14,3 bB	2,5 aA	2,2 bB	18,4 aA	12,6 bB	13,4 aA	7,6 aA	0,22 aB	0,36 aA	0,39 aA	0,36 aB

Values followed by lower case letters in the column (among genotypes) and capitals in the line (among irrigation level) do not differ significantly from each other, according to Tukey test at 5% probability.

We observed thar color tone (Hue angle) was both influenced by the genotype and water deficit treatment, specially on the last storage day (Tab. 2), were we found signifcant diferences in the ripening evolution. The Sense genotype under pre-harvest water deficit showed the reddish color tone and for WT genotype was the irrigated treatment.

Regarding β-carotene, on the harvest day (0 day), Sense genotype presented the highest contet, both for irrigated and water deficit treatment (Tab. 2). During low oxygen concentration period, there was no significant difference among treatments, as well as on the last storage day. On the first post-hypoxia day, Sense genotype under water deficit presented the highest content, demonstrating that the treatment and the genotype influenced the response.

On the harvest day, WT genotype under water deficit showed the highest lycopene content (Tab. 2). During hypoxia period, irrigated WT genotype showed the highest content compared to water deficit treatment. Sense genotype under water deficit showed higher lycopene content than WT genotype. On the fourth storage day, Sense genotype under water deficit presented the highest content, demonstrating that the genotype and the water deficit treatment influenced the response. On the last storage day, water deficit and high MT-sHSP23.6 expression influenced the response of Sense genotype. WT genotype showed higher content when irrigated, and higher than Sense in the same condition, demonstrating the influence of genotype.

Total phenols content, on the harvest day, Sense genotype presented lower content than WT genotype for both water deficit and irrigation, and WT genotype under pre-harvest water deficit showed the highest content (Tab. 2). During hypoxia period and on the first post-hypoxia day, the genotype with high MT-sHSP23.6 expression and water deficit influenced the most. On the last storage day, water deficit also influenced the response, being the WT genotype the one with the highest total phenols content.

Ascorbic acid content, on the harvest day, was influenced by water deficit treatment in both genotypes, being WT genotype the one with the highest content (Tab. 2). During hypoxia period, the genotype with high MT-sHSP23.6 expression (Sense) influenced the most, presenting higher ascorbic acid content in both treatments. Regarding WT genotype, water deficit treatment promoted the highest content. On the fourth and eighth storage days, the genotype influenced the most, being irrigated Sense genotype the one with the highest content on the day 4 and irrigated WT genotype on the day 8.

Table 2 – Averages of Hue angle, β-carotene, lycopene, total phenols and ascorbic acid, from genotypes with different MT-sHSP23.6 expression levels (WT and Sense), with or without irrigation (WD and Irrig.), evaluated on different hypoxia storage days (0, 3, 4 and 8 days). Multiple comparisons between genotypes within each level of irrigation and Irrigantion within each level of genotype.

		Hue angle		β-carotene		Lycopene		Total phenols		Ascorbic acid	
Days	Gen.	WD	Irrig.	WD	Irrig.	WD	Irrig.	WD	Irrig.	WD	Irrig.
0	Sense	110.7 bA	110.1 aA	1.0 aA	1.0 aA	0.1 bA	0.1 aA	95.3 bA	96.1 bA	30.4 bA	27.3 aB
	WT	112.0 aA	109.8 aB	0.8 bA	0.8 bA	0.4 aA	0.2 aB	104.7 aA	97.8 aB	37.7 aA	17.0 bB
3	Sense	110.9 bA	110.3 aA	1.0 aA	0.9 aA	0.3 aA	0.2 aA	106.6 aA	97.4 bB	34.5 aA	33.7 aA
	WT	113.4 aA	103.8 bB	1.0 aA	1.0 aA	0.1 bB	0.2 aA	105.8 bA	101.7 aB	26.6 bA	17.8 bB
4	Sense	98.2 bB	107.5 aA	1.5 aA	1.1 aB	2.3 aA	0.6 aB	131.3 aA	119.1 aB	30.0 bB	34.5 aA
	WT	108.7 aA	98.3 bB	1.0 bA	1.0 aA	0.3 bA	0.4 bA	114.3 bA	106.5 bB	31.8 aA	31.5 bA
8	Sense	48.4 bB	60.3 aA	1.3 aA	1.3 aA	4.3 aA	2.5 bB	133.6 bA	125.3 aB	28.0 bB	32.9 bA
	WT	56.4 aA	53.3 bB	1.3 aA	1.4 aA	2.8 bB	4.1 aA	135.2 aA	114.8 bB	31.1 aB	34.7 aA

Values followed by lower case letters in the column (among genotypes) and capitals in the line (among irrigation level) do not differ significantly from each other, according to Tukey test at 5% probability.

Regarding antioxidant activity, we observed a pattern in both DPPH and ABTS radical scavenging methods. In each storage period they presented similar reponses for both methods. On the first day (day 0) water deficit and genotype influenced the response, being WT genotype under water deficit the highest for DPPH and Sense under water deficit for ABTS. During hypoxia storage (day 3), water deficit influenced WT genotype, promoting higher antioxidant activity. Sense genotype presented the highest antioxidant activity in the irrigated treatment.

On the first post-hypoxia day (day 4), both genotypes presented higher antioxidant activity under water deficit, being the highest for WT genotype by the DPPH method and Sense genotype for ABTS method. On the last storage day (day 8), water deficit treatment influenced the response, as well as a higher MT-sHSP23.6 expression for both radical scavenging methods.

Table 3 - Averages of antioxidant activity of the DPPH and ABTS radical method, of genotypes with different MT-sHSP23.6 expression levels (WT and Sense), with or without irrigation (WD and Irrig.), evaluated on different hypoxia storage days (0, 3, 4 and 8 days). Multiple comparisons between genotypes within each level of irrigation and Irrigantion within each level of genotype.

		DPPH		ABTS	
Days	Gen.	WD	Irrig.	WD	Irrig.
0	Sense	59.9 bA	59.1 aA	37.0 aA	27.6 aB
	WT	63.0 aA	60.1 aB	26.6 bA	22.8 bB
3	Sense	64.7 bB	66.7 aA	30.2 aB	41.1 aA
	WT	66.3 aA	58.0 bB	26.7 bA	24.7 bA
4	Sense	69.9 bA	68.2 aB	46.2 aA	41.6 aB
	WT	72.0 aA	62.7 bB	33.6 bA	31.8 bB
8	Sense	77.3 bA	74.0 aB	54.8 bA	46.4 bB
	WT	78.5 aA	63.9 bB	56.0 aA	54.0 aB

Values followed by lower case letters in the column (among genotypes) and capitals in the line (among irrigation level) do not differ significantly from each other, according to Tukey test at 5% probability.

4.4 Discussion

Color tone is a good parameter to follow ‘Micro-Tom’ tomato fruits ripening evolution. The closer to 0°, the greater the tendency of red color tonality. Therefore, we observed that the Sense genotype under water deficit presented a greater ripening evolution. The red color of ripe tomato is mainly associated with lycopene content, being in agreement with the result obtained in this study.[26,27] Water deficit can promote an increment of tomato fruit ethylene content, which in turn may increase lycopene content.[27] It is worth to mention that WT genotype under water deficit did not show the same response, demonstrating that the overexpression of MT-sHSP23.6, associated with water deficit, increased lycopene accumulation.

We observed that the transformed genotype (Sense), under hypoxia period, both irrigated and with moderated water deficit, had the highest activity for all the enzymes analyzed, demonstrating that the overexpression of MT-sHSP23.6 influences the enzymatic antioxidant system during low oxygen storage. Interestingly, we observed that not always the treatments under water deficit showed the highest enzymes activity on the harvest day. Generally, when plants are subjected to an abiotic factor that can cause stress they present an increase in the activity of enzymes related to the ROS scavenging.[28,29] Probably the conditions imposed in the water deficit were not sufficient to create an increase in the activity of these enzymes, being the genotype a factor that influenced the most. The genotype with high MT-sHSP23.6 expression (Sense) had the highest enzyme activity on the harvest day, except for CAT activity.

The fourth storage day had the highest superoxide anion concentration for almost all treatments. Reestablishing normal oxygen conditions promote an increment in the respiration, leading to a higher accumulation of anion superoxide, originating of the electrons scape from the electron transport chain.[30] On the last day we observed that the genotype and water deficit influenced in the superoxide anion content, being the Sense genotype markedly the one with the lowest content.

A markedly increase in oxidative stress seems to occur on the last stages of normal fruit ripening, facilitating metabolic changes associated with ripening and fruit softening.[31] It was not observed in this study. The pre-harvest water deficit influenced the accumulation of H_2O_2 along the storage, being higher on the harvest day, and lower than irrigated at the end of storage period. Water deficit associated with low oxygen period may influence the antioxidant system at the end of storage period in tomato fruit, leading to a lower accumulation of H_2O_2 at the end of ripening. In agreement with our findings, studies with 'Micro-Tom' tomato under 10 days of water deficit showed that fruits on breaker stage had less H_2O_2 content than red ones, and compared to control, they presented lower content on the final ripening stage.[32]

Poor correlation was found for the enzymes and ROS analyzed, except for APX (r=-0.94 with H_2O_2) and GPOD (r=-0.88 with H_2O_2) in the WT genotype irrigated and CAT (r=-0.85) for the same genotype under water deficit. Plants have different mechanisms to deal with the excess of ROS and free radicals, not only the enzymatic antioxidant system. Small HSPs may have antioxidant activity[33], as do alternative oxidase, mitochondrial uncoupling protein, and compounds such as L-ascorbic acid, carotenoids and phenols.[6]

Pre-harvest abiotic factors, such as water deficit, can influence the phenotype at harvest, and when used moderately, might enhance fruit tolerance to different stresses through up-regulation of genes and pathways.[3,34] Water deficit is known to increase the accumulation of several non-enzymatic antioxidants in fruits. Applied moderately and in specific developmental stages, may improve fruit quality. During fruit ripening, moderate water deficit may increase the content of potentially bioactive phytochemicals, which in turn are very important for human health.[4,35]

β-carotene and lycopene presented higher content on the post-hypoxia period, specially the genotype with high MT-sHSP23.6 expression under water deificit. The sHSP proteins may act as molecular chaperones. Studies with chaperones Orange (OR) and ClpB3 showed that these proteins protects enzymes from the carotenoid biosynthetic pathway deoxyxylulose 5-phosphate synthase and phytoene synthase, and promote the differentiation of chromoplasts, preventing carotenoid degradation.[36]

During hypoxia and post-hypoxia period, water deficit treatments had higher accumulation of total phenols. Water deficit may influence carotenoids, phenols and ascorbic acid accumulation in different manners. There are at least two mechanisms that may occur and interact. One of them is caused by reduction in primary metabolites that are precursor of secondary metabolites biosynthesis, due to a reduction in leaf stomatal conductance, which consequently leads to a decrease in net photosynthesis. Other mechanism is due to a stress/oxidative sinalling, being ROS responsible for affect both direct and indirectly phenols, carotenoids and ascorbic acid biosynthesis.[35,37,38]

Ascorbic acid seems to be the most prominent antioxidant component analyzed on the harvest day, being directly influenced by water deficit. It is well known that ascorbic acid plays a important role, together with other antioxidant components, in protect plants from oxidative damage caused by abiotic stress factors.[32] The ascorbic acid acts as a sequestrant of reactive oxygen species, forming compounds with a lower reactivity, such as ascorbyl radical and dehydroascorbic acid. Also, together with APX, participates of the ascorbate-glutathione cycle H_2O_2-scavenging pathway.[39] During hypoxia period, the high MT-sHSP23.6 expression influenced the response. Both irrigated and under water deficit had the highest ascorbic acid content, compared to WT genotype.

We found higher antioxidant activity at the end of storage for water deficit treatments, together with higher content of phenols and lycopene. A Pearson's correlation analysis showed a high correlation for these components. WT genotype had r= 0.96 for DPPH and total phenols content. Also showed a high correlation of r= 0.97 for ABTS and lycopene content. The transformed genotype (Sense) had r= 0.93 for DPPH and total phenols content, and r= 0.96 for ABTS and lycopene content. These results demonstrate the biological activity of phenols and carotenoids as antioxidants, helping plant cells to maintain their redox balance.

4.5 Conclusions

High MT-sHSP23.6 expression and pre-harvest water deficit influenced the responses in the antioxidant system of tomato fruits subjected to low oxygen storage. The activity of antioxidant enzymes are markedly influenced by the high expression of MT-sHSP23.6 during hypoxia storage period. Water deficit had the major influence in the non enzymatic antioxidant system, promoting the highest accumulation, mainly for total phenols, ascorbic acid and antioxidant activity. More studies are necessary to understand how small heat shock proteins as MT-sHSP23.6 and the association with pre-harvest abiotic stress factors can influence fruit antioxidant system and ripening evolution.

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5 Considerações finais

Através da pesquisa realizada, observou-se que uma maior expressão do gene que codifica a proteína MT-sHSP23.6 influencia o sistema antioxidante de frutos de tomate ‘Micro-Tom’ submetidos à hipóxia pós-colheita, principalmente com relação às enzimas antioxidantes no período sob baixas concentrações de CO_2. Merecem destaque as enzimas ascorbato peroxidase e guaiacol perosidase, bem como os compostos fenólicos totais e ácido L-ascórbico.

No que diz respeito ao estudo com tratamento pré-colheita, a maior expressão de MT-sHSP23.6 e o déficit hídrico promoveram diferentes respostas no sistema antioxidante de frutos de tomate ‘Micro-Tom’ submetidos à condição de hipóxia, demonstrando que não se pode verificar a influência do tratamento com suspensão de água sem levar em consideração os diferentes níveis de expressão de MT-sHSP23.6 entre os genótipos e vice-versa. De maneira geral, o sistema antioxidante enzimático, no período de hipóxia, parece ser mais influenciado pela maior expressão de MT-SHSP23.6, enquanto o sistema antioxidante não enzimático parece ser mais influenciado pelo déficit hídrico.

A continuidade de pesquisas com plantas modificadas quanto à expressão do gene que codifica a proteína MT-sHSP23.6 se faz necessária, com a finalidade de compreender seu mecanismo de atuação no sistema antioxidante, e entender como esta proteína atua na fisiologia pré e pós colheita frente a fatores de estresse ambiental. Como contribuição para a área de Ciência e Tecnologia de Alimentos, esta Tese demonstrou que a proteína sHSP23.6 também desempenha papel importante na fisiologia pós-colheita de frutos, aumentando a atividade de enzimas e incrementando o acúmulo de compostos do sistema antioxidante em condições de armazenamento sob baixas concentrações de oxigênio.

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Apêndices

Apêndice A – Material suplementar do artigo "High MT-sHSP23.6 expression increases antioxidant system in 'Micro-Tom' tomato fruits during post-harvest hypoxia", publicado na revista Scientia Horticulturae

Table 1 - Color tone (ºHue) of 'Micro-Tom' tomato during post-harvest normoxia and hypoxia storage.

Genotype	**Hue Angle**			
	Normoxia (days)			
	0	**1**	**3**	**5**
WT	110.31±1.11^{Aa}	107.04±1.44^{Aa}	81.34±2.93^{Ab}	53.02±1.48^{Ac}
S	110.12±0.67^{Aa}	107.76±0.87^{Aa}	76.12±3.66^{Bb}	45.34±1.77^{Bc}
	Hypoxia (days)			
	0	**3**	**4**	**8**
WT	110.31±1.11^{Aa}	104.23±1,06^{Bb}	98.95±1,60^{Bc}	52.44±1,93^{Bd}
S	110.12±0.67^{Aa}	110.75±1.21^{Aa}	106.69±1.93^{Aa}	60.68±3.42^{Ab}

WT: wild type genotype; S: sense genotype. Values represent the mean ± SD (n = 4). Means followed by the same lowercase letter in the line, among storage days, do not differ by Tukey test (p≤0.05). Means followed by the same capital letter in the column, among genotypes, do not differ by Student t test (p≤0.05).

Table 2 - Antioxidant enzymes activity of ‘Micro-Tom’ tomato during post-harvest normoxia and hypoxia storage.

Genotype	Antioxidant enzymes			
	SOD			
	Normoxia (days)			
	0	**1**	**3**	**5**
WT	21.80±0.28^{Bb}	26.29±0.55^{Aa}	26.37±0.11^{Aa}	17.99±0.85^{Bc}
S	24.73±0.11^{Aa}	21.38±0.66^{Bb}	17.29±0.99^{Bc}	21.93±1.06^{Ab}
	Hypoxia (days)			
	0	**3**	**4**	**8**
WT	21.80±0.28^{Ba}	17.12±0.56^{Bb}	22.21±0.68^{ABa}	13.88±0.94^{Bc}
S	24.73±0.11^{Aa}	22.85±0.57^{Ab}	21.70±0.66^{Bb}	15.71±0.04^{Ac}
	CAT			
	Normoxia (days)			
	0	**1**	**3**	**5**
WT	2.51±0.04^{Ac}	3.29±0.04^{Aa}	3.12±0.04^{Bb}	2.32±0.08^{Ad}
S	2.25±0.07^{Bd}	2.47±0.02^{Bb}	3.36±0.04^{Aa}	2.36±0.04^{Ac}
	Hypoxia (days)			
	0	**3**	**4**	**8**
WT	2.51±0.04^{Ac}	2.72±0.05^{Bb}	3.40±0.04^{Aa}	2.18±0.03^{Bd}
S	2.25±0.07^{Bd}	3.41±0.03^{Aa}	2.52±0.05^{Bc}	2.78±0.01^{Ab}
	APX			
	Normoxia (days)			
	0	**1**	**3**	**5**
WT	22.54±0.77^{Aba}	20.26±1.01^{Bb}	22.90±0.09^{Ba}	10.73±0.71^{Bc}
S	16.58±0.70^{Bb}	22.99±0.56^{Aa}	24.04±1.02^{ABa}	11.72±1.04^{ABc}
	Hypoxia (days)			
	0	**3**	**4**	**8**
WT	22.54±0.77^{Aa}	11.85±1.01^{Bb}	21.42±1.02^{Ba}	12.63±0.64^{Bb}
S	16.58±0.70^{Bd}	48.58±0.46^{Aa}	27.63±1.14^{Ab}	20.73±0.94^{Ac}
	GPOD			
	Normoxia (days)			
	0	**1**	**3**	**5**
WT	8.52±1.19^{Ab}	10.81±1.02^{Bab}	12.14±1.13^{Aa}	3.80±0.09^{Ac}
S	8.49±0.75^{Ab}	9.69±0.61^{Bab}	11.69±2.02^{Aa}	5.50±0.61^{Ac}
	Hypoxia (days)			
	0	**3**	**4**	**8**
WT	8.52±1.19^{Aa}	6.71±0.85^{Bb}	8.85±0.66^{Aa}	7.61±0.74^{ABab}
S	8.49±0.75^{Ab}	13.01±0.41^{Aa}	6.97±0.83^{Bbc}	6.31±0.65^{Bc}

WT: wild type genotype; S: sense genotype. Values represent the mean ± SD (n = 4). Means followed by the same lowercase letter in the line, among storage days, do not differ by Tukey test (p≤0.05). Means followed by the same capital letter in the column, among genotypes, do not differ by Student t test (p≤0.05).

Table 3 - Superoxide anion and hydrogen peroxide content of ‘Micro-Tom’ tomato during normoxia and hypoxia storage.

Genotype	ROS			
	Superoxide anion			
	Normoxia (days)			
	0	**1**	**3**	**5**
WT	0.32±0.00^{Ab}	0.20±0.01^{Ad}	0.39±0.01^{Aa}	0.27±0.01^{Ac}
S	0.32±0.00^{Aa}	0.17±0.01^{Bc}	0.23±0.01^{Bb}	0.12±0.01^{Bd}
	Hypoxia (days)			
	0	**3**	**4**	**8**
WT	0.32±0.00^{Ad}	0.35±0.01^{Ac}	0.47±0.01^{Aa}	0.36±0.01^{Ab}
S	0.32±0.00^{Ab}	0.22±0.01^{Bc}	0.15±0.01^{Bd}	0.35±0.01^{Aa}
	Hydrogen peroxide			
	Normoxia (days)			
	0	**1**	**3**	**5**
WT	0.21±0.02^{Bb}	0.19±0.01^{Bb}	0.28±0.06^{Ab}	0.52±0.05^{Ba}
S	0.54±0.03^{Ab}	0.42±0.02^{Ac}	0.30±0.11^{Ad}	0.70±0.03^{Aa}
	Hypoxia (days)			
	0	**3**	**4**	**8**
WT	0.21±0.02^{Bb}	0.34±0.08^{Aa}	0.16±0.02^{Bb}	0.36±0.04^{Aa}
S	0.54±0.03^{Aa}	0.36±0.03^{Ab}	0.26±0.01^{Ac}	0.29±0.02^{Bbc}

WT: wild type genotype; S: sense genotype. Values represent the mean ± SD (n = 4). Means followed by the same lowercase letter in the line, among storage days, do not differ by Tukey test (p≤0.05). Means followed by the same capital letter in the column, among genotypes, do not differ by Student t test (p≤0.05).

Table 4 - Non-enzymatic antioxidant system of ‘Micro-Tom’ tomato during normoxia and hypoxia storage.

Genotype	Non-enzymatic antioxidants			
	β-carotene			
	Normoxia (days)			
	0	**1**	**3**	**5**
WT	0.83±0.08^{Bb}	0.92±0.06^{Bb}	1.39±0.05^{Aa}	1.31±0.05^{Ba}
S	0.99±0.08^{Ac}	1.21±0.01^{Ab}	1.34±0.07^{Aa}	1.44±0.08^{Aa}
	Hypoxia (days)			
	0	**3**	**4**	**8**
WT	0.83±0.08^{Bc}	1.03±0.04^{Ab}	1.05±0.09^{Ab}	1.39±0.04^{Aa}
S	0.99±0.08^{Abc}	0.94±0.07^{Ac}	1.08±0.06^{Ab}	1.32±0.03^{Aa}
	Lycopene			
	Normoxia (days)			
	0	**1**	**3**	**5**
WT	0.16±0.03^{Ad}	0.34±0.08^{Ac}	1.84±0.01^{Ab}	3.57±0.03^{Ba}
S	0.14±0.09^{Ac}	0.19±0.09^{Bc}	1.96±0.01^{Ab}	4.11±0.04^{Aa}
	Hypoxia (days)			
	0	**3**	**4**	**8**
WT	0.16±0.03^{Ac}	0.23±0.08^{Abc}	0.32±0.09^{Bb}	4.14±0.08^{Aa}
S	0.14±0.09^{Ac}	0.18±0.02^{ABc}	0.62±0.05^{Ab}	2.54±0.03^{Ba}
	Total phenols			
	Normoxia (days)			
	0	**1**	**3**	**5**
WT	95.70±3.00^{Ac}	112.46±4.70^{Ab}	144.44±0.90^{Aa}	138.46±11.20^{Aa}
S	95.61±3.23^{Ac}	111.71±2.42^{Ab}	140.79±4.00^{Aa}	132.90±4.94^{Aa}
	Hypoxia (days)			
	0	**3**	**4**	**8**
WT	95.70±3.00^{Ac}	100.11±2.95^{Abc}	105.24±2.81^{Bab}	112.31±4.07^{Ba}
S	95.61±3.23^{Ab}	99.64±4.10^{Ab}	119.77±1.23^{Aa}	123.60±2.37^{Aa}
	Ascorbic acid			
	Normoxia (days)			
	0	**1**	**3**	**5**
WT	16.61±1.02^{Bc}	27.80±0.76^{Bb}	29.11±0.17^{Bb}	34.15±1.13^{Ba}
S	27.33±0.72^{Ac}	32.79±0.85^{Ab}	36.03±0.60^{Aa}	36.78±0.82^{Aa}
	Hypoxia (days)			
	0	**3**	**4**	**8**
WT	16.61±1.02^{Bc}	17.79±0.39^{Bc}	31.53±0.50^{Bb}	34.70±0.47^{Aa}
S	27.33±0.72^{Ab}	33.74±0.77^{Aa}	34.14±0.96^{Aa}	32.56±0.91^{Ba}

WT: wild type genotype; S: sense genotype. Values represent the mean ± SD (n = 4). Means followed by the same lowercase letter in the line, among storage days, do not differ by Tukey test (p≤0.05). Means followed by the same capital letter in the column, among genotypes, do not differ by Student t test (p≤0.05).

Table 5 – Antioxidant activity of ‘Micro-Tom’ tomato during normoxia and hypoxia storage.

Genotype	Antioxidant activity			
	DPPH			
	Normoxia (days)			
	0	**1**	**3**	**5**
WT	61.22±2.59^{Ac}	71.11±1.85^{Ab}	82.65±3.50^{Aa}	76.84±1.12^{Aab}
S	59.13±0.60^{Ab}	65.54±2.74^{Ab}	77.38±2.79^{Aa}	72.95±3.53^{Aa}
	Hypoxia (days)			
	0	**3**	**4**	**8**
WT	61.22±2.59^{Ac}	57.98±0.62^{Bc}	62.69±0.12^{Bbc}	63.91±0.72^{Ba}
S	59.13±0.60^{Ab}	65.80±2.05^{Ab}	68.52±5.23^{Aab}	73.10±1.58^{Aa}
	ABTS			
	Normoxia (days)			
	0	**1**	**3**	**5**
WT	22.76±0.33^{Ac}	43.84±3.35^{Ab}	51.70±2.86^{Aa}	39.44±1.60^{Ab}
S	26.93±4.37^{Ad}	32.27±0.07^{Bc}	54.20±2.71^{Aa}	42.37±1.59^{Ab}
	Hypoxia (days)			
	0	**3**	**4**	**8**
WT	22.76±0.33^{Ac}	24.61±2.45^{Bc}	31.78±3.67^{Bb}	54.63±4.04^{Aa}
S	26.93±4.37^{Ac}	40.68±1.05^{Ab}	41.17±1.16^{Aab}	46.43±0.65^{Ba}

WT: wild type genotype; S: sense genotype. Values represent the mean ± SD (n = 4). Means followed by the same lowercase letter in the line, among storage days, do not differ by Tukey test (p≤0.05). Means followed by the same capital letter in the column, among genotypes, do not differ by Student t test (p≤0.05).