A COMPREHENSIVE STUDY OF THE EFFECT OF METEOROLOGICAL CONDITIONS ON FRUIT ABSCISSION AND METAMITRON THINNING EFFICACY IN *MALUS DOMESTICA* BORKH.

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TESE ELABORADA PARA OBTENÇÃO DO GRAU DE DOUTOR EM ENGENHARIA AGRONÓMICA

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JURI

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Abstract

Metamitron is an apple (Malus domestica Borkh.) fruit thinner that acts by reducing the photosynthetic capacity of trees. Thinning is a common practice among apple growers however, the effect of meteorological conditions, per si, and after metamitron spraying, is far from elucidated. In order to investigate the triggered physiological and biochemical processes, trials with shading nets (SN) during 5 days and high nighttime temperature (HNT) during 5 nights, along with high humidity (HH) for 3 h prior spraying were set in three locations, within 3 years. Lower irradiance caused a momentaneous photosynthetic decrease that led to reduced levels of leaf sucrose and sorbitol and consequent increases in fruit abscission rates. HNT pathway is a slight increment in the respiratory mechanisms, but mainly in the whole tree metabolic activity, finally resulting in a shortage in sucrose and sorbitol levels, reducing fruit growth and promoting higher abscission rates. Metamitron acts through a photosynthetic inhibition, including reductions in RuBisCO total activity, translating in less CH production. In addition, it stimulates vegetative growth, consuming CH and slowing down fruit growth rate, and finally enhancing of abscission. The combined treatments resulted in the strongest thinning efficacy, through a suppression of the CH production caused by metamitron in combination with low radiation and or through enhanced expenditure cause by HNT, frequently over-thinning. The effect of HH may enhance fruit drop under humid climates and in younger trees, more susceptible to variations in CH content. This work shows meteorological conditions, namely cloudy days, periods of HNT and increased humidity levels, may affect metamitron thinning efficacy and must be considered in order to decide which rate to apply to achieve an optimum crop load.

Key-words: abscission, carbohydrates, nighttime temperature, photosynthesis, radiation

Resumo

O metamitrão é um agente de monda química usado em macieiras (*Malus domestica* Borkh.) que reduz a capacidade fotossintética das árvores. A monda química é uma prática comum entre os produtores, mas há ainda muitas dúvidas quanto ao efeito das condições meteorológicas na abscisão, per si, e após a aplicação de metamitrão. Para clarificar os processos fisiológicos e bioquímicos desencadeados por estas condições, foram implementados ensaios com redes de sombreamento (SN) durante 5 dias e com temperaturas nocturnas altas (HNT) durante 5 noites, assim como com humidade alta (HH) durante as 3 h que antecedem a aplicação de metamitrão, em três locais, ao longo de 3 anos. Menores níveis de radiação causaram um decréscimo fotossintético momentâneo, reduzindo os níveis foliares de sacarose e sorbitol, e aumentando a queda de frutos. A HNT levou a um aumento ligeiro dos mecanismos respiratórios, mas principalmente de toda a actividade metabólica da árvore. HNT reduziram os níveis de sacarose e sorbitol, promovendo o abrandamento da taxa de crescimento dos frutos e o aumento da taxa de abscisão. O metamitrão inibiu a taxa de fotossíntese e reduziu a actividade total da RuBisCO, traduzindo-se numa menor produção de fotoassimilados. Adicionalmente, estimula o crescimento vegetativo, abrandando o crescimento dos frutos, estimulando a sua queda. A eficácia de monda mais alta foi observada no tratamento combinado, devido ao efeito de supressão da produção de fotoassimilados causado pelo metamitrão em combinação com a baixa radiação, ou pelo aumento do gasto energético causado pela HNT, provocando frequentemente excesso de monda. A HH potencia o efeito do metamitrão em climas húmidos e árvores jovens. Este trabalho demonstra que as condições meteorológicas, nomeadamente dias nublados, temperaturas nocturnas altas e humidade elevada, afectam a eficácia do metamitrão e devem ser consideradas aquando da decisão da dose a aplicar para obter uma carga óptima.

Palavras-chave: abscisão, fotoassimilados, fotossíntese, temperatura nocturna, radiação

Resumo Alargado

A comprehensive study of the effect of meteorological conditions on natural fruit abscission mechanisms and metamitron thinning enhancement in *Malus domestica* Borkh.

A macieira (*Malus Domestica* Borkh.) é uma árvore que frequentemente vinga demasiados frutos e, apesar de ter mecanismos naturais de abscisão de frutos que permitem uma regulação da carga, estes não são suficientes para normalizar a alternância da produção e obter frutos com calibre e caracteristicas organolépticas adequadas para o consumo em fresco. Por isso, a monda química é uma prática comum entre os produtores, tendo no entanto resultados muito inconstantes, pois a eficácia dos agentes de monda é afectada por elevado número de factores. O metamitrão é um inibidor de fotossíntese utilizado como agente de monda química que através da redução da produção de fotoassimilados, aumenta a taxa de abscissão. A sua eficácia depende das condições meteorológicas, justificando uma investigação aprofundada dos seus efeitos na abscissão natural e na eficácia do metamitrão, assim como uma caracterização das alterações fisiológicas e bioquímicas provocadas por este factores, nos dias seguintes à imposição dos tratamentos. Neste âmbito, foram delineados ensaios de três tipos: instalação de redes de sombreamento durante 5 dias, aumento da temperatura nocturna durante cinco noites e aumento da humidade relativa durante três horas. Estes tratamentos foram avaliados per si, e em combinação com a aplicação de 247,5 ppm de metamitrão. Estes ensaios foram repetidos em dois anos, e em três localizações na Europa, utilizando as cultivares: 'Gala', 'Golden', 'Braeburn' e 'Elstar'.

A análise foliar de metamitrão e do seu principal metabolito, desamino-metamitrão, clarificaram o efeito das condições meteorológicas na sua absorção. A redução de radiação em 50%, levou a um aumento significativo da absorção de metamitrão e à diminuição da sua degradação. A molécula de metamitrão degrada-se através de uma reação de fotólise. Consequentemente, debaixo das redes de sombreamento, a molécula de metamitrão manteve-se intacta e apta para absorção durante um maior período de tempo, aumentando a absorção e atrasando o processo de degradação. Já a temperatura nocturna não teve quaquer efeito na absorção, enquanto que o aumento da humidade relativa pode potenciar a absorção de metamitrão em determinadas circunstâncias, nomeadamente em climas mais húmidos, como Sint-Truiden, em que a folha se devolve com características mais propensas a permitir a absorção de compostos químicos, e árvores mais jovens. Os nossos resultados confirmaram que a baixa radiação e o metamitrão afectam a taxa de fotossíntese e condutância estomática 2 a 5 dias após a imposição dos tratamentos. Concomitantemente, a actividade total da enzima RuBisCO foi reduzida, para cerca de metade dos valores de controlo, de forma similar, com e sem redes de sombreamento, 5 dias após os tratamentos. Demonstraram ainda que nem o aumento de humidade relativa nem o aumento da temperatura nocturna afectam a taxa de fotossíntese e a condutância estomática. Contudo, nos tratamentos combinados, observou-se uma relação entre o aumento de absorção de metamitrão e uma maior inibição da taxa fotossintética.

Os resultados do ensaio de temperatura nocturna em condições de campo levantaram questões relacionadas com o seu efeito nos mecanismos fotossíntético e respiratórios. Através de um ensaio de fitoclima que simulou as condições de campo, foi possível clarificar o efeito da temperatura nocturna na taxa de fotossíntese, capacidade fotossintética, actividade da enzima RuBisCO, a taxa de transporte de electrões e fluorescência da clorofila a. A aplicação de metamitrão reduziu significativivamente todos estes parâmetros e aumentou a fluorescência a, 4 dias após a aplicação. O aumento de temperatura per si, e em combinação com metamitrão, levou a um incremento e a um atenuamento do efeito do metamitrão, respectivamente, nestes parâmetros. Através da avaliação da resposta da taxa de transporte de electrões à aplicação de metamitrão, foi possível confirmar a afectação do fotossistema II, com e sem o complexo de oxidação da água, em detrimento do fotossistema I, tendo a actividade do último sido significativamente potenciada sob condições de temperatura nocturna alta.

A escassez de fotoassimilados é uma resposta comum a alterações ambientais, como baixa radiação ou a temperaturas nocturnas altas, e à redução das taxas fotossínteticas. Este trabalho permitiu concluir que períodos de pelo menos 5 dias com 50% menos irradiância e de 5 noites com um aumento de temperatura para cerca de 15 ºC, promovem alterações diminutas nos níveis de glucose e frutose, independentemente da localização e dia. No entanto, resultam em reduções significativas dos níveis de sacarose e sorbitol, entre 2 a 5 dias após a imposição dos tratamentos. O mesmo foi observado após a aplicação de metamitrão. As maiores reduções de sacarose (mais pronunciadas) e de sorbitol foram observadas nos tratamentos que combinaram metamitrão e baixa radiação ou temperatura nocturna alta. No caso dos tratamentos de aumento de temperatura nocturna, as reduções de açucares solúveis foram mais extremas em amostras colhidas antes do nascer do sol. A humidade alta, per si, não alterou os níveis de açucares solúveis ao nível da folha. De acordo com os restantes parâmetros obtidos, nomeadamente maior absorção de metamitrão e maior inibição de fotossíntese, a maior redução de açucares solúveis (sacarose e sorbitol) foi observada no tratamento que combinou metamitrão e humidade alta. Já em Lleida e Girona, o aumento da humidade não teve qualquer efeito em nenhum dos parâmetros mencionados.

Dado que a temperatura nocturna de 15 ºC potenciou todos os parâmetros fotossintéticos, mesmo sob o efeito de metamitrão, foi conduzida uma investigação mais detalhada, no ensaio de fitoclima, dos seus efeitos ao nível do metabolismo respiratório. O aumento da temperatura nocturna aumentou significativamente a taxa respiratória no 1º dia após a sua imposição, no entanto, não houve diferenças nos restantes dias do ensaio. A actividade da enzima PK baixou a 15 ºC, mas a actividade da MDH aumentou significativamente, justificando o grande decréscimo observado antes do nascer do sol, que se manteve em amostras colhidas ao meio-dia. Após a aplicação de metamitrão e exposição a 7,5 ºC, a actividade de ambas as enzimas decaíu, mas aquando da exposição a 15 ºC, a MDH manteve a sua actividade durante toda a experiência assim como a PK de dia 7 a 14 após aplicação. Apesar destes resultados, conclui-se que, de uma forma geral, a exposição a temperaturas nocturnas de 15 ºC aumenta o metabolismo da árvore, estimulando o crescimento e desenvolvimento, resultando num maior

consumo de fotoassimilados. Desta forma, aumenta a competição e contribui para um balanço de carbohidratos negativo.

A análise do estado oxidativo ao nível da folha permitiu concluir que apesar de alguns aumentos moderados nos níveis de H_2O_2, nenhum dos tratamentos promoveu alterações ao nível da membrana. No entanto, registaram-se aumentos na actividade das enzimas APX, CAT, GR e SOD após aplicar metamitrão, per si, ou combinado com temperaturas nocturnas altas. O sombreamento atenuou os efeitos de stress oxidativo e, em combinação com metamitrão, apenas foram observados aumentos moderados na actividade da CAT e GPX. Todos os tratamentos promoveram um aumento dos níveis de glutationa e reduziram o conteúdo de ascorbato, contribuindo para a manutenção do estado oxidativo das células.

Nesta fase de crescimento exponencial dos frutos, há uma competição muito grande entre os frutos e a parte vegetativa da árvore. Os resultados obtidos permitiram concluir que o metamitrão estimula o crescimento vegetativo, aumentando assim a competição por fotoassimilados. Por outro lado, a escassez de fotoassimilados observada nas árvores tratadas com metamitrão ou aquecidas durante a noite, resultou num abrandamento significativo da taxa de crescimento dos frutos, que possivelmente estimulou a formação da zona de abcsissão.

A rede de sombreamento, o aumento da temperatura nocturna e o metamitrão potenciaram a queda natural de frutos. O efeito da radiação provocou uma taxa de abscissão mais alta que o próprio metamitrão, enquanto que a temperatura nocturna alta conduziu a quedas semelhantes ao efeito do mesmo. Todos estes tratamentos promoveram aumentos no tamanho e na qualidade do fruto, no entanto, foi a combinação de metamitrão com sombreamento ou com temperaturas altas que promoveu as taxas de abscissão mais altas, e por vezes, situações de excesso de monda, devido às fortes perdas de produtividade. Em concordância com o aumento de absorção, inibição de fotossíntese e de redução de sacarose, apenas em Sint-Truiden houve uma tendência para um menor número de frutos por árvores no tratamento combinado de metamitrão e humidade alta que, no entanto, resultou em aumentos do tamanho e qualidade do fruto significativos. Estes resultados demonstram que um aumento de humidade pode potenciar o efeito do metamitrão em climas húmidos, com folhas com características mais propensas à absorção, mas também que árvores mais jovens, com menos reservas, são mais sensíveis a variações de fotoassimilados.

A análise dos açucares solúveis em todos os tratamentos assim como a avaliação das taxas de abscissão permitiu estabelecer uma forte correlação entre a percentagem de queda de frutos à colheita e a percentagem de redução de sacarose, ambos comparativamente ao controlo.

Este trabalho permitiu concluir que as condições meteorológicas podem, por si só, aumentar a queda natural de frutos. Em combinação com metamitrão, devem ser seriamente avaliadas, pois podem potenciar a sua eficácia e levar a resultados indesejados de excesso de monda e perda de produção. Mais ainda, concluiu-se que os níveis foliares de sacarose são bastante promissores como biomarcador, tendo-se revelado como um bom indicador da eficácia do metamitrão.

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Abbreviations

6-BA	Benzyladenyne
A_{max}	Photosynthetic capacity
APX	Ascorbate peroxidase
AsA	Ascorbic acid
ATP	Adenosine triphosphate
CAT	Catalase
CH	Carbohydrate
Chl	Chlorophyll
CTR	Control
DAFB	Days after full bloom
DAS	Days after spraying
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
DCPIP	2,6-Dichlorophenolindophenol
DHA	Dehydroascorbic acid
DPC	1,5-Diphenylcarbazide
DW	Dry weight
F_0	Minimal fluorescence from the antennae
F_s/F_m'	Predictor of the rate constant of PSII inactivation
F_v/F_m	Photochemical efficiency of PSII
F_v'/F_m'	PSII photochemical efficiency of energy conversion under light exposure
FW	Fresh weight
GPX	Glutathione peroxidase
GR	Glutathione redutase
g_s	Stomatal conductance
GSH	Reduced glutathione
GSSG	Oxidized glutathione
H_2O_2	Hydrogen peroxide
HH	High humidity
HNT	High nighttime temperature
HPLC	High performance liquid chromatography
IRGA	Infrared Gas Analyzer
LC-MS/MS	Liquid chromatography with tandem mass spectrometry
MDA	Malondialdehyde
MDH	NADH-dependent malate dehydrogenase
MET	Metamitron

MV	Methyl viologen
NAA	Naphtaleneacetic acid
NADP	Nicotinamide adenine dinucleotide phosphate
NSC	Non-structural carbohydrates
OEC	Oxygen-evolving complex
PGR	Plant growth regulators
PI	Photo-inhibition
PK	Pyruvate kinase
Pn	Photosynthetic rate
POD	Guaiacol peroxidase
PPFD	photosynthetic photon flux density
ppm	Parts per million
PS	Photosystem
PTFE	Polytetrafluoroethylene
PVPP	Polyvinylpolypyrrolidone
$\mathbf{Q_A}$	Plastoquinone A
$\mathbf{Q_B}$	Plastoquinone B
$\mathbf{q_L}$	Photochemical quenching based on the concept of interconnected PSII antennae
$\mathbf{R_d}$	Dark respiration
ROS	Reactive oxygen species
RuBisCO	Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase
RWC	Relative water content
SE	Standard error
SN	Shading net
SOD	Superoxide dismutase
TBA	Thiobarbituric Acid
TCA	Trichloroacetic acid
$\mathbf{V_i}$	Initial activity
$\mathbf{V_{max}}$	Maximum activity
$\mathbf{V_t}$	Total activity
$\mathbf{Y_{(II)}}$	Photosynthetic quantum yields of non-cyclic electron transfer
$\mathbf{Y_{(NO)}}$	Non-regulated energy dissipation (heat and fluorescence) of PSII
$\mathbf{Y_{(NPQ)}}$	Photoprotective regulated energy dissipation of PSII

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CHAPTER 1

General Introduction

1. General Introduction

1.1 Introduction

1.1.1 Apple Production in the World

The apple tree (*Malus domestica* Borkh.) is a deciduous tree of the Rosaceae family, subfamily Pomoideae. There are nearly 8000 varieties of apple identified in the world, most of them, autochthone, grown and maintained in collection orchards to preserve its genome (Garkava-Gustavsson et al., 2011). Nowadays, apple trade and consumption are reduced to a small group of cultivars grown in commercial quantity, obtained by breeding programs, with the top ten compromising almost 90% of the total crop production. The most cultivated varieties in Europe are 'Golden Delicious', 'Gala', 'Idared', 'Red Delicious' and 'Jonagold' (WAPA, 2013). These varieties became well known due to its organoleptic characteristics and resistance to mechanical transport, allowing providing the consumer with apples throughout the year. Apple breeding programs are constantly evolving and since in the past years the use of pesticides has been widely questioned, the focus is now on obtaining disease resistant cultivars.

In 2018, the apple was the third most produced fruit in the world, reaching more than 86 million tons over almost 5 million hectars (FAO, 2020). Despite the fact that China is the greater producer in the world, producing around 60% of the total world production, Switzerland, New Zealand and Chile are the countries that reach the highest yields.

Apples can be used for fresh consumption, or for industry, namely juice, dried apple, jam, cider, and so on. According to the Portuguese Ministry of Agriculture, in Portugal 75% of apple production is for the fresh market, in which the flawless appearance, including size and color, along with taste and texture typical of each cultivar, are important characteristics valued by consumers.

1.1.2 Fruit Growth Development and Abscission Regulation

The growth of apple fruits has been described as simple sigmoidal (Tromp and Wertheim, 2005) and occurs generally in two phases: the phase of cell division and cell expansion for approximately 4 to 5 weeks after fertilization, followed by the phase of only cell expansion. The first phase is characterized by a rapid increase in flesh volume, mainly as the result of cell division (Gillapsy et al., 1993) and in the second phase fruit growth slows down and it is mainly characterized by expansion in longitudinal, radial and tangential planes until it reaches its final size (Bain and Robertson, 1951; Westwood, 1978).

Usually, the apple tree sets to many fruits and has self-regulating mechanisms to control crop load through physiological processes such as flower and fruit abscission. According to Tromp and Wertheim (2005), there are several natural drop periods: (1) flower drop - shortly after flowering non-pollinated or non-fertilized ovaries are shed, (2) June drop - young fruitlets are shed due to competition for carbohydrates between clusters and fruitlets within a cluster, and between fruitlets and shoots and (3) pre-harvest drop.

The natural degree of abscission at June drop depends on several factors. Fruit position within the cluster has a great influence, lateral fruits are more subjected to abscission compared to king fruits (Bangerth, 2000; Dal Cin et al., 2007; Jakopič et al., 2015). Besides the cluster, the position in the tree has also a major importance, the fruitlets developing in one-year-old branches and the ones close to the trunk have a higher probability to fall (Bangerth, 2000). Shoot growth might also contribute to enhance fruit abscission, since during the early phase of fruit development there is a strong competition between the vegetative parts of the tree and the fruitlets (Miller et al., 2015). According to Forshey and Elfving (1989), Bepete and Lakso (1998) and Lakso et al. (2001), in apple, shoot growth has priority over fruit growth for carbohydrate partitioning, when light levels during the first 40 DAFB are limiting, which can highly contribute to increase fruit fall. In addition, orchard-related factors, such as rootstock, tree age, vigor, bloom density, crop load, and presence of pollinators, should be considered due to their effects on carbohydrate availability (Jones et al., 2000; Doerflinger et al., 2015). Finally, the genetic difference between cultivars is another factor influencing the abscission rate (Wertheim, 2000).

According to a model described by Botton et al. (2011), apple fruitlet abscission takes place in four main steps, corresponding to the four structural levels where the key events may occur, which are the tree, the fruit cortex, the seed and the abscission zone. Considering transcriptomic data at fruit level, it was proposed that the early reaction of the cortex of abscising fruitlets to abscission induction is a result of a successive chain of events, including an increase of sucrose and reactive oxygen species production, increased abscisic acid production and enhanced ethylene biosynthesis (Botton et al., 2011; Eccher et al., 2013, 2015). According to this approach, the cortex would be the place where the primary abscission signal is generated, whereas the seed would function as a modulator of the physiological response, translating this signal to the abscission zone.

1.1.3 The Thinning Practice

Apple is a biennial bearing tree characterized by heavy bloom in the ‘on’ year, which generally leads to an overset of fruit, and an ‘off’ year, with less flower buds than desired (Greene, 2002). As the fresh fruit’s market is very demanding, to maximize profit, the grower has to produce large fruits of uniform size along with high organoleptic characteristics. Therefore, the need of applying additional artificial methods such as chemical, mechanical or manual thinning are often practiced by growers in order to achieve an optimum crop load. Thinning balances sink strength of the tree (flowers and fruits), and source capability to provide the required photosynthates, and the result is a more regular production over the year accompanied by an increase in the percentage of fruits with good market value.

Manual thinning is time consuming and highly expensive, and it’s mainly used for crop load adjustments after mechanical or chemical thinning (Costa et al., 2013). Mechanical thinning has the advantage of not depending on weather conditions and can adapt to most orchards by adjusting the rotation speed to the different types of canopies and flower intensities. Nevertheless, there are disadvantages such as entangled strings that need to be manually

untied, the risk of branch, shoot, leaf and fruitlet damage. Finally, it increases risk of disease spread, namely fire blight, due to the physical contact between the trees and the machine （Seehuber et al., 2013)

Shading is an environmental friendly thinning technique that has been used in organic farms leading to the same results in both fruit quality and biennial bearing effect than with other types of thinning. The physiological basis behind it is the shortage in carbohydrates induced by low radiation (Zibordi et al., 2009). The problem concerns the duration of shading period and the timing of the treatment, factors that have a great influence on the thinning results (Basak, 2011) and are not yet defined. Besides the positive results, it is a difficult and expensive technique to approach in commercial orchards.

1.1.3.1 Chemical Thinning and Metamitron

Chemical thinning is the most practical and cost-effective way of thinning however, results are inconsistent between years and orchards, often leading to situations of under thinning or over-thinning. Due to the apprehension of over-thinning, the grower does not like to take risks and often prefers to spray a lower rate of the chosen thinner. This leads to many situations of under-thinning that require almost invariably hand thinning to achieve optimum crop load (Costa et al., 2013). Considering treatment timing chemical thinners may be applied over a relatively wide range of time, from 6 mm until reach a diameter of up to 25 mm king fruit diameter. However, usually the later the thinning the lower the efficacy induced by the thinning agent (Greene, 2002; Costa et al., 2013).

There are several thinning agents in the market, but the two main used types are plant growth regulators (PGR) and photosynthesis inhibitors. PGR such as benzyladenyne (6-BA) and naphtaleneacetic acid (NAA) influence the complex balance between ethylene and auxins (Wouters, 2014), as well as, the competition between the flower cluster and vegetative shoot (Dal Cin *et al*., 2007). Photosynthesis inhibitors such as herbicides (Byers et al., 1990) are also potential thinning agents for their capacity to promote fruitlets abscission.

Metamitron (4-amino-3-methyl-6-phenyl-1,2,4-triazin-5(4H)-one) is a systemic chemical compound of the triazinone group of herbicides, only mobile in xylem, that, at a low dose, inhibits photosynthesis (Schmidt and Fedtke, 1977). Since in most fruit growing countries in Northern Europe temperatures required by PGR efficacy are rare in the short thinning window, metamitron has become one of the most used thinners in intensive apple growing systems. With its use becoming more common, many dose related questions emerged along with doubts regarding the physiological effects in the tree. Metamitron acts by inhibiting photosystem (PS) II whose application disrupts the thylakoid electron transport up to 60% (McArtney et al., 2012), by binding on D1 protein and blocking the normal transfer of electrons between the primary ($Q_{A)}$ and secondary quinones (Q_B) of PSII (Fig. 1.1) (Guidi and Degl'Innocenti, 2011). This will lead to the closure of the reaction centres, reducing the efficacy of photosynthesis (Maxwell and Johnson, 2000; Abbaspoor et al., 2006). Finally, carbon fixation and ATP production are

reduced, blocking the production and import of assimilates to the fruits, restricting carbohydrate (CH) availability and eventually causing a negative carbohydrate balance that enhances natural shedding (Byers et al., 1991; Lakso and Grapadelli, 1992; Abbaspoor et al., 2006; Elsysy et al., 2019).

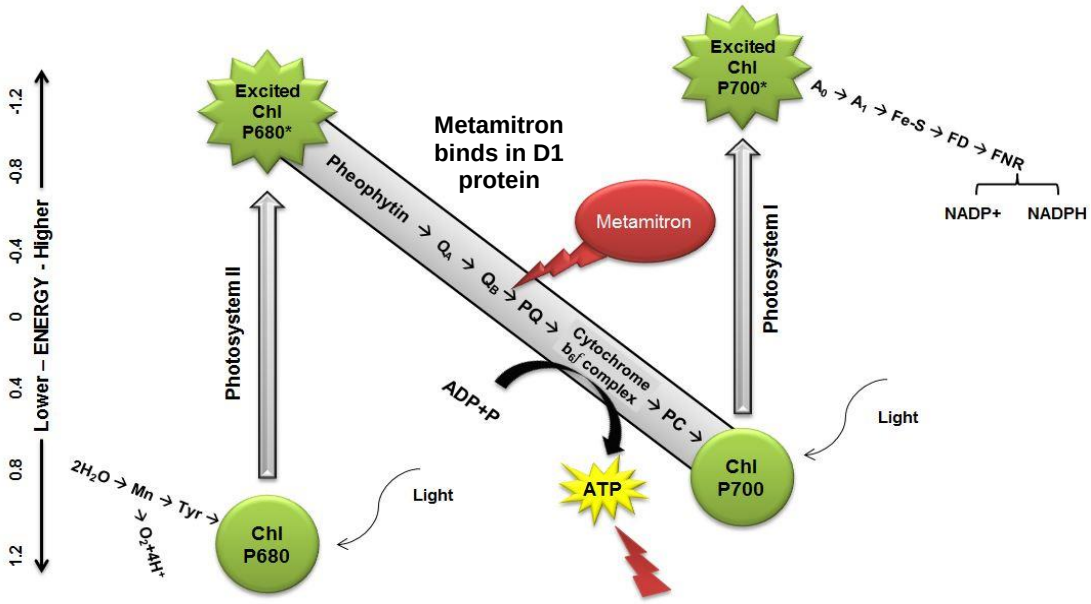

Figure 1.1 – The site of action of metamitron and consequent NADP+ and NAPDH affection represented on the Z-scheme (adapted from Govindjee and Wilbert Veit, 2010)

The application window of metamitron is from 8 to 14 mm king fruit diameter for maximum response but some thinning can be achieved up to 20 mm (McArtney and Obermiller, 2012). Nevertheless, for optimum metamitron results it should be applied between 12 to 14 mm diameter, when fruitlets no longer depend from trunk reserves and are only dependent on photoassimilates originated from shoot leaves photosynthesis (Gonzalez et al. 2019).

Metamitron is a selective herbicide used in sugar beet crops due to the ability of these plants to inactivate it within its leaves by a deamination reaction (Schmidt & Fedtke, 1977). This reaction results in only slight modifications in the main compound, namely the rupture of the connection N-NH_2, under a photolysis reaction which occurs in the presence of light, oxygen and water. This reaction forms deaminated compounds as main degradation metabolites, namely desamino-metamitron, which is not capable of inhibiting the photosystem activity (Palmer et al., 1997; Kouras, 2012). Since metamitron UV maximum absorption wavelength is 306 nm, located in the ultra-violete, direct photolysis reaction can occur in the field.

1.1.4 Precision Crop Load Management

The efficacy of metamitron is strongly dependent on a complex interaction between cultivar, tree vigor, time of application, mode of application and environmental conditions (Racskó, 2006; Wouters, 2014). Temperature, humidity and radiation levels are also three very important factors to take into account, not only during the spraying but also in the following days (Stover and Greene, 2005). These factors have a great impact not only on metamitron absorption but also on carbohydrate balance and, consequently, on metamitron thinning efficacy (Fig. 1.2). A carbohydrate surplus will lead to a lower fruit fall rate, while a deficit will result in fruit growth decline and stimulation of the formation of an abscission zone, resulting in a higher efficacy of the thinning compound (Lordan et al., 2019).

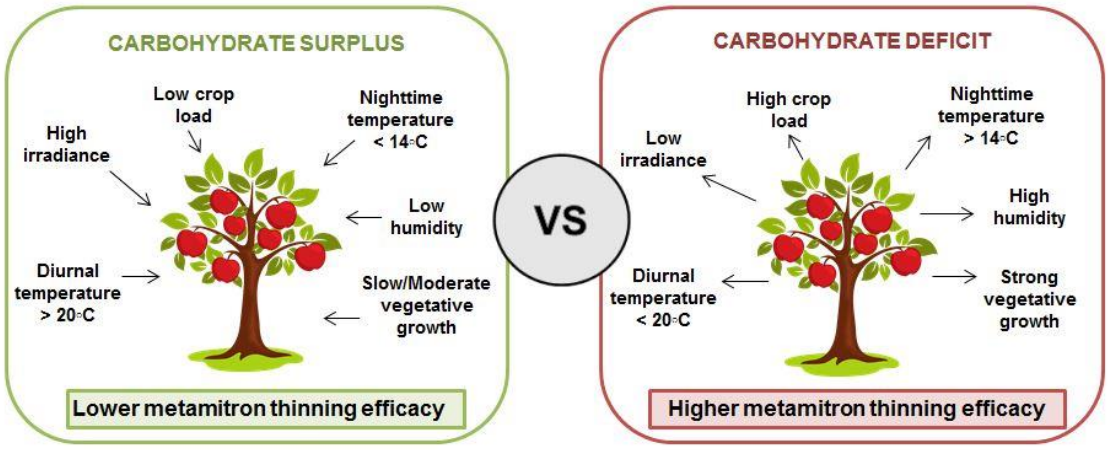

Figure 1.2 – Factors that contribute to a carbohydrate surplus and carbohydrate deficit and consequent metamitron expected thinning efficacy.

Several studies had proved that by shading apple trees for a certain number of days at a specific post-bloom period and with a large reduction in light availability, it is possible to induce fruit drop due to the restriction in carbohydrate availability caused by the limited light reaching the leaves (Kondo and Takahashi, 1987; Byers et al., 1990, 1991; Lakso and Corelli Grapadelli, 1992; Zibordi et al., 2009; Basak, 2011; Elsysy et al., 2019). The hypothesis is that periods of low radiation after metamitron application may create a negative carbohydrate balance that will enhance its efficacy. As a support for this statement, several works done with shading nets demonstrated similar results to chemical thinning, showing that carbon balance in the tree seemed to be the most important factor that influences fruit abscission (Byers et al., 1990; Corelli Grappadelli et al., 1994, Zibordi et al., 2009; Domingos, 2016). For ‘Gala’ cultivar, blocking 70% of solar radiation during 10 days when fruits were in the 9-14 mm stage promoted a reduction of 25% in yield per tree, as compared to a control, while by spraying 350 ppm of metamitron no differences from control were seen (Basak, 2011). According to Corelli

Grapadelli et al. (1994), shade periods delay markedly the onset of export of photosynthates, leading to fruitlet starvation and consequent shedding. Hence, when a period of cloudy sky follows metamitron application, the tree photosynthetic performance will be additionally lowered, limiting availability of carbohydrates and increasing fruit abscission due to increased competition between fruitlets and vegetative growth.

Diurnal and nighttime temperatures also have an effect on CH balance. Higher diurnal temperature promotes higher photosynthetic rates and consequently more photoassimilate production. On the other side, there is evidence that periods of high nighttime temperature stimulate its consumption by enhanced dark respiration (Robinson and Lakso, 2011; Jing et al., 2016). The result can be an enhancement of fruit abscission or even over-thinning, as it was demonstrated by Stern et al., (2014) in apple trees. Additionally, it has been reported that trees exposed to high night temperatures in the range of 15-16 ºC (at least 4-5 ºC above normal conditions) have higher dark respiration rates (Kondo and Takahashi, 1987). As sucrose is the respiration substrate, an increased consumption of this soluble sugar is expected (Rosa et al., 2009). Sink organs depend on the delivery of sucrose (or other forms of carbohydrates) by the phloem for their growth and development (Lemoine et al., 2013). This is perceived by the fruitlet cortex in the first place and provides a stimulus for fruit abscission. Therefore, changes in nighttime temperature are thought to have an important role in carbon balance and, consequently, in fruit abscission rate, although this remains to be completely elucidated. Moreover, not much is known about the combined effect of metamitron and high nighttime temperature.

It is known that relative humidity affects the drying period at the leaf surface after a chemical application. The longer the drying period, the greater the chemical penetration, with the epicuticular wax and cuticle becoming softer and more predisposed to absorption (Orbovic et al., 2001). Consequently, a high relative humidity may enhance metamitron absorption, and thus photosynthesis inhibition. Therefore, sugar production may be reduced and fruit drop may be enhanced by metamitron application under high relative humidity conditions.

There are many factors to take into consideration in the decision making process of metamitron spraying. It is very difficult for the grower to assess all of them and decide when to apply metamitron and at what concentration. For this reason, models for carbohydrate estimation have been developed in the past years (Lakso and Johnson, 1990; Lakso et al., 2001; Doerflinger et al., 2015; Lordan et al., 2019). These models mostly rely on tree phenology and meteorological conditions to calculate the carbohydrate demand of the tree and its production. However, there are not studies in apple tree that evaluate the direct effect of radiation, nighttime temperature and humidity, per si, or combined with metamitron on non-structural sugars. This study was already raised in citrus (Stander et al., 2018) in which they verified a decrease in soluble sugar content after the application of metamitron. A study that correlates the decrease in leaf non-structural sugars with the final decrease of fruits will certainly help to make better

predictions of the metamitron thinning efficacy and provide the grower with a better spraying rate to an obtain optimal crop load adjustment.

1.1.5 Tree Physiological and Biochemical Response to Metamitron and Meteorological Conditions

Abiotic stresses trigger many physiological, biochemical, and molecular responses that influence various aspects of plant metabolism. Furthermore, under stress conditions, stress-related hormones such as abscisic acid and ethylene are produced, play an important role in sugars-sensing, and are particularly important and interconnected molecules that trigger abscission mechanisms (Rosa et al., 2009). In a context of fruit abscission, factors that lead to a negative carbohydrate balance can be considered as stresses, namely metamitron, low radiation, high nighttime temperature and high humidity.

The efficacy of metamitron is dependent on carbohydrate balance in the tree by the time of spraying, daily level of carbon assimilation and allocation of assimilated carbohydrates between competing sinks (McArtney et al., 2012). Different thinning results when spraying the same dose of metamitron can be explained by meteorological conditions that can change the efficacy of metamitron from no thinning at all to an over thinning effect due to its effect on CH balance (Stovar and Greene, 2005). Another reason is the fact that the variability of the uptake of a chemical compound is highly dependent on the meteorological conditions before, during, and after application (Robinson et al., 2013; Lakso and Robinson, 2015). It is widely known that metamitron reduces the photosynthetic rate (Brunner, 2014; Gabardo et al., 2017), but it is not clear how its combination with certain meteorological parameters affect it, and it is likely that an increase in its absorption caused by certain meteorological conditions may cause an even stronger photosynthesis reduction. According to Koch (2004), around 80% of the CO_2 assimilated during photosynthesis is channelled into synthesis of sucrose, which is the major form of exportation of organic carbon from source to sink organs. As so, the photosynthesis block caused by metamitron combined with other abiotic conditions that influence carbon balance seems to be of great importance when evaluating metamitron thinning efficacy.

The effect of metamitron on fruit growth rate on several apple cultivars has been described by Rosa et al. (2018), who observed no changes in growth rate of ‘Gala’ and an increase in ‘Red Delicious’ and ‘Pink Lady’ a few days after a two time 165 ppm spraying of metamitron. Gabardo et al. (2017) refer to a decrease in ‘Maxi Gala’ growth rate 7 days after a 350 ppm metamitron application. After increasing nighttime temperature 27 and 34 DAFB, Kondo and Takahashi (1987) observed a reduction in apple fruit growth rate on the 4th day after the beginning of increased nighttime temperature, as compared with fruits exposed to natural environmental conditions. However, there is no knowledge about how radiation and humidity may affect fruit growth, or about its affect in vegetative development.

Plant strategies to cope with stresses normally involve wide and integrated stress avoidance and tolerance mechanisms, as reactive oxygen species (ROS) production, which appear as the

more common consequences of exposure to abiotic stresses (Bechtold and Field, 2018). The uncontrolled ROS production is extremely harmful to organisms, especially when its level exceeds the capability of the defence mechanisms to control them, causing oxidative stress (Sharma *et al*., 2012). In the chloroplast the over production of ROS is frequently a consequence of a disruption in thylakoid electron transport chain, as is the case of the impairment between Q_A and Q_B caused by metamitron (Foyer and Noctor, 2000; Noctor et al., 2002). In this context, the evaluation of the oxidative stress status of the plant might improve the understanding of how the plants react to the extra stress caused by metamitron application.

1.2 Objectives and Thesis Outline

The aim of the research here described was to determine the physiological and biochemical changes occurring during the period of about two weeks after changing environmental conditions and/or applying metamitron, which have a profound impact on fruit abscission and in the quality of apple at harvest. In addition, we aim to evaluate the effect of the meteorological conditions in the few hours before and after metamitron application on metamitron absorption and degradation throughout time and explore a correlation with the thinning efficacy observed at harvest.

Separate trials were designed across three different locations in Europe - Girona and Lleida (Spain) and Sint-Truiden (Belgium) - in several cultivars - ‘Golden’, ‘Gala’, ‘Braeburn’ and ‘Elstar’ - and repeated for at least two years. We resort to shading nets, not to explore a new thinning technique, but to create a procedure to simulate low radiation conditions. Also, we built structures placed over the trees, heaters and thermostats that allowed increasing nighttime temperature, and repeated the same trial design in growth chambers, in order to reduce the field variability and look deeper into physiological and biochemical changes caused by this meteorological change. Finally, to increase relative humidity, we set structures with foggers or sprinklers to increase relative humidity.

Evaluating the interactions between these meteorological parameters and the metamitron thinning effect is, therefore, relevant for understanding reason(s) for variable thinning efficacies and finally, provide a more accurate dosage recommendation.

To accomplish the above mentioned aims, we intend to fulfil the following specific research objectives:

- To quantify metamitron leaf absorption, and assess, if and how, radiation, nighttime temperature and humidity affect its absorption, and identify its possible influence on thinning efficacy. Also, to evaluate its degradation by means on desamino-metamitron assessment.
- To ascertain the main pathways involved in fruit abscission in several apple cultivars, specifically targeting the period immediately after meteorological conditions changing and/or metamitron application. This involves gas exchanges, RuBisCO activity, non-structural

sugars and shoot and fruit growth. In addition, evaluate if these parameters could be good indicators of the final thinning efficacy observed at harvest.

- To explore the variations of non-structural sugars not only at midday, as in the radiation and humidity trials, but also before sunrise in the nighttime temperature trials, allowing to make a deeper and more accurate assessment of how a period of increased temperature during the night affect carbohydrate balance. To try to establish a relationship between the soluble sugar variations and the final number of fruits at harvest.
- To evaluate how radiation, nighttime temperature and humidity affect natural fruitlet abscission and if and how they enhance metamitron thinning efficacy, in order to increase the knowledge of thinning with metamitron and provide the growers and advisors with information to provide a better metamitron dose to achieve an optimum crop load.
- To evaluate if these meteorological conditions affect the tree oxidative status and to characterize it in terms of lipid peroxidation by means of MDA and H_2O_2 quantification, antioxidative products such as ascorbate and glutathione and finally, possible up-regulation of antioxidative enzyme activity.
- Finally, to clarify the results of field nighttime temperature trials, a trial with potted trees, under controlled environmental conditions, with increased nighttime temperature was set to understand the exact photosynthetic components affected by metamitron, assessing its impact on leaf gas exchanges, photosynthetic capacity, electron transport rate, activity of photosynthetic enzymes and on membrane level, but mainly, to clarify the effects of high nighttime temperature alone and combined with metamitron on dark respiration and on respiratory enzymes.

To achieve these goals, four types of approaches were designed and developed in each chapter, where a detailed introduction on each topic can be found, according to the thesis organization described in Fig 1.3.

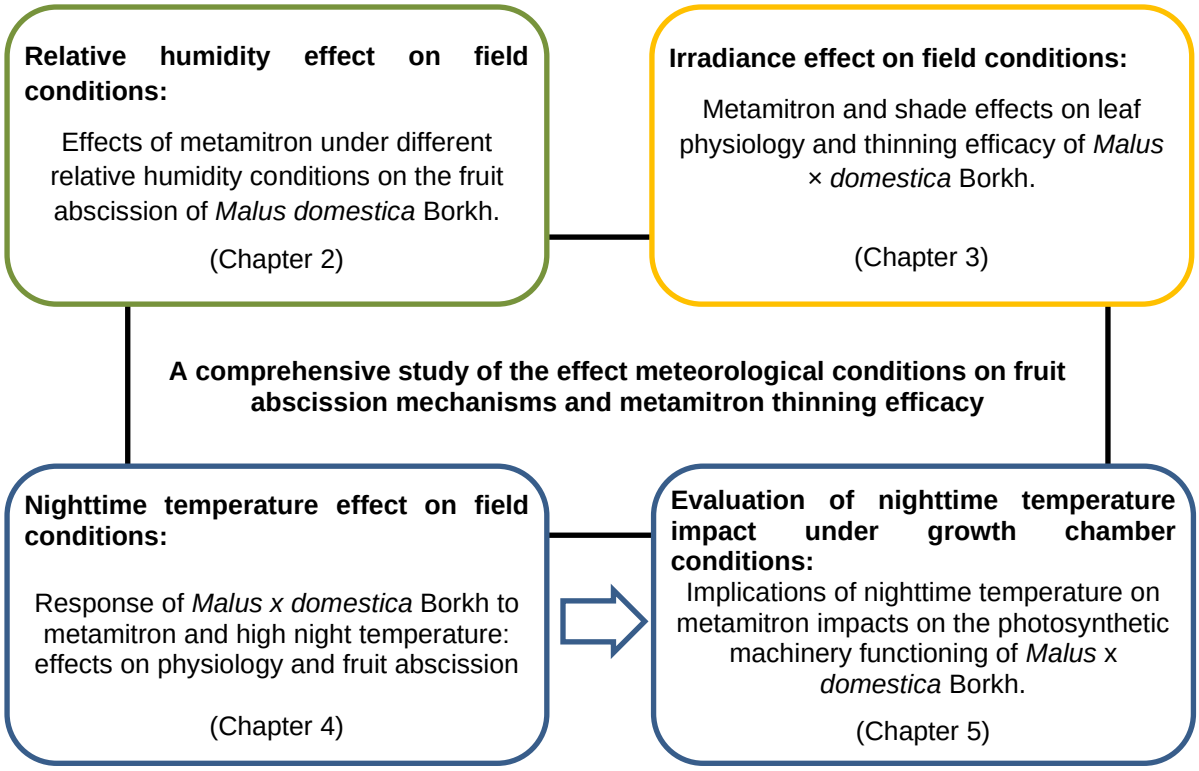

Figure 1.3 – Diagram of thesis organization. Advancing in knowledge about the effect of relative humidity (chapter 2), irradiance (chapter 3) and nighttime temperature (chapters 4 and 5) on natural fruit abscission regulation and enhancement of metamitron thinning efficacy.

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CHAPTER 2

Effects of Metamitron under Different Relative Humidity Conditions on the Fruit Abscission of *Malus domestica* Borkh. Cultivars

Foggers in Belgium (left side) and sprinklers in Lleida (right side) to increase relative humidity

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2. Effects of Metamitron under Different Relative Humidity Conditions on the Fruit Abscission of Malus domestica Borkh. Cultivars

Abstract: Metamitron is an apple (*Malus domestica* Borkh.) fruit thinner that acts by reducing the photosynthetic capacity of trees. Relative humidity may influence thinning efficacy; however, the broadness of this effect is not yet fully understood. Trials were set in Sint-Truiden (Belgium) in 2018 and Lleida and Girona (Spain) in 2019, using 4-year-old cvs. Braeburn and Elstar trees in Belgium, and 16-year-old cv. Golden Reinders trees in Spain. Four treatments were implemented at the stage of 12–14 mm fruit diameter: (a) CTR—control, trees under natural environmental conditions; (b) HH—high humidity, trees submitted to artificially increased air relative humidity applied for 3 h prior to the beginning of the experiment; (c) MET—trees sprayed with 247.5 mg/L metamitron; (d) MET + HH—trees submitted to the combination of increased humidity (HH) and metamitron (MET) application. In Belgium, metamitron absorption by leaves was greater than in Spain. This might be related to morphological characteristics of the leaves which developed under greater natural relative humidity levels in Belgium than in Spain. Compared to MET alone, 'Braeburn' and 'Elstar' demonstrated significantly greater metamitron absorption, 59% and 84%, respectively, under MET + HH, accompanied by declines in leaf net photosynthesis (10% and 32%, respectively) and sucrose (31% and 26%, respectively). At harvest, MET + HH treatment reduced yield by 24% and 32% in 'Braeburn' and 'Elstar', respectively, when compared with MET alone. A large reduction (considered over thinning) in the yield of 'Elstar' occurred. In contrast, metamitron absorption by 'Golden Reinders' using MET alone was similar to MET + HH; however, there was a slight foliar sugar reduction in the latter treatment. In addition, both treatments enhanced shoot growth and increased fruit abscission with similar improvements in fruit weight and size. In this study, high relative humidity enhanced fruit thinning efficacy under certain circumstances, such as age or genetic predisposal, which left the tree more susceptible to a negative carbohydrate balance. For instance, 'Braeburn' and 'Elstar' were easier to thin when compared to 'Golden Reinders'. In addition, this study raises a question that requires further research regarding the impact of HH before and after spraying as well as its effect in combination with higher temperatures.

Keywords: absorption; carbohydrate balance; photosynthesis; sucrose; sorbitol; thinning

2.1 Introduction

Apple (*Malus domestica* Borkh.) growers' profits are increasingly dependent on management strategies that prioritize apple fruit quality for fresh consumption, such as fruit size, to increase the economic value of the apples. One of the most important practices to achieve good fruit size is thinning. There are several thinning techniques, although the chemical strategy is the most widely used. The efficacy of a chemical compound can change between years, orchards, and cultivars (Lakso et al., 2001; Robinson and Lakso, 2004). Thus, unveiling the factors that can

directly enhance the efficacy of chemical thinners is of extreme importance to provide a practical way of predicting the effects and obtaining a result as close as possible to the optimal goal.

Metamitron, a systemic triazinone herbicide, is one of the most common thinners used in intensive apple growing systems. Metamitron acts by inhibiting photosystem (PS) II and disrupts thylakoid electron transport by blocking the electron transfer between the primary and secondary quinones of PSII (Abbaspoor et al., 2006; Guidi and Degl'innocenti, 2001), leading to the closure of the reaction centers and, ultimately, inhibiting photosynthetic carbon fixation (Abbaspoor et al., 2006; Maxwell and Johnson, 2000). This will restrict carbohydrate availability, causing a negative carbohydrate balance and an enhancement of fruit drop (Byers et al., 1991; Lakso and Corelli Grapadelli, 1992; Zibordi et al., 2009).

Shoot growth might also contribute to a negative carbohydrate balance, as during the early phase of fruit development, there is a strong competition between the vegetative parts of the tree and the fruitlets (Miller et al., 2015). In the apple tree, shoot growth has priority over fruit growth for carbohydrate partitioning during the first 40 days after full bloom (DAFB), which strongly contributes to a greater fruit fall under high vegetative growth (Forshey and Elfving, 1989; Bepete and Lakso, 1998; Lakso et al., 2001). In addition, orchard-related factors, such as rootstock, tree age, vigor, bloom density, crop load, and presence of pollinators, should also be considered when deciding the thinning strategy due to their effects on carbohydrate availability (Jones et al., 2000; Doerflinger et al., 2015).

Cultivar traits can lead to different responses to thinning applications (Jones et al., 2000; Rosa et al., 2018); therefore, adjustments are often made depending on the cultivar thinning susceptibility, which can be classified as easy ('Braeburn'), average, or difficult ('Golden Reinders') to thin (Washington State University, 2020). Associated with specific plant responses, the variability of a chemical compound's uptake is highly dependent on the meteorological conditions before, during, and after application, namely the air relative humidity (Robinson et al., 2013; Lakso and Robinson, 2015).

A high relative humidity affects the drying period at the leaf surface after chemical application. The longer the drying period, the greater the chemical penetration, with the epicuticular wax and cuticle becoming softer and more predisposed to absorption (Orbovic et al., 2001; New England – Tree Fruit Management Guide, 2020). In addition, a high relative humidity maintains the turgor pressure of the guard cells, keeping the stomata open, which could also allow for a greater absorption of chemicals. Absorption occurs through a diffusion process, from a more concentrated area (leaf surface) to a less concentrated area, through water with a low diffusion resistance.

If the vapor pressure deficit (VPD) on the leaf surface is low, stomatal conductance is higher (Hernandez et al., 2016; Merilo et al., 2018), and the water gradient will facilitate the penetration of the chemical. Along with the possible entry via open stomata, the diffusion process happens rapidly (Schulze et al., 1972; Patin and Blatt, 2018). Consequently, a high relative humidity may

enhance metamitron absorption, and thus photosynthesis inhibition, increasing the competition between fruitlets for the reduced available photosynthates. Therefore, as glucose is necessary for sucrose and sorbitol synthesis, we hypothesized that a decrease in these sugars may be enhanced by metamitron application under high relative humidity conditions, resulting in a more pronounced thinning effect.

Meteorological conditions play a crucial role in a tree's carbon balance (Lafer, 2010; Robinson, 2016; Lordan et al., 2019) and could change the efficacy of metamitron from no thinning response to over-thinning. Evaluating the interactions between relative humidity and the metamitron thinning effect is, therefore, relevant for understanding reason(s) for variable thinning efficacies, along with other aspects of the tree response through characterization of physiological and biochemical parameters for the single and combined applications of metamitron and high air relative humidity.

2.2 Materials and Methods

2.2.1. Trial Design and Plant Material

2.2.1.1. Plant Material

In 2018, two trials were performed in Sint-Truiden, Belgium, and, in 2019, one trial was performed in Girona and one in Lleida, Spain. Trials were held in the experimental orchards of the PCFruit Research Station—Proefcentrum Fruittteelt vzw, Sint-Truiden, Flanders, Belgium (50°45′49" N/05°09′26" E, 97 m altitude), with 'Braeburn' and 'Elstar' trees in separate orchards, grafted on M9 rootstocks, planted in 2014 (4 years old), spaced 3.5 × 1.0 m, with 'Granny Smith' as the pollinator. The two orchards were located in the same field, side by side.

In 2019, the trials were performed in the experimental orchards of the IRTA—Lleida research station, Mollerussa, northeast Spain (41°61′96. 37" N/0°87′66" E, 245 m altitude), and at the IRTA Más Badia research station, Girona, northeast Spain (42°03′97" N/3°03′13" E, 12 m altitude). In both locations, 'Golden Reinders' apple trees were used, planted in 2003 (16 years old). In Lleida, the trees were grafted on M9 rootstocks, spaced 4 × 1.4 m, with a canopy height of 3 m and 'Gala Brookfield' as the pollinator, while in Girona, the trees were grafted on M9 NAKB rootstock, spaced 3.8 × 1.1 m, with a canopy height of 2.5 m and 'Granny Smith' as the pollinator. Both orchards were trained as a central leader system.

For biochemical evaluations, the leaves were quickly cleaned with a water-wet tissue before being frozen in liquid N_2. All leaves were finely powdered with a mortar and pestle in liquid N_2 and kept at −80 °C until analysis.

2.2.1.2 Treatment Implementation and Experimental Design

Four treatments were implemented: (a) CTR—control, corresponding to trees under natural environmental conditions; (b) HH—high humidity, trees submitted to artificially increased air relative humidity applied for 3 h prior to the beginning of the experiment; (c) MET—trees

receiving 247.5 mg/L metamitron applied as described below; (d) MET + HH—trees receiving the combination of increased air relative humidity (HH) and metamitron (MET) application.

The MET application was performed at a fruit diameter of 12–14 mm. All sprays were made using the recommended dose of 247.5 mg/L metamitron—as the active ingredient of Brevis® (ADAMA, Telaviv, Israel)—per 1000 L ha^{-1}, using a hand-gun sprayer. Metamitron and/or relative humidity treatments were imposed between the 29th of April and the 16th of May.

In Sint-Truiden, to raise the relative humidity, a plastic cover was placed over the trees. The relative humidity was increased by two centrifuge humidifiers of type EPA with foggers with an output of 2–20 L/hour (JDK, Sint-Truiden) placed in the middle of the area. Due to the high relative humidity outside on the application day (65.9%), in addition to the 3 h of increased relative humidity prior spraying, it was also increased for 1 h after the metamitron was applied. In Girona and Lleida, a similar plastic cover was used, but the high relative humidity was achieved with irrigation sprinklers Super 10 (Novagric, Almeria, Spain), placed at a 1 m distance on both sides of the trees. The irrigation system was connected to a water tank coupled to a tractor working at 4.5 bar.

To monitor the environmental conditions in each trial, sensors for the temperature and relative humidity record were installed inside and outside the structures on both sides and in the middle (with and without HH); in each case in the upper (2 m) and lower (1 m) levels of the trees. In Girona, six EasyLog USB Data Loggers (Lascar Electronics, Wiltshire, UK) were used; in Lleida, six Testo 177-h1 sensors were used (Testo, Titisee-Neustadt, Germany); and six Testo 174H sensors (Testo, Titisee-Neustadt, Germany) were used in Sint-Truiden.

The initial number of flower clusters per tree was homogeneous among treatments in each orchard. The experimental design in each orchard was a randomized complete block, with four blocks each with four trees per treatment in each block, in which the two central trees of each set of four were measured, for a total of eight measured trees per treatment.

2.2.2 Metamitron Concentration

Leaf samples for the metamitron and its main metabolite concentration, desamino-metamitron, were collected two days after spraying (DAS). Each sample was a pool of three shoot leaves from the top, middle, and bottom part of each tree, with one sample taken from the eastern and another from the western side of the canopy, for a total six repetitions per treatment.

Metamitron extraction was conducted according to the QuEChERS method (Lesueur et al., 2008) (quick, easy, cheap, effective, rugged, and safe) using 500 mg fresh weight (FW) of frozen leaf powder and 3 mL of acetonitrile. The samples were shaken manually for 1 min, after which, 1.95 g of extraction Supel™ QuE Citrate Extraction Tube (Sigma-Aldrich, Missouri, United States of America) was added, containing 1.2 g of magnesium sulfate, 0.3 g of sodium chloride, 0.15 g of sodium citrate dibasic sesquihydrate, and 0.3 g of sodium citrate tribasic dehydrate. The samples were further shaken manually for 1 min and centrifuged (6000× *g*, 5 min, 4 °C). An aliquot of 1.2 mL of the supernatant was transferred to a 2 mL Supel™ QuE

Verde clean-up tube (Sigma), vortexed, and further centrifuged (6000× *g*, 5 min, 4 °C). The obtained supernatant was filtered (0.45 µm filter, PTFE), and injected on a LC-MS/MS (Waters, USA). Standard curves were used for the quantification of metamitron (Sigma-Aldrich, Missouri, United States of America) and desamino-metamitron-desamino (LGC Standards, USA).

2.2.3 Leaf Net Photosynthesis

Leaf net photosynthesis measurements (P_n) were performed in Sint-Truiden five and seven days after spraying (DAS) in recently fully developed shoot leaves at about 1.5 m in height with a portable Infrared Gas Analyzer (IRGA) LCi Ultra Compact Photosynthesis System (ADC BioScientific, Hoddesdon, UK), under ambient conditions of irradiance, air humidity, temperature, and CO_2 supply, between 10:00 and 12:00. In each of the four blocks, one evaluation in the eastern and one in the western side of the canopy were performed, totaling eight measurements per treatment.

2.2.4 Leaf Soluble Sugars

Leaf sampling for non-structural sugar quantification was performed five and seven days after spraying (DAS) in Sint-Truiden and 2, 5, and 10 DAS, in Lleida and Girona, always between 11:00 and 12:30 h. In Sint-Truiden, six repetitions of a pool with two shoot leaves and two cluster leaves were used. In Lleida and Girona, sampling consisted of ten separate shoot leaves per treatment. The quantification of sucrose, fructose, glucose, and sorbitol was based on the method described by Ramalho et al. (2013), using about 150 mg FW frozen leaf material. The separation of sugars was performed using a Sugarpak1 column (300 × 6.5 mm, Waters) at 90 °C, with H_2O (containing 50 mg EDTA-Ca L^{-1}) as the eluent, at a flow rate of 0.5 mL min^{-1} in an HPLC system equipped with a refractive index detector (Model 2414, Waters, Milford, USA). Standard curves of each sugar were used for quantification.

2.2.5 Shoot Growth

Shoot growth was measured in Girona and Lleida using eight bourse shoots per block (total 32 shoots per treatment). The shoot length was measured on the day of metamitron application and 10 days after spraying (DAS).

2.2.6 Yield Parameters

All fruit was harvested from each tree at harvest. The number of fruit per tree, yield, fruit weight, and distribution per fruit size were determined using a commercial sorting machine (Maf Roda Agrobotic, Montauban Cedex, France).

2.2.7 Statistical Analysis

Data were subjected to an analysis of variance, through a one-way ANOVA, to evaluate the differences between treatments on one single day after spraying, or a two-way ANOVA to evaluate the differences between the four treatments, across the several days after spraying.

Means were compared by Tukey's Honestly Significant Difference (HSD) test at α = 0.05. Each ANOVA was performed independently for each of the trials. The statistical analysis was performed using Statistix 9 (Analytical Software, Tallahassee, FL, USA).

2.3 Results

2.3.1 Environmental Conditions

A brief characterization of the environmental conditions in the four trials is shown in Table 2.1. For the global irradiance, the night temperature before application, and the diurnal temperature on the day of application, the values were very homogeneous between the two years and locations. The average night temperature after application was always lower than 13.5 °C, although it was higher in Girona by 3 °C compared with the other locations. The highest relative humidity increase was registered in the trial performed in Girona (35.3%).

Table 2.1 - Summary of the meteorological conditions in each trial: the cultivar, fruit diameter at the time of metamitron application, average of daily irradiance during 5 days after spraying (DAS) (MJ/m^2), average night-time temperature from 20:00 to 8:00 h during 5 nights before and after spraying (°C), average diurnal temperature from 8:00 to 20:00 h on the spraying day (°C), and average air relative humidity during the 3 h prior spraying (%) in environmental conditions (Control) and high relative humidity conditions (HH).

Location/ Cultivar	Fruit Diameter (mm)	Global Irradiance MJ/m^2 5 Days After	Night Temperature °C 5 Nights Before	Night Temperature °C 5 Nights After	Diurnal Temperature °C Application Day	Relative Humidity %	
		Control	Control	Control	Control	Control	HH
			2018				
Sint-Truiden 'Braeburn'	14 ± 0.1	21.7 ± 0.6	12.1 ± 0.6	9.7 ± 0.9	17.2 ± 0.9	65.9 ± 3.6	73.9 ± 2.7
Sint-Truiden 'Elstar'	13 ± 0.1	21.7 ± 0.6	12.1 ± 0.6	9.7 ± 0.9	17.2 ± 0.9	65.9 ± 3.6	73.9 ± 2.7
			2019				
Girona 'Golden Reinders'	14 ± 0.1	21.4 ± 2.4	12.0 ± 0.9	13.4 ± 0.4	16.9 ± 0.8	40.8 ± 1.0	76.1 ± 3.7
Lleida 'Golden Reinders'	12 ± 0.2	21.4 ± 1.9	11.1 ± 1.1	10.3 ± 1.1	18.5 ± 1.0	44.3 ± 1.4	58.0 ± 3.3

In Sint-Truiden, the relative humidity was increased by an average of 8% for 3 h prior to spraying. However, as described above, the relative humidity was also increased during the 1 h after spraying, from 48.6% to 68.6%. Along with the relative humidity increase, there was an

average temperature increase of 7.5 °C (Figure 2.1). In the case of Lleida and Girona, the relative humidity was raised 13.7% and 35.3%, respectively, and the temperature inside the structure was 1 °C less in both locations.

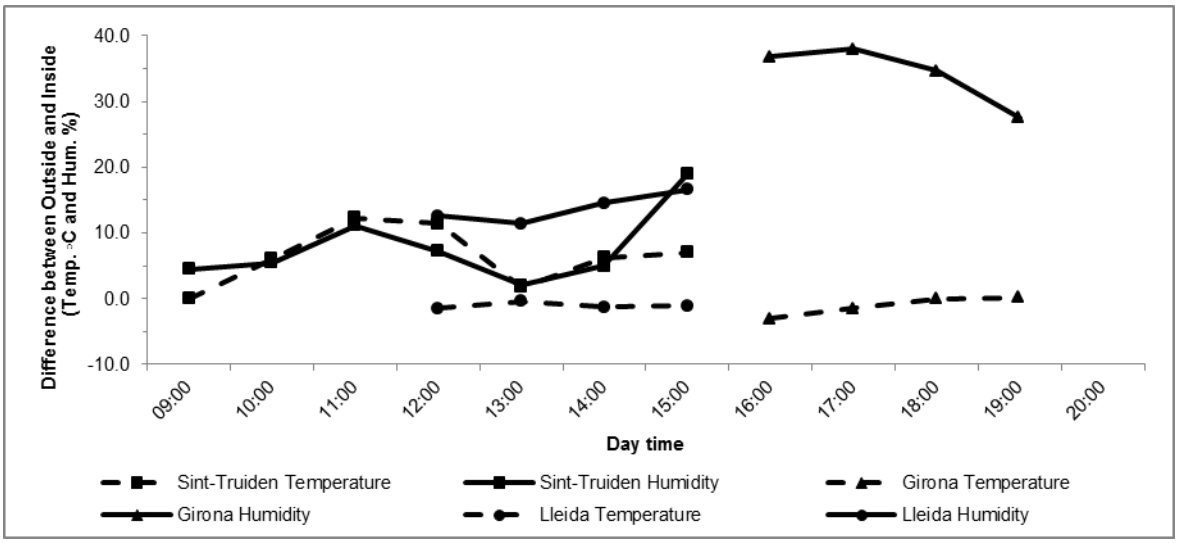

Figure 2.1 - Difference in relative humidity and temperature between the outside and the inside of the plastic covered trees during the humidity increase period in Sint-Truiden, Belgium, in 2018 (▪), and Girona (Δ), and Lleida (●), Spain, in 2019. The solid line represents the air relative humidity (%), and the dashed line represents temperature (°C).

2.3.2 Metamitron Concentration in Leaves

In the trials in Sint-Truiden, the increased relative humidity significantly increased the metamitron absorption (MET + HH), 59% and 84%, and increased desamino-metamitron formation, 49% and 58%, in 'Braeburn' and 'Elstar', respectively, as compared to MET alone (Figure 2.2), observed by 2 DAS when the maximal absorption is expected to occur, as previously found in our preliminary experiments (data not shown). In Girona and Lleida, the metamitron and desamino-metamitron content did not significantly differ in the MET or MET + HH treatments. Metamitron absorption by 'Golden Reinders' (Lleida and Girona) was similar to that by 'Elstar' (Sint-Truiden); however, the desamino-metamitron content was higher when compared with the trials in Sint-Truiden. Among the cultivars, 'Braeburn' showed 50% higher absorption than the other cultivars.

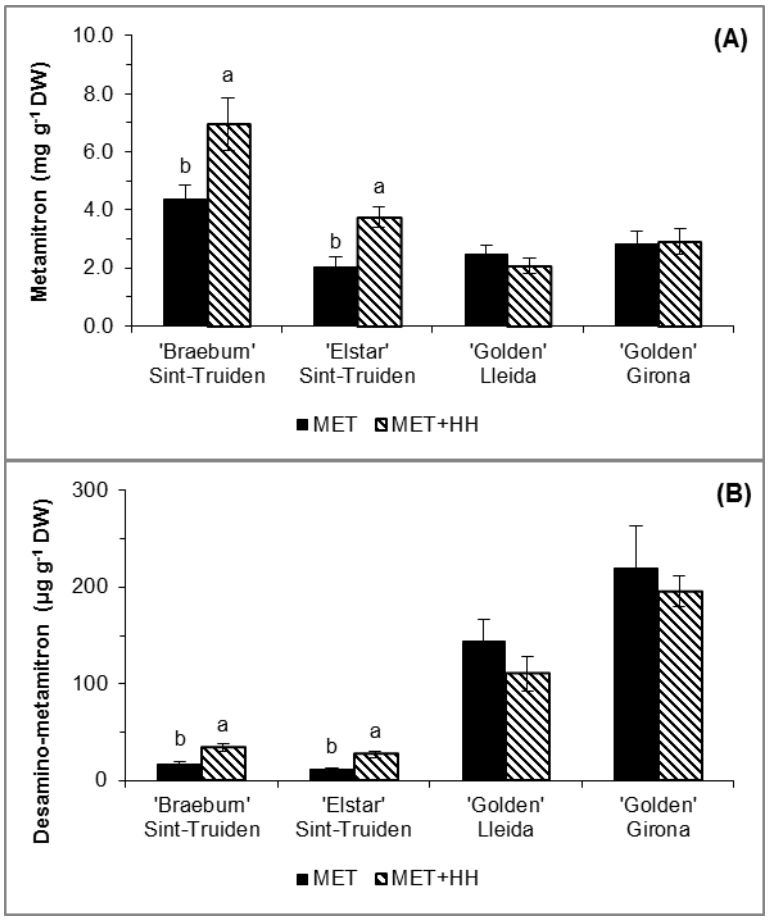

Figure 2.2 - (A) Metamitron and (B) desamino-metamitron evaluated 2 days after spraying (DAS), in the trials with 'Braeburn' and 'Elstar' apples in Sint-Truiden, Belgium, and with 'Golden Reinders' apple in Girona and Lleida, Spain. For each parameter, the mean values ± SE (n = 6) with different letters express significant differences between treatments within each cultivar/location using Tukey's HSD test (α-value ≤ 0.05). No letters indicate no significant difference between means.

2.3.3 Leaf Net Photosynthesis

The HH treatment did not affect the P_n values of the cultivars 'Braeburn' and 'Elstar'; however, MET significantly decreased P_n by 62% and 50% at 5 DAS and 33% and 35% at 7 DAS, in 'Braeburn' and 'Elstar', respectively, as compared to the CTR (Figure 2.3). The majority of the apparent MET recovery was related to a P_n reduction in CTR and HH from 5 to 7 DAS, due to cloudy conditions.

Concerning MET + HH, 'Braeburn' and 'Elstar' exhibited a tendency to a further P_n decrease than compared with MET 5 and 7 DAS, although this was significant only for the latter.

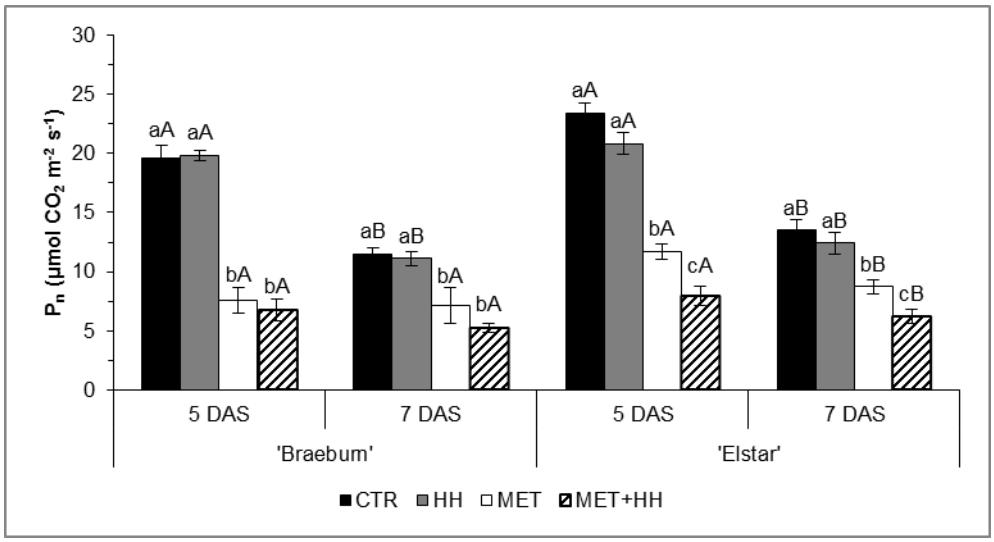

Figure 2.3 - Leaf net photosynthesis (Pn) (µmol CO_2 m^{-2} s^{-1}) evaluated 5 and 7 days after spraying (DAS), in the trials with 'Braeburn' and 'Elstar' apples in Sint-Truiden, Belgium. For each parameter, the mean values ± SE (n = 8) followed by different letters express significant differences between treatments within each day (a, b, and c), or between days within each treatment (A, B), using Tukey's HSD test (α-value ≤ 0.05). Treatments were control (CTR), increased relative humidity for 3 h prior spraying (HH), metamitron application at 247.5 mg/L (MET), and their combination (MET + HH).

2.3.4 Leaf Soluble Sugars

The HH treatment led to only a few modifications in the leaf soluble sugar content. The only variation registered was an increase in sorbitol in 'Braeburn', both at 5 and 7 DAS (Table 2.2). Metamitron (MET) significantly reduced the sucrose, fructose, sorbitol, and total sugar content at 5 and 7 DAS in 'Braeburn'. 'Elstar' showed similar trends, with significant reductions in sucrose (5 DAS), fructose (7 DAS), as well as of sorbitol, and the total sugars on both days (Table 2.2). In 'Golden Reinders' in Girona and Lleida, MET also reduced the sucrose content to one third and one fourth at 2 DAS, in Girona and Lleida, respectively, compared to the CTR (Table 2.3). At 5 DAS, these differences were somewhat reduced in Girona, whereas, in Lleida, the trees still presented a reduced content of sucrose of 50% compared to the CTR (Table 2.3). A similar reduction was observed in the sum of all analyzed sugars in Lleida and Girona (2 DAS) and in sorbitol and total sugars, in Lleida and Girona, respectively (5 DAS). However, the effect on these sugars was not as strong as with sucrose. In general, the lowest sugar contents were observed between 5 and 7 DAS. In contrast, the fructose content increased significantly in 'Golden Reinders' at Lleida and Girona at 2 and 5 DAS.

The combined exposure to metamitron and high relative humidity conditions (MET+HH) significantly exacerbated the MET impact on sucrose reduction in both cvs. at 5 DAS and on 'Elstar' at 7 DAS (Table 2.2). Sucrose content was significantly lower in 'Braeburn' (31%) and 'Elstar' (26%) than under MET. For many other sugars, values observed in the MET + HH

treatment did not always differ from the MET alone treatment (Tables 2.2 and 2.3). Additionally, at 7 DAS, 'Elstar' presented significantly lower values for all sugars except fructose under MET+HH conditions when compared with MET. Notably, in Lleida, differences observed among the four treatments until 5 DAS were no longer present at 10 DAS for all of the sugars.

Table 2.2 - The sucrose, glucose, fructose, sorbitol, and total sugar concentration in the leaves (mg g^{-1} dry weight (DW)) evaluated 5 and 7 days after spraying (DAS) in trials with 'Braeburn' and 'Elstar' apples in Sint-Truiden, Belgium.

Treatment[z]	5 DAS									
	Sucrose		Glucose		Fructose		Sorbitol		Total	
					'Braeburn'					
CTR	32.8±2.3	aA[y]	28.7±2.4	NS	3.2±0.5	aA	85.7±5.4	bB	150.6±10.1	aA
HH	32.0±1.6	aA	31.9±1.8		3.1±0.3	aA	98.4±3.5	aB	166.2±6.7	aA
MET	23.4±2.2	bA	28.2±0.9		2.3±0.4	bA	75.8±5.2	cB	131.7±7.6	bA
MET+HH	17.7±1.8	cA	31.4±2.4		3.0±0.6	aA	70.0±3.6	cA	122.0±6.2	bA
					'Elstar'					
CTR	36.6±1.8	aA	39.6±2.4	aA	7.8±0.5	aA	100.7±2.8	aA	184.7±4.6	aA
HH	34.0±1.5	aA	40.9±2.3	aA	6.8±0.7	aA	99.6±3.3	aA	181.2±6.1	aA
MET	30.2±2.5	bA	39.8±2.6	aA	6.4±0.5	aA	82.6±3.4	bA	159.0±8.2	bA
MET+HH	22.6±1.2	cA	39.4±1.9	aA	3.4±0.5	bA	82.2±3.6	bA	147.6±4.8	bA
	7 DAS									
	Sucrose		Glucose		Fructose		Sorbitol		Total	
					'Braeburn'					
CTR	15.7±0.6	abB	30.9±1.3	NS	2.4±0.2	aA	98.0±2.1	bA	147.1±3.4	bA
HH	19.7±2.3	aB	34.2±2.5		2.6±0.7	aA	122.5±6.5	aA	179.0±9.3	aA
MET	13.1±0.8	bB	28.6±2.8		1.9±0.4	bB	86.0±5.0	cA	129.7±6.7	cA
MET+HH	10.6±1.6	bB	28.0±3.5		1.6±0.4	bB	77.3±7.3	cA	117.5±11.8	cA
					'Elstar'					
CTR	29.4±4.1	aA	39.4±2.4	aA	9.3±1.9	aA	102.6±3.2	aA	191.0±4.9	aA
HH	31.0±1.2	aA	38.3±2.2	aA	6.1±0.4	abA	98.4±3.7	aA	173.9±7.0	bA
MET	25.9±1.1	aA	34.1±0.8	aA	5.2±0.5	bA	88.3±4.0	bA	153.5±5.2	cA
MET+HH	16.5±2.9	bB	26.7±4.1	bB	4.1±0.6	bA	64.9±10.2	cB	112.3±17.6	dB

[z] Treatments were control (CTR), increased relative humidity for 3 h prior spraying (HH), metamitron applied at 247.5 mg/L (MET), and their combination (MET + HH).

[y] For each parameter, the mean values ± SE (n = 6) followed by different letters indicate significant differences between treatments within each day (a, b, and c), or between days within each treatment (A, B), using Tukey's HSD test at $\alpha \leq 0.05$. NS indicates no significant difference among values.

Table 2.3 - The sucrose, glucose, fructose, sorbitol, and total sugar concentration in the leaf (mg g^{-1} DW) evaluated 2, 5, and 10 days after spraying (DAS) in the trials with 'Golden Reinders' apple in Lleida and Girona, Spain.

Treatment[z]	2 DAS									
	Sucrose		Glucose		Fructose		Sorbitol		Total	
Lleida										
CTR	19.0±1.3	aB[y]	27.5±1.2	aA	7.2±0.5	bA	62.1±3.7	aB	122.3±2.5	aA
HH	21.5±1.4	aB	26.6±1.4	aA	8.0±0.7	bA	66.2±2.3	aB	115.8±3.9	abA
MET	14.8±1.6	bA	25.2±1.4	aA	9.8±1.0	aA	56.3±3.6	aB	106.1±3.7	bB
MET+HH	15.2±2.6	bA	25.0±1.3	aA	8.7±0.8	abA	60.4±3.1	aB	110.5±2.6	bB
Girona										
CTR	16.6±0.9	aA	21.7±1.0	aA	7.2±0.4	bA	53.8±2.0		99.3±2.1	aA
HH	13.7±1.0	aA	20.6±1.2	aA	7.2±0.4	bA	60.0±2.7		101.6±2.5	aA
MET	9.7±0.7	bA	19.7±0.7	aA	9.2±0.6	aA	55.1±1.2		93.8±1.7	bA
MET+HH	10.0±1.0	bA	18.1±0.8	aA	10.3±1.0	aA	60.9±1.8		99.2±2.0	aA
	5 DAS									
	Sucrose		Glucose		Fructose		Sorbitol		Total	
Lleida										
CTR	29.3±1.9	aA	21.8±0.5	abB	6.2±0.6	bA	76.3±3.2	aA	129.7±5.8	aA
HH	31.7±2.2	aA	23.8±0.9	aA	5.9±0.2	bB	75.2±3.7	aA	133.2±5.8	aA
MET	16.5±2.5	bA	19.3±0.9	bB	9.6±1.2	aA	68.2±6.6	bA	120.7±4.7	abA
MET+HH	15.3±1.8	bA	19.2±0.7	bB	8.7±0.8	aA	62.6±3.6	bB	105.8±4.5	bB
Girona										
CTR	13.4±0.9	aA	24.3±0.6	aA	7.2±0.4	bA	58.6±1.9		103.7±2.5	aA
HH	13.5±0.6	aA	21.6±0.7	abA	5.9±0.4	bA	55.3±2.0		96.3±1.9	abA
MET	8.8±1.0	bA	19.8±1.0	bA	8.4±0.7	aA	56.7±2.7		93.6±3.2	bA
MET+HH	9.4±0.8	bA	19.5±0.9	bA	7.1±0.8	bB	54.4±2.5		88.7±1.9	bB
	10 DAS									
	Sucrose		Glucose		Fructose		Sorbitol		Total	
Lleida										
CTR	16.8±0.7	aB	23.0±1.1	aB	6.6±0.6	aA	80.7±2.9	aA	126.0±2.0	aA
HH	17.5±0.8	aB	24.0±0.9	aA	7.3±0.4	aA	81.1±3.2	aA	132.0±2.7	aA
MET	15.8±0.8	aA	24.3±1.0	aA	7.5±0.5	aB	76.6±2.8	aA	124.1±3.0	aA
MET+HH	16.8±0.9	aA	24.2±0.9	aA	6.8±0.5	aB	78.6±3.1	aA	126.3±3.0	aA

[z] Treatments were Control (CTR), increased relative humidity for 3 h prior spraying (HH), metamitron applied at 247.5 mg/L (MET), and their combination (MET+HH). [y] For each parameter, the mean values ± SE (n = 6) followed by different letters indicate significant differences between treatments within each day (a and b), or between days within each treatment (A, B), using Tukey's HSD test at $\alpha \leq 0.05$. NS indicates no significant difference among values.

2.3.5 Shoot Growth and Yield Parameters

The increase of relative humidity (HH) did not alter the daily shoot growth rate; however, both MET and MET+HH treatments induced significant increases of 40% in 'Golden Reinders' at

Lleida (both treatments), and 120% in MET, and 96% in MET+HH in Girona compared to the CTR (Figure 2.4).

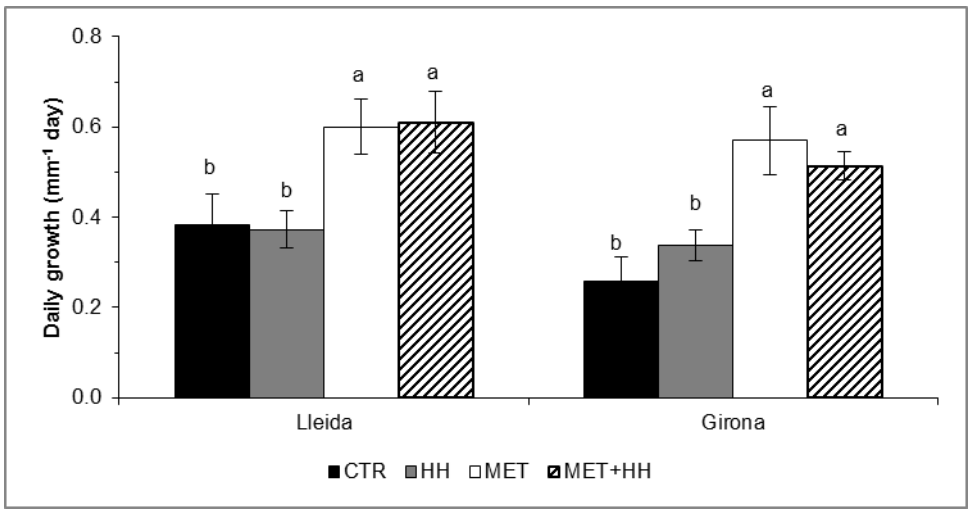

Figure 2.4 - The daily shoot growth rate (mm^{-1} day) evaluated 10 days after spraying (DAS), in the trials with 'Golden Reinders' apple at Lleida and Girona, Spain. Mean values ± SE (n = 32) followed by different letters express significant differences between treatments within each day using Tukey's HSD test ($\alpha \leq 0.05$). Control (CTR), increased relative humidity for 3 h prior spraying (HH), metamitron 247.5 mg/L (MET), and their combination (MET+HH).

The HH conditions did not significantly affect the yield parameters, regardless of the year and cultivar (Table 2.4). In contrast, MET significantly decreased the number of fruits and the yield and, consequently, improved the fruit quality in all the parameters analyzed in Sint-Truiden in 'Braeburn' and 'Elstar', while in Girona and Lleida, in 'Golden Reinders', the same tendency was observed although not significantly (except for an increase in the percentage of fruits in the fruit size class >70 mm, in Girona). MET strongly reduced the number of fruits per 100 flower clusters, 60% and 51%, in 'Braeburn' and 'Elstar', respectively, with a consequent improvement in the percentage of fruits larger than 70 mm of 20% in both cultivars.

In 'Golden Reinders', the tree response to metamitron was lower, resulting in smaller reductions in the number of fruits per 100 flower clusters, ranging between 10% to 20% in both locations. The outcome in the fruit size was significant, resulting in 39% and 25% more fruits larger than 70 mm, in Girona and Lleida, respectively. In Sint-Truiden, MET+HH promoted an even greater reduction of the fruit yield per tree and a greater fruit quality improvement. The latter contributed a further decrease in the number of fruits per 100 flower clusters of 31% and 47% in 'Braeburn' and 'Elstar', respectively, compared with MET alone. Consequently, a strong yield reduction and quality increment was observed in the other parameters. In the trials conducted in Lleida and Girona, there were no differences observed between MET applied alone and the combined exposure (MET+HH). Although the variation patterns were quite similar to those found in Sint-

Truiden, in most cases, there were no significant differences between all treatments and their respective CTR.

Table 2.4 - The number of fruits per 100 flower clusters, fruit weight (g), yield per tree (kg), and fruits in the fruit size class >70 mm (%) at harvest, in trials with 'Braeburn' and 'Elstar' apples performed in Sint-Truiden, Belgium, and with 'Golden Reinders' in Lleida and Girona, Spain.

Location	Cultivar Year	Treatment [z]	Fruits/100 flower clusters		Fruit weight (g)		Yield/tree (kg)		% fruits > 70 mm	
Sint-Truiden	'Braeburn' 2018	CTR	62.0±5.4	a[y]	166.0±4.4	b	14.7±0.9	a	75.9±3.3	b
		HH	58.0±5.5	a	170.1±4.5	b	14.4±1.3	a	79.9±4.6	b
		MET	27.1±5.7	b	214.8±7.8	a	7.4±0.9	b	94.4±4.6	a
		MET+HH	20.6±4.4	b	241.8±5.4	a	7.8±1.3	b	98.9±1.1	a
	'Elstar' 2018	CTR	104.3±12.2	a	121.0±6.5	b	9.8±0.6	a	67.9±9.1	b
		HH	101.0±14.2	a	125.3±6.7	b	8.8±1.2	a	60.3±5.4	b
		MET	62.4±12.3	ab	151.0±5.1	a	6.9±1.0	ab	83.9±2.6	a
		MET+HH	42.5±4.2	b	157.1±4.5	a	4.7±0.6	b	87.7±3.0	a
Girona	'Golden Reinders' 2019	CTR	141.6±8.5	NS	126.7±5.0	NS	48.2±3.5	NS	22.6±6.3	b
		HH	143.2±7.9		129.4±4.8		41.1±4.1		28.4±4.5	b
		MET	126.7±9.0		141.6±8.5		38.1±4.0		43.0±2.6	a
		MET+HH	129.4±4.8		143.2±2.9		34.7±2.6		42.6±3.4	a
Lleida	'Golden Reinders' 2019	CTR	137.8±6.9	NS	113.1±4.7	NS	45.1±3.5	NS	44.1±7.3	
		HH	127.0±9.5		118.8±3.2		45.2±4.1		46.2±3.4	
		MET	106.4±6.2		129.4±2.8		39.1±0.8		61.3±2.0	
		MET+HH	104.0±14.6		127.6±4.9		38.1±3.3		58.3±4.0	

[z] Treatments were Control (CTR), increased relative humidity for 3 h prior spraying (HH), metamitron applied at 247.5 mg/L (MET), and their combination (MET+HH). [y] For each parameter, the mean values ± SE (n = 8) followed by different letters indicate significant differences between treatments using Tukey's HSD test at $\alpha \leq 0.05$. NS indicates no significant difference among values.

2.4 Discussion

2.4.1 Metamitron Absorption

There are many factors affecting leaf chemical uptake dynamics, with relative humidity being one of them (Robinson et al., 2013; Lakso and Robinson, 2015; New England – Tree Fruit Management Guide, 2020; Orbovic et al., 2001). High relative humidity conditions can affect the leaf morphological structure, reducing the thickness of the parenchyma and both the adaxial and abaxial epidermis (Nemeskéry et al., 2009; Locatelli et al., 2019), leaving a softer and thinner (less waxy and more permeable) cuticle, which is more prone to chemical absorption. Previous observations (Orbovic et al., 2001) confirmed these aspects as a 50% increase of urea leaf absorption was observed after a relative humidity increase from 35% to 50%. In addition,

the relative humidity influences the number of stomata in the leaf and its state, and drier conditions result in an increase in the stomatal number and closure (Schulze et al., 1972; Nemeskéry et al., 2009).

High relative humidity conditions allow a liquid layer to remain longer on the leaf surface, promoting a chemical in solution to be absorbed over a longer period of time by increasing the drying period (Byers, 2002). This was verified by Orbovic et al. (2001) who observed the longer persistence of a wet layer of urea solution for 24 h on grapefruit leaves when the relative humidity levels were 80–100%. Leaves developed under more humid and cold climates, such as Sint-Truiden, Belgium, by comparison with Lleida and Girona, Spain, may have different morpho-structural characteristics, such as those described above, which allow for a greater absorption, justifying the increase of absorption in this location.

These characteristics may also explain the higher concentration of metamitron in leaves from the combined treatment (MET+HH) observed in both cultivars in Sint-Truiden, but not in Lleida and Girona. The temperature increase that occurred only in Sint-Truiden (Figure 2.1) may have also influenced absorption. An increase in urea absorption was previously verified when the temperature was 28 °C compared to 19 °C (Orbovic et al., 2001). In Sint-Truiden ('Braeburn' and 'Elstar'), an increase in temperature occurred during the HH period, from 16 to 23 °C, which may additionally explain the contrast of no extra absorption caused by HH at Lleida and Girona. This may also be related to 'Golden Reinders' leaf characteristics, which may be less susceptible to morphological changes under different relative humidity conditions, producing more metamitron degradation.

2.4.2 Leaf Net Photosynthesis

Metamitron significantly reduced the P_n by 50–60% (5 DAS); a greater reduction than the 19% decrease observed by Brunner (2014) using 'Golden Delicious' apple with a similar 247.5 mg/L application, or compared with the 30% reduction by 3 DAS of a 350 mg/L dose in 'Fuji Suprema' apple trees (Gabardo et al., 2017). In the latter study, a full P_n recovery was observed by 7 DAS, whereas our data by that time still showed P_n values one third lower in MET than in the CTR. The combined treatment (MET+HH) resulted in a tendency for lower P_n rates in 'Braeburn' and significantly lower P_n in 'Elstar' at 5 and 7 DAS, which is in line with the greater absorption of metamitron in the MET+HH treatment (evaluated at 2 DAS) (Figure 2.2) as compared with the single MET application in these cultivars.

2.4.3 Leaf Soluble Sugars

Photosynthesis is active primarily in mature leaf mesophyll cells, and sucrose - the primary photosynthate - is transported to meristems and sink organs. Sugar sensing and signalling are involved in the control of growth and development during the entire plant cycle, ensuring the optimal use of carbon and an appropriate response of the metabolism to specific situations, such as the carbohydrate shortage caused by photoinhibiting thinners and, at the limit, triggering fruit abscission (Jones et al., 2000;Lakso and Robinson, 2015).

The strong P_n reduction after metamitron application would have limited the photoassimilate production in accordance with the lower sucrose and sorbitol content found for the MET and MET+HH treatments, especially at 5 DAS (Tables 2.2 and 2.3). By interrupting the thylakoid electron transfer (Abbaspoor et al., 2006), metamitron impairs ATP and $NADPH_2$ and, therefore, CO_2 fixation. In this case, a shortage in the soluble sugar production can be expected, as reported by (Stander et al., 2018) after applying 75, 150, and 300 mg/L metamitron in mandarin. A reduction in the total sugars was found at 1 DAS, which persisted until 7 DAS with the 300 mg/L application, leading to a 25% reduction in the number of fruits. In our study, we saw the strongest reduction in sucrose and sorbitol, at 5 days after a single application of MET, resulting in a sucrose decrease of 27%, 28% and 44%, in 'Braeburn', 'Elstar', and 'Golden Reinders' and a sorbitol decrease of 12% and 18% in 'Braeburn' and 'Elstar'.

In the cultivars 'Braeburn' and 'Elstar', the minimum values of sucrose and sorbitol were observed between 5 and 7 DAS after application of MET prior to a 3 h period of high relative humidity (MET+HH). This treatment, resulted in 25% less sucrose content than MET alone in both cultivars, at 5 DAS, and 36% less sucrose content than MET on 'Elstar', at 7 DAS. The differences between the content of sorbitol in MET and MET+HH were only significant in 'Elstar' at 7DAS, with the latter treatment showing 27% sorbitol than MET alone. These results in sugar shortage are in line with the absorption increase and consequent stronger P_n inhibition in MET + HH.

2.4.4 Shoot Growth and Thinning Efficacy

In the early phase of fruit development, there is a strong sink competition for carbohydrates between the vegetative parts of the tree (shoots) and the small fruitlets. Under a situation of strong vegetative growth, a negative carbohydrate balance and induced fruit abscission is more likely (Forshey and Elfving, 1989). Under limited sugar availability, during the first 40 DAFB, shoot growth has priority over fruit growth for carbohydrate partitioning (Lakso et al., 2001; Forshey and Elfving, 1989; Bepete and Lakso, 1998); therefore, this response has a cost, and may contribute to a negative CH balance that can enhance fruit abscission.

In the present study, the shoot growth increased after metamitron application, that is, in a soluble sugar limiting context, confirmed by the general sucrose and sorbitol reduction (Tables 2.2 and 2.3), although starch remobilization from the tree structures into soluble sugars might have compensated in some extent for the decline of photosynthate production (MacNeill et al., 2017; Dong and Beckles, 2019; Breen et al., 2020).

The shoot growth could be interpreted as a tree response to MET application by producing more fully-functioning leaves capable of restoring the tree photosynthetic capacity, which would have aggravated the competition with the developing fruits, and contributed to the reduction of the number of fruits per 100 flower clusters in all cultivars in the MET treatment. This was stronger in 'Braeburn' and 'Elstar', which was likely linked to higher metamitron absorption (Figure 2.2). This greater effect on fruit numbers in these cultivars promoted a significant

improvement in the fruit quality (fruit weight and percentage of fruits in the fruit size class >70 mm) while reducing the global yield per tree to about half and 2/3, respectively.

These findings were in line with those reporting a decrease in the number of fruits per tree to about half, and a rise in the average fruit weight and size, accompanied with a 2/3 reduction in yield in the cultivar 'Fred Hough' apple after spraying with 384 mg/L metamitron (Petri et al., 2016), and with a 11% and 39% decrease in 'Golden Reinders' after a 247.5 mg/L application at 12 mm (Brunner, 2014), in both cases with a reduction in yield.

When the metamitron application was performed after a 3 h exposure to high relative humidity conditions (MET + HH) in the present study, the cultivars responded in a different way. In 'Braeburn' and 'Elstar', this treatment promoted 24% and 32% less fruit per 100 flower clusters with consequent improvements in the fruit weight and fruit size, whereas in 'Golden Reinders', there was no extra thinning response compared to MET sprayed under environmental conditions. As described in Section 2.4.1 above, leaf morphology varies from cultivar to cultivar, resulting in certain cultivars that are more susceptible to increased absorption under high relative humidity than others. In addition, 'Braeburn' and 'Elstar' were also exposed to 1 h of high relative humidity after spraying (average 18.4%, although half of the 3 h average in Girona, 35%) and higher temperatures (7 °C), which might indicate that temperature and relative humidity after spraying have a greater effect on the metamitron efficacy.

The genetic factors, which determine the cultivars that are more prone to thinning, are also expressed as cultivar susceptibility. 'Braeburn' is classified as an easy cultivar to thin, and 'Golden Reinders' is considered more difficult to obtain a thinning response (Washington State University, 2020), which is in line with our results. In brief, the thinning efficacy is a result of many environmental and orchard factors that interact with each other. A tree's age influences the vigour and tree carbohydrate reserves, which can make the difference between a strong thinning efficacy and resistance in response to the application of a thinner. The 'Braeburn' and 'Elstar' trees were 4 years old, consequently with less reserves and less vigour and were likely more susceptible to thinning. On the other side, the 'Golden Reinders' trees were 16 years old, with a larger trunk diameter, meaning that many carbohydrate reserves were stored, positively influencing the carbohydrate balance and, hence, increasing the resilience to environmental changes, such as high relative humidity. Such trees were apparently less sensitive to metamitron, which affects the thinning response.

2.5 Conclusions

Environmental conditions change from region to region, which can be translated as different leaf morphological and structural characteristics with different absorption capacities. In Sint-Truiden particularly with 'Braeburn' apple, there was more absorption than in Girona and Lleida under ambient relative humidity, likely due to a higher relative humidity during leaf development. Absorption was enhanced by short-term high relative humidity conditions. This resulted in

greater P_n inhibition and a strong reduction in the leaf soluble sugar content, primarily for sucrose and sorbitol.

This reduction, together with the tree age, resulted in a large fruit abscission in MET and MET + HH, the latter with a tendency for lower numbers of fruit, a higher fruit weight, size improvements, and with a stronger yield reduction compared with MET alone, an over thinning response. Although with similar tendencies and together with the permanence of shoot growth, less metamitron absorption (and higher degradation) in 'Golden Reinders', along with the older tree age, would explain the smaller impact on fruit thinning and yield reduction, fruit weight, and % of fruits over 70 mm.

This study indicated that relative humidity for at least 3 h prior to metamitron application may enhance metamitron thinning efficacy under specific circumstances, such as greater humidity and colder temperatures that leave a tree more susceptible to thinning and including the cultivar susceptibility and tree age. In addition, this raises the question of how temperature, in combination with high relative humidity (either before or after spraying), can affect the thinning results, which should be further explored.

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CHAPTER 3

Metamitron and shade effects on leaf physiology and thinning efficacy of *Malus × domestica* Borkh.

Shading nets placed in Girona (2017) to reduce irradiance and simulate cloudy days

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3. Metamitron and shade effects on leaf physiology and thinning efficacy of *Malus* × *domestica* Borkh.

Abstract: Thinning strategies, namely shade or photosynthetic inhibitors, rely on the reduction of carbon supply to the fruit below the demand, causing fruit abscission. In order to clarify the subject, seven field trials were carried out in Lleida, Girona, and Sint-Truiden (2017 + 2018), using orchards of 'Golden' and 'Gala' apple trees. At the stage of 9–14-mm fruit diameter, four treatments were implemented: (A) CTR-control, trees under natural environmental conditions; (B) SN–shaded trees, trees above which shading nets reducing 50% of irradiance were installed 24 h after metamitron application date—without application of metamitron—and removed after five days; (C) MET-trees sprayed with 247.5 ppm of metamitron; (D) MET + SN–trees submitted to the combined exposure to metamitron application and shading nets. Low radiation significantly increased metamitron absorption (36–53% in the three locations in 2018) and reduced its degradation. Net photosynthesis and stomatal conductance were strongly reduced in all treatments, with minimum values 2 days after spraying (DAS) and incomplete recovery 10 DAS in MET + SN. All treatments resulted in leaf sucrose and sorbitol decreases, leading to a negative carbon balance. SN and MET + SN promoted the highest thinning efficacy, increasing fruit weight and size, with MET + SN causing over-thinning in some trials. Leaf antioxidant enzymes showed moderate changes in activity increases under MET or MET + SN, accompanied by a rise of glutathione content and a reduction in ascorbate, however without lipid peroxidation. This work shows that environmental conditions, such as cloudy days, must be carefully considered upon metamitron application, since the low irradiance enhances metamitron efficacy and may cause over-thinning.

Keywords: carbohydrate balance; fruit abscission; photosynthesis; reactive oxygen species; RuBisCO; shading

3.1 Introduction

Annual apple (*Malus domestica* Borkh.) production has increased steadily, becoming the third most produced fruit in the world in 2018, with 86 million metric tons (FAO, 2020). In times in which farmers must meet the high quality criteria of the market, thinning is one of the most important management practices to achieve apple quality for fresh consumption and, consequently, economic sustainability. The thinning strategy needs to be adjusted every year, depending on the fruit set and desired crop load. However, the results can strongly differ between years and regions (Lakso et al., 2001; Robinson and Lakso, 2004) in some cases, in an unpredictably manner.

Nowadays, there are several widely used chemical thinning agents, including metamitron. This triazinone herbicide is a systemic xylem-translocated compound, which inhibits photosystem (PS) II by blocking the electron transfer between the primary and secondary quinones, leading

to the closure of the reaction centres and disrupting thylakoid electron transport (Abbaspoor et al., 2006; Guidi and Degl'Innocenti, 2001), which, ultimately, will greatly reduce photosynthetic carbon fixation (Abbaspoor et al., 2006; Maxwell and Johnson, 2000). The use of shading nets is another thinning technique used in several crops and in organic farming, namely in grapes (Domingos et al., 2016) and apples (Zibordi et al., 2009; Peifer et al., 2020). The role of light in apple production was frequently studied (Flore and Lakso, 1989). Studies have shown that by significantly reducing light availability to apple trees through shading, for a certain number of days at a specific post-bloom period, fruit drop will be promoted due to a restriction in carbohydrate availability caused by the limited leaf C-assimilation (Zibordi et al., 2009; Byers et al., 1991; Lakso and Corelli-Grapadelli, 1992; Elsysy et al., 2019). Under light, the photochemical reactions provide reducing power, nicotinamide adenine dinucleotide phosphate (NADPH), and chemical energy molecules, adenosine triphosphate (ATP), both of which are essential for sugar production resulting from the carboxylation capability of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), and Calvin–Benson cycle functioning.

Both metamitron and shading nets can significantly reduce the photosynthetic rate in apple trees, by inhibiting the photosynthetic apparatus (Brunner, 2014), and by reducing the amount of light energy reaching the chloroplast, which will reduce glucose and sorbitol synthesis (Hansen, 1978). This last monosaccharide is necessary to form sucrose, the main form of transport of assimilated carbon within the plant, from source to sink organs. Additionally, sorbitol synthesis could also be affected. This polyol represents the highest percentage of non-structural sugars in apple trees, being as well a primary product of photosynthesis in the Rosaceae family (Escobar-Gutiérrez, 1996). However, unlike sucrose, which is synthesized and utilized by leaves of all ages, sorbitol is synthesized in leaves, but is metabolized only in sink tissues (Loescher et al., 1982).

It is increasingly accepted that thinning efficacy is directly related to carbohydrate balance, leading to the development of several models for thinning estimation (Lakso and Johnson, 1990; Robinson and Lakso, 2011; Robinson et al., 2017). A carbohydrate surplus will lead to a lower fruit drop rate, while a deficit will result in fruit growth decline and stimulation of the abscission zone formation, promoting the efficacy of the thinning compound (Lordan et al., 2019). Meteorological conditions also play a very important role on the tree's carbon balance (Lordan et al., 2019; Robinson, 2016; Lafer, 2010), thus with the potential to change the efficacy of metamitron from no thinning at all to an over thinning effect (Penzel and Kröling, 2020). A study of the interactions between cloudy weather, fruit set, and chemical thinning application methods is therefore relevant to unveil the variability of thinning efficacy.

Several abiotic stressors that impair the photochemical energy use can increase cell oxidative conditions (Bechtold and Field, 2018). Metamitron also reduces the light energy use by blocking PSII function, what might contribute to an over reduction of the photosynthetic apparatus, thus increasing the probability of electron acceptance by molecular oxygen and a greater reactive oxygen species (ROS) production in the chloroplast (Foyer and Noctor, 2000; Noctor et al.,

2002; Fortunato et al., 2010). In fact, plant strategies to cope with abiotic stresses normally involve a wide set of responses, being the control of reactive oxygen species (ROS) one of the most important (Sharma et al., 2012). This involves enzymatic and non-enzymatic antioxidants that direct and indirectly allow the plants to cope with the ROS imbalance, thus avoiding oxidative damage. The ascorbate–glutathione pathway, whose enzymes act directly on ROS, such as H_2O_2, and also regenerate reduced forms of non-enzymatic antioxidants (e.g., ascorbate, glutathione), is a very important way of maintaining the plant oxidative status.

On the other hand, the reduction of irradiance reaching the photosynthetic apparatus by means of shading nets would reduce the probability of oxidative stress occurrence. Therefore, the evaluation of the balance of oxidative stress and their control mechanisms in apple leaves might contribute to improve the understanding regarding the implications of the single or combined use of metamitron and shade treatments. Overall, in this study, we characterize some physiological and biochemical responses of the apple leave/tree to the single and combined use of these thinning agents. It is also our goal to evaluate the effect of low irradiance (cloudy days) in fruit abscission and how it can enhance metamitron thinning efficacy, in order to provide the grower with more detailed information about time intervals with negative carbon balance which, depending on the crop load goal, acts as perfect thinning windows or may cause over-thinning.

3.2 Materials and Methods

3.2.1 Plant Material and Experimental Design

3.2.1.1 Plant Material

Trials were performed in experimental orchards of *Malus x domestica* in Lleida and Girona (Spain) in 2017, and in Lleida, Girona, and Sint-Truiden (Belgium) in 2018. In Lleida, trials were carried out in the experimental orchards of IRTA, Mollerussa, northeast of Spain (41° 61′ 96, 37″ N/0° 87′ 06, 66″ E, 245 m altitude), using ‘Gala Brookfield’ trees, grafted on M.9 NAKB, spaced 4 m × 1.4 m, with a canopy height of 3 m, planted in 2003, with ‘Fuji’ as pollinator. In Girona, trials were carried out in IRTA Más Badia, northeast of Spain (42°03′12. 97″ N/3°03′46. 13″ E, 12 m altitude), using ‘Golden Reinders’ trees, grafted on M9 NAKB, spaced 3.8 m × 1.1 m, with a canopy height of 2.5 m, planted in 2003, with ‘Granny Smith’ as pollinator. In Sint-Truiden, trials were performed on the orchards of PCFruit Research Station–Proefcentrum Fruitteelt vzw, Belgium (50° 45′ 49″ N/05° 09′ 26″ E, 96 m altitude), using ‘Golden Delicious’ trees, grafted on M9, spaced 3.5 m × 1.5 m, with a canopy height of 3 m, planted in 2005, without pollinator.

For biochemical evaluations, the leaves were cleaned with a water-wet tissue before being frozen in liquid N_2. All leaves were then finely powdered with a mortar and pestle in liquid N_2 and kept at −80 °C until analysis.

3.2.1.2 Treatment Implementation

The shade treatment was imposed by using shading nets installed at 4 m high, covering the whole canopy until the ground, on Eastern and Western sides of tree, which reduced the photosynthetic photon flux density (PPFD) by 50%, evaluated by two Watchdog 3670I Silicon pyranometers placed above and under the net and a Testo 1000 Microstation (Spectrum Technologies, Inc., Aurora, USA) (Figure 3.1). The shading nets were placed 24 h after metamitron application, to ensure that absorption was not affected by low radiation and/or temperature, maintained during 5 days, and were removed at the end of the fifth day.

Figure 3.1 - Shading nets installed in Girona, IRTA Más Badia orchards, in 2017.

Spraying of metamitron, the active ingredient of Brevis® (ADAMA, Telaviv, Israel), was carried out always in the early morning with the recommended dose of 247.5 ppm per 1000 L ha^{-1}, using a hand-gun sprayer. The moment of single application was determined by average fruit diameter: between 9–10 or 13–14 mm in 2017 (in two distinct trials) and around 14 mm in 2018.

Four treatments were established: (A) CTR-control, corresponding to trees under natural environmental conditions; (B) SN–shaded trees, trees above which shading nets were installed 24 h after metamitron application date—without application of metamitron—and removed after five days; (C) MET-trees sprayed with 247.5 ppm of metamitron, applied as referred above; (D) MET + SN–trees submitted to the combined exposure to metamitron application (MET) and shading nets placement during 5 days after metamitron application (SN). Metamitron and/or shade treatments were implemented between the 18th of April and the 18th of May.

To monitor the environmental conditions in each trial, temperature and relative humidity sensors placed in both sides of the blocks and in the middle (with and without shading net), in each case, in the upper (2 m) and lower (1 m) level of the trees. In Lleida, six Testo 177-h1 (Testo, Titisee-Neustadt, Germany) were used; in Girona, six EasyLog USB Data Logger (Lascar Electronics, Wiltshire, England) were used; and six Testo 174H sensors (Spectrum Technologies, Inc., Aurora, USA) were used in Sint-Truiden.

The initial number of flower clusters was similar among treatments (data not shown). Four blocks were established along two rows of the orchard, each 150-m long, in a randomized

complete block design, with several trees between them with no treatments assigned and no observations done. The blocks were interspersed within the two rows, assuring that there were no blocks for observation immediately on the side row, avoiding the edge effect. Each block was constituted by four trees per treatment, but only the 2 central ones were used, performing a total of 8 observed trees per treatment, except in Girona 2017, where 4 trees per treatment was used.

3.2.2 Metamitron Leaf Analysis

In 2017, leaf samples for metamitron and desamino-metamitron concentration were collected 2, 4, 6, and 9 days after spraying (DAS) in leaves of the 9–10 mm fruit diameter trial in Lleida, whereas in 2018, the samples were taken 2 DAS in all locations. Each sample was a pool of three shoot leaves from the top, middle, and bottom part of each tree, with three samples being taken from the Eastern and three from Western side of the canopy, for a total of six repetitions per treatment.

Metamitron extraction was conducted according to the QuEChERS method (Lesueur et al., 2008) using 500 mg fresh weight (FW) of frozen leaf powder and 3 mL of acetonitrile. The samples were shaken manually for 1 min, after which, 1.95 g of extraction Supel™ QuE Citrate Extraction Tube (Sigma-Aldrich, St. Louis, USA) was added, containing 1.2 g of magnesium sulfate, 0.3 g of sodium chloride, 0.15 g of sodium citrate dibasic sesquihydrate, and 0.3 g of sodium citrate tribasic dehydrate. The samples were further shaken manually for 1 min and centrifuged (6000× *g*, 5 min, 4 °C). An aliquot of 1.2 mL of the supernatant was transferred to a 2 mL Supel™ QuE Verde clean-up tube (Sigma-Aldrich, St. Louis,, USA), vortexed, and further centrifuged (6000× *g*, 5 min, 4 °C). The obtained supernatant was filtered with a polytetrafluoroethylene (PTFE) 0.45 µm filter, and injected. Standard curves were used for the quantification of metamitron (Sigma-Aldrich, St. Louis,, USA) and desamino-metamitron-desamino (LGC Standards, Middlesex, USA).

3.2.3 Leaf Gas Exchanges

Leaf gas exchanges measurements included net photosynthesis rate (P_n), and stomatal conductance to water vapour (g_s), and were obtained using a portable Infra-Red Gas Analyzer (IRGA) LCi Ultra Compact Photosynthesis System (ADC BioScientific, UK), under ambient conditions of irradiance, temperature (between 17–25 °C), humidity, and CO_2 supply (400 ± 20 ppm), between 10–12 h. In each of the four blocks, two evaluations in the Eastern and two in the Western side of the canopy were performed, in recently fully developed shoot leaves at ca. 1.5 m height, totaling 8 leaves per treatment. In 2017, gas exchanges measurements were taken in Lleida and Girona 2, 4, 6, 9, and 11 DAS, while in 2018, they were taken in Sint-Truiden 2, 5, and 10 DAS.

3.2.4 Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase Activity

For ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO, EC number: 4.1.1.39), one shoot leaf per tree, making a total of four samples per treatment, were sampled 5 DAS in Sint-Truiden, in 2018, and from each leaf, ten 0.5 cm^2 leaf discs (80 mg FW) were cut, immediately frozen in liquid N_2 and stored at −80 ◦C.

3.2.4.1 Extraction

Leaf material was homogenized in a cooled pestle and mortar, along with quartz sand, and 1% (w/v) insoluble polyvinylpyrrolidone, in 1 mL of ice-cold extraction buffer 50 mM Bicine-KOH (pH 8.0), containing 1 mM EDTA, 5% (w/v) polyvinylpyrrolidone, 6% polyethylene glycol (PEG4000), 10 mM DTT, 50 mM β-mercaptoethanol, and 1% (v/v) protease inhibitor cocktail for plant extracts (Sigma Aldrich, Germany). The homogenates were centrifuged (14,000× *g*, 5 min, 4 °C), and the clear supernatant was immediately used for RuBisCO activities evaluation by the incorporation of $^{14}CO_2$ into acid-stable products at 25 °C, following Perchorowicz, et al. (1982).

3.2.4.2 Total Activity Evaluation

The assay medium for enzyme activity determination contained 100 mM Bicine-NaOH pH 8.2, 40 mM $MgCl_2$, 100 mM $NaHCO_3$ and 10 mM $NaH^{14}CO_3$ (7.4 kBq μmol^{-1}). For total activity (V_t), 450 µL of assay medium and 25 µL of extract was incubated for 3 min at 25 °C for carbamylation, after which 25 µL of 0.4 mM RuBP was added. The reaction was allowed for 1 min, after which it was stopped with 200 µL 10 M HCOOH (formic acid). Samples were then kept overnight in an oven at 70 °C until total medium evaporation and the residue rehydrated with 500 µL of ultrapure water and 5 mL of scintillation liquid (Ultima Gold™, Sigma-Aldrich, St. Louis, USA). Radioactivity due to ^{14}C incorporation in the acid-stable products was measured by liquid scintillation counting using a scintillation spectrometer LS 7800 (Beckman Instruments Inc., Indianapolis, USA).

3.2.5 Leaf non-Structural Sugars

In 2017, sampling was performed between 11:30 and 13:00, at 2, 4, 6, and 9 DAS only from the 9–10-mm application in Lleida for starch analysis, while in 2018, that was done at 2, 5, and 10 DAS for soluble sugars in all trials. In 2017, four repetitions per treatment, one per block, in pools of 2 shoot leaves and 2 spur leaves were used. In 2018, the number of repetitions was increased to six.

3.2.5.1 Soluble Sugars

Quantification of sucrose, fructose, glucose, and sorbitol was based on the method described by Ramalho et al. (2013) using 150 mg FW frozen leaf material. The separation of sugars was performed using a Sugarpak1 column (300 × 6.5 mm, Waters) at 90 °C, using H_2O (containing 50 mg EDTA-Ca L^{-1}) as eluent, at a flow rate of 0.5 mL min^{-1} in an HPLC system equipped with

a refractive index detector (Model 2414, Waters, USA). Standard curves of each sugar were used for quantification.

3.2.5.2 Starch

Starch quantification was performed based on a previous report (Stitt et al., 1978) using ca. 150 mg FW frozen leaf material. The glucose derived from starch was enzymatically determined, with readings at 340 nm using a spectrophotometer UV-VIS Helios (Thermo Fisher, Waltham, USA).

3.2.6 Leaf Oxidative Status Evaluation

Sampling was performed 5 DAS in Sint-Truiden, in 2018, between 10–12 h. Each sample was a pool of three shoot leaves (one sample per block, totaling 4 samples per treatment) that was frozen in liquid nitrogen and stored at −80 °C until analysis.

3.2.6.1 Lipoperoxidation and H_2O_2 Content

Sample extraction was performed using 200 mg FW frozen material, homogenized with 2.0 mL of 0.1% trichloroacetic acid (TCA), and centrifuged (12,000× *g*, 15 min, 2 °C). Lipid peroxidation was estimated by measuring malondialdehyde (MDA) content, using the thiobarbituric acid (TBA) method, as described by Demiral and Turkan (2005). After extraction, 4 mL of 20% TCA containing 0.5% TBA was added to a 1 mL aliquot of the supernatant. This mixture was heated (95 °C, 30 min) followed by quick cooling in an ice bath and centrifugation (10,000× *g*, 15 min, 2 °C). The amount of MDA was calculated from the coefficient of absorbance at 532 nm after subtracting the non-specific absorption at 600 nm. The extinction coefficient 155 mM^{-1} cm^{-1} for MDA was used.

Hydrogen peroxide (H_2O_2) content was measured using the method described previously Singh et al. (2006). To a 50 µL aliquot of the supernatant obtained in the extraction, 959 µL of 100 mM phosphate buffer, pH 7.6, and 1 mL of 1 M potassium iodide were added. The absorbance of the supernatant was measured at 390 nm and for quantification, we used a standard curve of hydrogen peroxide (0, 1.1, 2.2, 3.3, 4.4, and 5.5 µg mL^{-1}).

3.2.6.2 Antioxidative Enzyme Assays

For catalase (CAT), guaiacol peroxidase (GPOD), superoxide dismutase (SOD), and glutathione redutase (GR) 200 mg FW frozen material were homogenized in 2 mL of cold 100 mM Tris-hydrochloric acid (HCl) buffer, pH 7.8, containing 3 mM dithiothreitol, 1 mM EDTA, 2% (w/w) insoluble PVPP and centrifuged (12,000× *g*, 20 min, 4 °C). For ascorbate peroxidase (APX) activity determinations, 10 mM of ascorbate was added to the previously described solution. For glutathione peroxidase (GPX) activity determinations, 0.1% (w/v) Triton X-100, 5 mM cysteine, and 0.1 mM Phenylmethanesulfonyl fluoride were added to the solution described for CAT, SOD, GPOD, and GR. The resulting supernatant was used for determination of enzymatic activity (4 replicates were used for each determination). Absorbance was measured

in a Hitachi (U-2000 UV/Vis, Hitachi, Japan) spectrophotometer, at ca. 25 °C. The enzyme activity was expressed as unit g^{-1} FW.

Catalase

CAT activity (EC 1.11.1.6) was evaluated as described earlier by Aebi (1983), with some changes, by following the decrease in absorbance at 240 nm for 2 min in a solution containing 10 mM of H_2O_2 in 50 mM phosphate buffer, pH 7.0. Enzymatic activity was defined as the consumption of 1 µmol H_2O_2 per min and per cm^3 using a coefficient of absorbance of 39.4 mM^{-1} cm^{-1}.

Guaiacol Peroxidase

Guaiacol peroxidase (GPOD) activity (EC 1.11.1.7) was determined following the increase of absorbance at 470 nm, according to a modification of methodology described previously (Gajewska et al., 2006), using a reaction mixture containing 30 mM 2-methoxyphenol (guaiacol) and 4 mM H_2O_2 in 0.2 M sodium acetate buffer, pH 6.0. Enzymatic activity was defined as the consumption of 1 µmol of guaiacol per min and per mL using a coefficient of absorbance for tetraguaiacol of 26.6 mM^{-1} cm^{-1}.

Glutathione Reductase

Glutathione reductase (GR) activity (EC 1.8.1.7) was determined using a modified Shanker et al. (2004) method, measuring the increase in absorbance at 412 nm, using a reaction mixture containing 3 mM 5,5′-dithio-bis(2-nitrobenzoicacid) (DTNB), 2 mM nicotinamide adenine dinucleotide phosphate (NADPH) and 20 mM oxidized glutathione (GSSG) in 100 mM phosphate- ethylenediaminetetraacetic acid (EDTA) buffer, pH 7.6, and 1mM EDTA. Enzymatic activity was defined as the consumption of 1 µmol of GSSG per min and per mL using a coefficient of absorbance of 6.2 mM^{-1} cm^{-1}.

Superoxide Dismutase

Superoxide dismutase (SOD) activity (EC 1.15.1.1) was determined using a modified Rubio et al. (2002) method, following the variation of absorbance at 550 nm, using a reaction mixture with 0.1 mM EDTA, 0.5 mM Xantine and 0.05 mM of ferricytochrome c in 100 mM phosphate buffer, pH 7.6, and 1 U mL^{-1} xantine-oxidase. Enzymatic activity was defined as µmol of ferricytochrome c reduction by superoxide radical min^{-1}.

Ascorbate Peroxidase

Ascorbate peroxidase (APX) activity (EC 1.11.1.11) was determined according to a previous study (Sharma and Dubey, 2004), in a reaction mixture containing 0.25 mM ascorbate and 0.3 mM hydrogen peroxide in 50 mM phosphate buffer, pH 7.0, following the decrease in absorbance at 290 nm. Enzymatic activity was defined as the consumption of 1 µmol ascorbate per min and per mL using a coefficient of absorbance of 2.8 mM^{-1} cm^{-1}.

Glutathione Peroxidase

Glutathione peroxidase (GPX) activity (EC 1.11.1.9) was determined according to Aravind and Prasad (2005), in a reaction mixture containing 1.14 mM sodium chloride, 2 mM reduced glutathione, 2.5 mM hydrogen peroxide, 2 mM NADPH in 50 mM Tris-HCl buffer, pH 7.9. Enzymatic activity was defined as the glutathione-peroxidase necessary to reduce 1 µmol NADPH per min and per mL at room temperature using a coefficient of absorbance of 6.2 mM^{-1} cm^{-1}.

3.2.6.3 Non-Enzyme Antioxidants Quantification

For glutathione and ascorbate evaluations, samples of 100 mg of powdered frozen leaf were homogenized in 0.5 mL of ice-cold 6% meta-phosphoric acid, pH 2.8, containing 1 mM EDTA and 1% activated charcoal powder for chlorophyll removal. Homogenates were centrifuged (27,000× *g*, 15 min, 4 °C), and the obtained supernatant was stored at -80 ◦C prior to glutathione and ascorbate analysis.

Glutathione

The quantification of reduced (GSH) and oxidized (GSSG) glutathione was based on the method described previously by Anderson et al. (1992). Total glutathione was measured spectrophotometrically at 412 nm in a microplate reader Synergy HT (BioTek Instruments, Winooski, USA). Oxidized glutathione (GSSG) was measured by incubating the diluted sample in 0.5% 2-vinylpyridine for 1 h at 25 °C and then proceeding as described above. Reduced glutathione (GSH) was determined as the difference between total glutathione and GSSG.

Ascorbate

The quantification of ascorbic (AsA) and dehydroascorbic (DAsA) acids was based on a method adapted from a previous study (Okamura, 1980), as described earlier (Carvalho and Amâncio, 2002). Absorbance was recorded at 525 nm in a microplate reader Synergy HT (BioTek Instruments, Winooski, USA). Concentration of AsA was determined using a calibration curve of AsA in the range of 10–60 mM prepared in 5% metaphosphoric acid. The concentration of DAsA was calculated by subtracting the AsA concentration measured from the total ascorbate assayed.

3.2.7 Yield Parameters

All fruits were picked from each observed tree at harvest, on one time. The number of fruits per tree, yield, fruit weight, and distribution per fruit size was determined using a commercial sort machine (Maf Roda Agrobotic, Montauban Cedex, France).

3.2.8 Statistical Analysis

The various measured and calculated parameters were subjected to an analysis of variance, through a one-way ANOVA, to evaluate the differences between treatments on one single day after spraying, or a two-way ANOVA to evaluate the differences between the four treatments,

across the several days after spraying, followed by a Tukey's test for mean comparisons. Each ANOVA was performed independently for each of the trials. For metamitron leaf concentration and non-structural sugars data analysis six samples were used and a completely randomized design analysis was performed. A 95% confidence level was adopted for all tests. The statistical analysis was performed using Statistix 9 (Analytical Software, Tallahassee, FL, USA).

3.3 Results

3.3.1 Environmental Conditions

A brief characterization of the environmental conditions in the seven performed trials is shown in Table 3.1. Global irradiance values were quite homogeneous within all trials. The 2018 trial in Girona stands out due to the higher night temperature values, above 14 °C, registered before the application. In all the other trials carried out, the sensors installed inside and outside the shading nets showed no relevant differences neither in humidity nor in temperature (≈5% and 0.5 °C, respectively), meaning that the only different parameter was irradiation, which was reduced by half.

Table 3.1 - Summary of meteorological conditions ± SE in trials performed in each year and location and fruit diameter at the time of metamitron application: average of daily irradiance 5 days after spraying (DAS) (MJ m−2), average night-time temperature from 20:00–8:00 h (°C), 5 nights before and after spraying, and average air relative humidity (%) during the 3 h prior to spraying in natural environmental conditions (Control) and under the shading nets (SN).

Location	Fruit Diameter (mm)	Global Irradiance MJ/m²—5 Days after		Night Temperature °C—5 Nights before	Night Temperature °C—5 Nights after	Diurnal Temperature ◦C—5 Days after	Relative Humidity %
		Control	SN	Control	Control	Control	Control
2017							
Lleida	10 ± 0.4	24.8 ± 2.1	12.4 ± 1.2	11.1 ± 0.4	7.9 ± 0.5	14.3 ± 0.4	71.7 ± 1.1
	13 ± 0.4	17.7 ± 1.9	8.8 ± 0.9	7.5 ± 0.5	8.0 ± 0.6	13.0 ± 0.3	56.0 ± 3.4
Girona	9 ± 0.7	20.7 ± 1.8	10.4 ± 0.9	9.3 ± 0.6	10.4 ± 0.4	13.3 ± 0.4	47.3 ± 3.1
	13 ± 0.2	19.2 ± 1.2	9.6 ± 0.6	10.4 ± 0.4	8.2 ± 0.7	11.0 ± 0.4	46.3 ± 3.0
2018							
Lleida	14 ± 0.3	17.5 ± 1.2	8.8 ± 0.6	10.2 ± 0.2	11.8 ± 0.5	12.7 ± 0.5	61.5 ± 2.1
Girona	14 ± 0.3	19.3 ± 1.3	9.7 ± 0.6	15.5 ± 0.7	11.8 ± 0.4	14.6 ± 0.3	69.1 ± 1.9
Sint-Truiden	14 ± 0.4	22.1 ± 1.4	11.1 ± 0.7	11.5 ± 0.2	11.6 ± 0.3	14.3 ± 0.6	60.5 ± 1.7

3.3.2 Metamitron Absorption and Degradation to Desamino-Metamitron

To evaluate the metamitron impacts it is crucial to determine its absorption by the leaves and its permanence/degradation along time. For the applied dose, MET treatment reached 2 mg g^{-1} dry weight (DW) in leaf biomass of metamitron 2 DAS, but the combined MET + SN treatment showed a significantly higher content of 1/3 considering an average of the three locations (Figure 3.2).

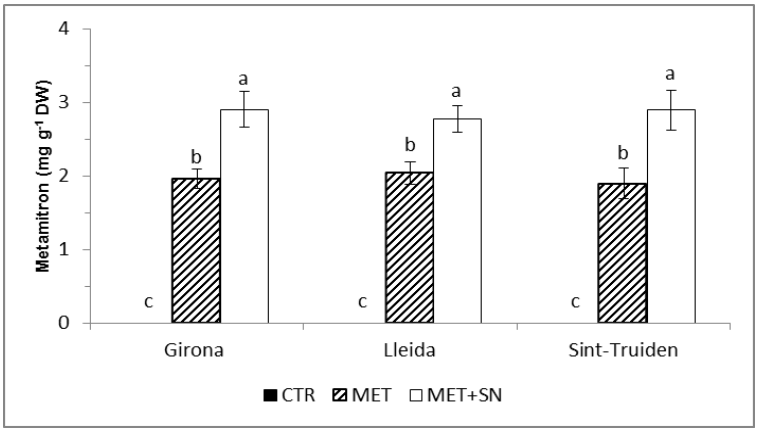

Figure 3.2 - Metamitron content (mg g^{-1} DW), evaluated 2 DAS, in the trials of 2018 in Girona ('Golden Reinders'), Lleida ('Gala Brookfield') and Sint-Truiden (Golden Delicious'). For each parameter, the mean values ± SE (n = 6) followed by different letters express significant differences between treatments within each cultivar after a Tukey's HSD test (p-value ≤ 0.05). SN – Shading net; MET – Metamitron.

The pattern of variation of MET and MET + SN was similar during the whole experiment for desamino-metamitron, the main degradation product of metamitron (Figure 3.3). However, for MET in a higher extent (usually more than double) along this period. Desamino-metamitron highest values were observed at 6 DAS, decreasing afterwards in both treatments.

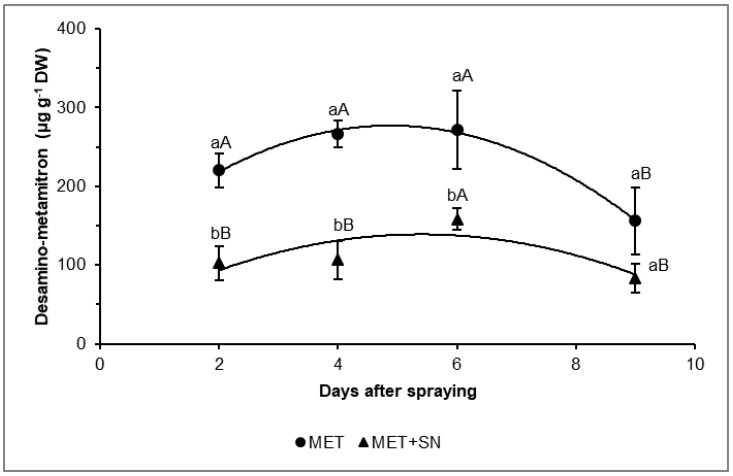

Figure 3.3 - Desamino-metamitron content evaluated 2, 4, 6, and 9 DAS, for metamitron with (MET + SN-▲) or without (MET-●) shading nets, in the trials of 2017, in Lleida ('Gala Brookfield'). For each parameter, the mean values ± SE (n = 8) followed by different letters express significant differences between treatments within each day (a and b), or between days within each treatment (A and B), after a Tukey's HSD test (p-value ≤ 0.05). SN – Shading net; MET – Metamitron, DAS – Days after spraying.

3.3.3 Leaf Gas Exchanges

P_n and g_s were similar among the treatments applied at 9–10 mm and 13 mm, allowing combining the data from those trials for each site (Figure 3.4). The recovery period was evaluated 9 DAS in the 9–10 mm fruit diameter metamitron application and 11 DAS in the 13 mm, and the analysis made separately (data not shown).

Overall, the treatments of metamitron and/or shade imposed a reduction on P_n and g_s until 6 DAS in both locations. In detail, SN significantly reduced P_n and g_s in Lleida (50% and 55%) (Figure 4 a and 4b) and Girona (24% and 53%) (Figure 4c and 4d), respectively, 2 DAS. Lowered P_n and g_s values were mostly maintained 4 DAS, but a strong increase to values close to their controls was observed 6 DAS, that is, just one day after shade removal.

MET significantly reduced P_n at 2 and 4 DAS, 35 and 42%, and 39 and 80%, in Lleida and Girona, respectively, as compared to their control values on the same days. Moreover, although in Lleida the P_n values in the MET treatment were invariant until 6 DAS, in Girona, minimum P_n values were observed 4 DAS with a tendency to recovery afterwards. MET further caused g_s reductions in both sites, with maximal declines at 4 DAS of 42 and 40% of their respective controls.

The MET + SN treatment caused the most significant reduction in P_n and g_s 2 and 4 DAS, to half of CTR values, or even to a greater extent. Furthermore, 2 DAS, the values were usually lower than those of MET. Although a tendency to recover was observed after shade removal, in MET + SN treatment, P_n and g_s did not differ from those from MET alone, showing that 6 DAS

metamitron was leading the impact of MET + SN treatment. Evaluation of the leaf gas exchanges for longer periods in the Lleida experiments showed that at 9 DAS MET plants still represented 30% (P_n) and 25% (g_s) lower values than CTR, but 11 DAS differences between treatment become absent (data not shown). Measurements performed in Sint-Truiden in 2018 were in line with those of Lleida in 2017, although at 10 DAS (thus, five days after the removal of the shading nets), only the MET + SN maintained significant reduced P_n values (data not shown).

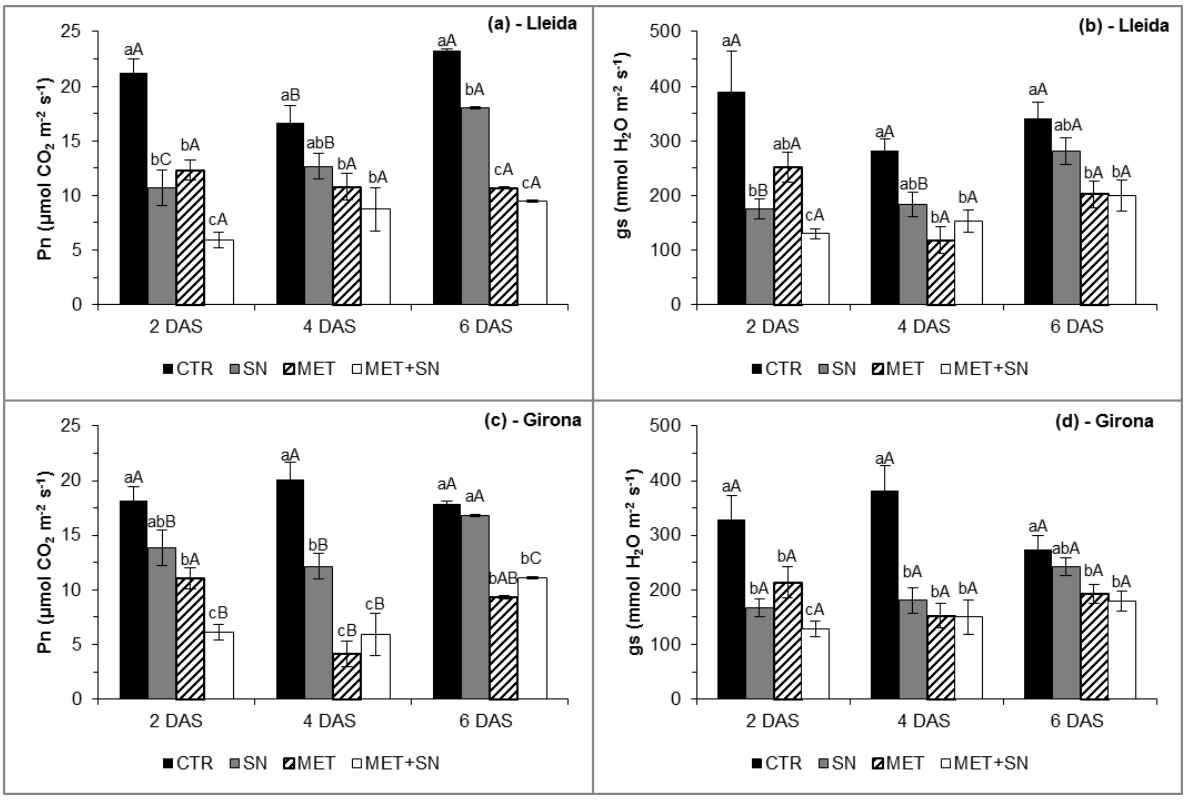

Figure 3.4 - Net CO_2 gas exchange (P_n) (µmol CO_2 m^{-2} s^{-1}) (a and c) and stomatal conductance to water vapor (g_s) (mmol H_2O m^{-2} s^{-1}) rate (b and d), in Lleida and Girona, respectively, evaluated 2, 4, and 6 days after shade (DAS) installation, in the 2017 trials in Lleida ('Gala Brookfield') and Girona ('Golden Renders'). Shading nets were removed 5 DAS. For each parameter, the mean values ± SE (n = 16) followed by different letters express significant differences between treatments within each day (a, b, and c), or between days within each treatment (A, B, and C), after a Tukey's HSD test (p-value ≤ 0.05). SN – Shading net; MET – Metamitron, DAS – Days after spraying.

3.3.4 RuBisCO Activity

Total activity of RuBisCO (V_t) was significantly reduced to less than half as CTR, in MET and MET + SN treatments, without significant differences between them (Table 3.2).

Table 3.2 - RuBisCO total activity (V_t) (µmol CO_2 m^{-2} s^{-1}) evaluated 5 DAS, in the trial of 2018 in Sint-Truiden ('Golden Delicious'). For each parameter, the mean values ± SE (*n* = 4) followed by different letters express significant differences between treatments within each cultivar after a Tukey's HSD test (*p*-value ≤ 0.05). SN – Shading net; MET – Metamitron.

	V_t (µmol CO_2 mg^{-1} Chl)	
CTR	20.7 ± 1.4	a
SN	19.8 ± 0.9	a
MET	8.4 ± 0.3	b
MET + SN	8.3 ± 0.8	b

3.3.5 Leaf Sugars

SN treatment significantly reduced sucrose content in Lleida and Girona at 2 DAS (36 and 53%) and in all locations at 5 DAS (between 47–62%) (Table 3). A similar trend was observed for sorbitol, although less striking than sucrose, significant in Sint-Truiden at 2 DAS (36%) and in all locations at 5 DAS (between 22–34%). Glucose also showed significant a reduction in Lleida, with 21% less content than CTR. At 10 DAS, there were no differences from CTR likely due to the removal of the nets at 5 DAS.

The MET treatment showed only non-significant impacts 2 DAS. However minimum levels were reached at 5 DAS, and sucrose (34–59%), fructose (24–44%), sorbitol (22–24%), and total sugars (21–24%) were frequently significantly reduced, as compared to CTR. At 10 DAS, Lleida trees still presented reduced content of sucrose, whereas Sint-Truiden all evaluated sugars presented values similar to control.

The combined MET + SN treatment showed a consistent tendency to cause greater impact that the single treatments in all days and trials, although minimum values were reached at 5 DAS. By this time, MET + SN treatment promoted significant reductions in sucrose (62–78%), sorbitol (29–42%), and total sugars (30–38%) in the three locations, as compared to the control. In addition, this treatment resulted in 70% less glucose in Lleida and 53 and 64% less fructose content in Lleida and Sint-Truiden, respectively, comparing to the respective controls. At 10 DAS, the trees still presented a reduced content of sucrose of 80% compared to the CTR.

Table 3.3 **-** Main soluble sugars content (mg g^{-1} DW) of apple leaves: sucrose, glucose, fructose, sorbitol, and total sugars at 2, 5, and 10 DAS in Lleida ('Gala Brookfield'), Girona ('Golden Reinders') and Sint-Truiden ('Golden Delicious') in the 2018 trials. Shading net was removed 5 DAS. For each parameter, the mean values ± SE (n = 6) followed by different letters express significant differences between treatments within each day (a, b, and c), or between days within each treatment (A and B), after Tukey's HSD test (p-value ≤ 0.05). No letters indicate a p-value > 0.05. SN – Shading net; MET – Metamitron, DAS – Days after spraying.

	2 DAS									
	Sucrose		**Glucose**		**Frutose**		**Sorbitol**		**Total**	
					Lleida					
CTR	16.6 ± 1.2	aA	31.5 ± 2.8	aA	2.9 ± 0.5		91.0 ± 5.3	aA	141.9 ± 7.9	aA
SN	10.7 ± 1.0	bA	33.2 ± 2.9	aA	2.0 ± 0.5		77.6 ± 4.2	aA	123.5 ± 6.9	aA
MET	12.7 ± 0.5	aA	31.5 ± 2.3	aA	2.3 ± 0.2		68.1 ± 9.9	aB	114.6 ± 13.5	aA
MET+SN	10.9 ± 1.7	bA	32.0 ± 3.8	aA	1.7 ± 0.6		79.8 ± 6.4	aA	124.4 ± 12.1	aA
					Girona					
CTR	19.6 ± 1.9	aA	40.5 ± 4.1	abA	2.3 ± 0.7		88.9 ± 7.8	abA	151.3 ± 12.9	abA
SN	9.3 ± 0.9	bA	37.9 ± 1.5	bA	3.1 ± 0.4		71.3 ± 4.5	bcA	121.6 ± 5.9	bA
MET	14.2 ± 2.0	abA	48.4 ± 4.0	aA	4.8 ± 0.7		101.0±10.1	aA	168.3±15.1	aA
MET+SN	6.8 ± 1.0	cA	35.8 ± 2.1	cA	3.6 ± 0.6		65.3 ± 6.0	cA	111.5 ± 8.4	cA
					Sint-Truiden					
CTR	24.8 ± 3.0	aA	44.7 ± 4.7		6.2 ± 0.6	aA	120.0±10.9	aA	195.8 ± 14.6	aA
SN	21.1 ± 8.6	aA	33.7 ± 5.0		4.4 ± 0.3	aA	77.4 ± 4.6	bAB	136.6 ± 10.5	bA
MET	17.5 ± 1.0	aA	42.6 ± 4.4		6.2 ± 0.8	aA	99.4 ± 6.3	abA	165.7 ± 10.7	abA
MET+SN	11.9 ± 1.1	aA	41.0 ± 2.7		5.5 ± 1.2	aA	78.4 ± 4.4	bAB	136.7 ± 8.5	bB
	5 DAS									
	Sucrose		**Glucose**		**Frutose**		**Sorbitol**		**Total**	
					Lleida					
CTR	12.9 ± 0.9	aAB	36.3 ± 1.6	aA	2.2 ± 0.4		87.4 ± 2.6	aA	138.9 ± 4.9	aA
SN	4.9 ± 0.5	cA	28.6 ± 1.8	aA	1.5 ± 0.1		58.5 ± 2.7	cB	93.5 ± 3.5	cB
MET	6.7 ± 0.5	bB	31.6 ± 1.7	aA	1.8 ± 0.1		69.4 ± 2.5	bB	109.5 ± 2.3	bA
MET+SN	2.9 ± 0.6	dB	10.5 ± 0.6	bB	3.2 ± 0.2		50.9 ± 2.7	cB	67.5 ± 2.6	dB
					Girona					
CTR	15.5 ± 1.4	aA	28.1 ± 2.1	aB	3.1 ± 0.5		70.5 ± 3.7	aA	117.2 ± 6.5	aA
SN	6.1 ± 0.2	bB	25.0 ± 1.8	aB	3.2 ± 0.2		46.6 ± 2.0	bB	78.9 ± 3.0	bB
MET	6.7 ± 1.4	bB	25.5 ± 3.3	aB	2.9 ± 0.8		53.8 ± 5.7	abB	89.0 10.4	abB
MET+SN	5.2 ± 0.5	bA	23.1 ± 1.1	aB	2.2 ± 0.3		42.8 ± 4.5	cB	73.3 5.0	bB
					Sint-Truiden					
CTR	16.6 ± 1.1	aA	39.7 ± 3.1		5.0 ± 1.1	aA	85.4 ± 4.4	aB	146.7 ± 8.7	aA
SN	7.7 ± 1.1	cB	35.2 ± 1.7		3.4 ± 0.5	abA	66.4 ± 4.0	abB	112.1 ± 6.3	abB
MET	11.0 ± 1.5	bB	30.8 ± 1.1		3.8 ± 0.7	abA	66.9 ± 3.9	abB	112.5 ± 5.0	abB
MET+SN	6.4 ± 0.6	cB	35.7 ± 2.6		1.8 ± 0.3	bB	59.9 ± 2.7	bB	102.8 ± 4.8	bA

Table 3.3 – *Cont.*

	10 DAS									
	Sucrose		**Glucose**		**Frutose**		**Sorbitol**		**Total**	
					Lleida					
CTR	10.8 ± 0.8	aB	14.2 ± 2.5	aB	2.5 ± 0.4		89.9 ± 4.8	aA	117.4 ± 7.8	aA
SN	7.9 ± 0.8	bA	11.2 ± 1.5	aB	2.2 ± 0.4		87.0 ± 2.7	aA	108.4 ± 2.8	aAB
MET	2.1 ± 0.3	cAB	15.6 ± 1.0	aB	3.7 ± 0.7		80.9 ± 4.0	aA	101.4 ± 4.3	aA
MET+SN	5.0 ± 0.8	bB	12.7 ± 1.3	aA	3.4 ± 0.6		75.1 ± 5.4	aA	95.3 ± 6.6	aAB
					Sint-Truiden					
CTR	15.3 ± 1.8	aA	45.9 ± 2.2		3.4 ± 0.6	aA	102.6 ± 4.0	aAB	167.1 ± 7.3	aA
SN	11.2 ± 0.8	aAB	32.5 ± 1.8		3.2 ± 0.2	aA	93.8 ± 3.4	bB	140.7 ± 4.9	bA
MET	12.5 ± 0.6	aAB	37.5 ± 2.5		3.0 ± 0.5	aA	103.6 ± 6.7	aAB	156.6 ± 9.0	aA
MET+SN	12.4 ± 1.0	aA	35.4 ± 3.0		3.5 ± 0.2	aAB	98.8 ± 4.0	aA	150.0 ± 7.5	abA

Regarding the insoluble sugar starch, all treatments promoted content reduction up to 6 DAS (significantly until 4 DAS). Maximal declines (greater than 70%) were observed at 4 DAS, as compared to the respective control value (Table 3.4). The starch content was similar among the treatments at 9 DAS, although related to a strong starch reduction in CTR plants, as compared with the previous days.

Table 3.4 - Leaf starch content (mg g^{-1} DW) at 2, 4, 6, and 9 DAS (9–10-mm application trial) in the trial of 2017, in Lleida ('Gala Brookfield'). Shading net was removed 5 DAS. For each parameter, the mean values ± SE (n = 4) followed by different letters express significant differences between treatments within each day (a and b), or between days within each treatment (A and B), after Tukey's HSD test (p-value ≤ 0.05). No letters indicate a p-value > 0.05. SN – Shading net; MET – Metamitron, DAS – Days after spraying.

	Starch (mg g^{-1} DW)							
	2 DAS		**4 DAS**		**6 DAS**		**9 DAS**	
CTR	10.5 ± 1.7	aA	8.6 ± 2.1	aA	11.0 ± 4.2	aA	3.4 ± 2.0	aB
SN	3.2 ± 0.7	bB	2.6 ± 0.9	bB	7.3 ± 2.1	aA	3.8 ± 1.3	aB
MET	3.9 ± 0.6	bA	2.1 ± 0.9	bA	4.9 ± 0.9	aA	5.3 ± 1.9	aA
MET + SN	4.8 ± 2.1	bA	2.2 ± 0.5	bA	8.0 ± 3.7	aA	2.6 ± 0.7	aA

3.3.6 Leaf Oxidative Status

3.3.6.1 Lipid Peroxidation

The MDA and H_2O_2 content were not affected regardless of treatment, as compared to CTR values (Figure 3.5a and 3.5b).

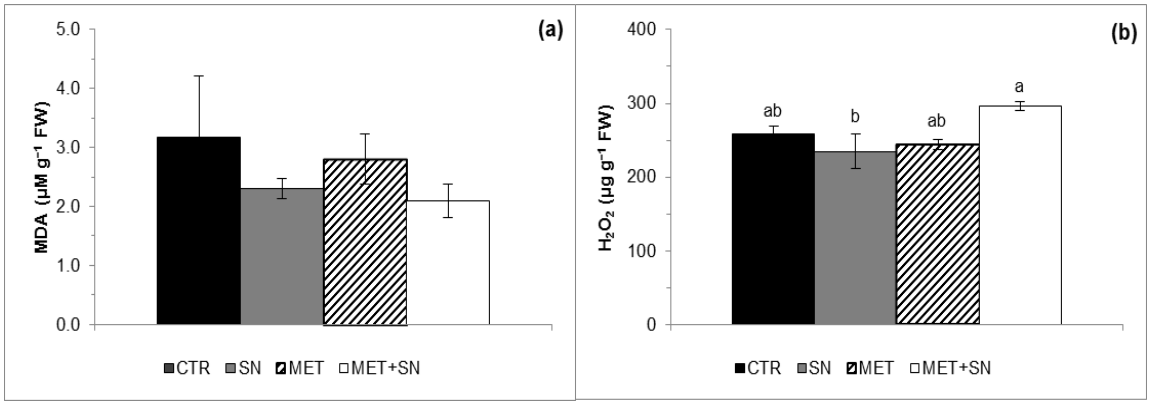

Figure 3.5 **-** Leaf average contents of malondialdehyde (MDA) (µM g^{-1} FW) (a) and hydrogen peroxide (H_2O_2) (µg g^{-1} FW) (b) evaluated 5 DAS, in the trial of 2018 in Sint-Truiden ('Golden Delicious'). For each parameter, the mean values ± SE (*n* = 4) followed by different letters express significant differences between treatments within each cultivar after a Tukey's HSD test (*p*-value ≤ 0.05). No letters indicate *p*-value > 0.05. SN – Shading net; MET – Metamitron.

3.3.6.2 Antioxidative Enzyme Activity

Changes in the activity of the studied antioxidative enzymes were observed 5 DAS, differently among treatments (Figure 3.6). MET promoted greater activity values in APX, whereas MET + SN induced maximal values for CAT, GR, and GPX. By contrast, these two treatments resulted in the lowest activity of SOD. POD activity differed significantly in SN (decreased) and MET (increased). The SN imposition did not significantly affect the activity of any of the studied enzymes.

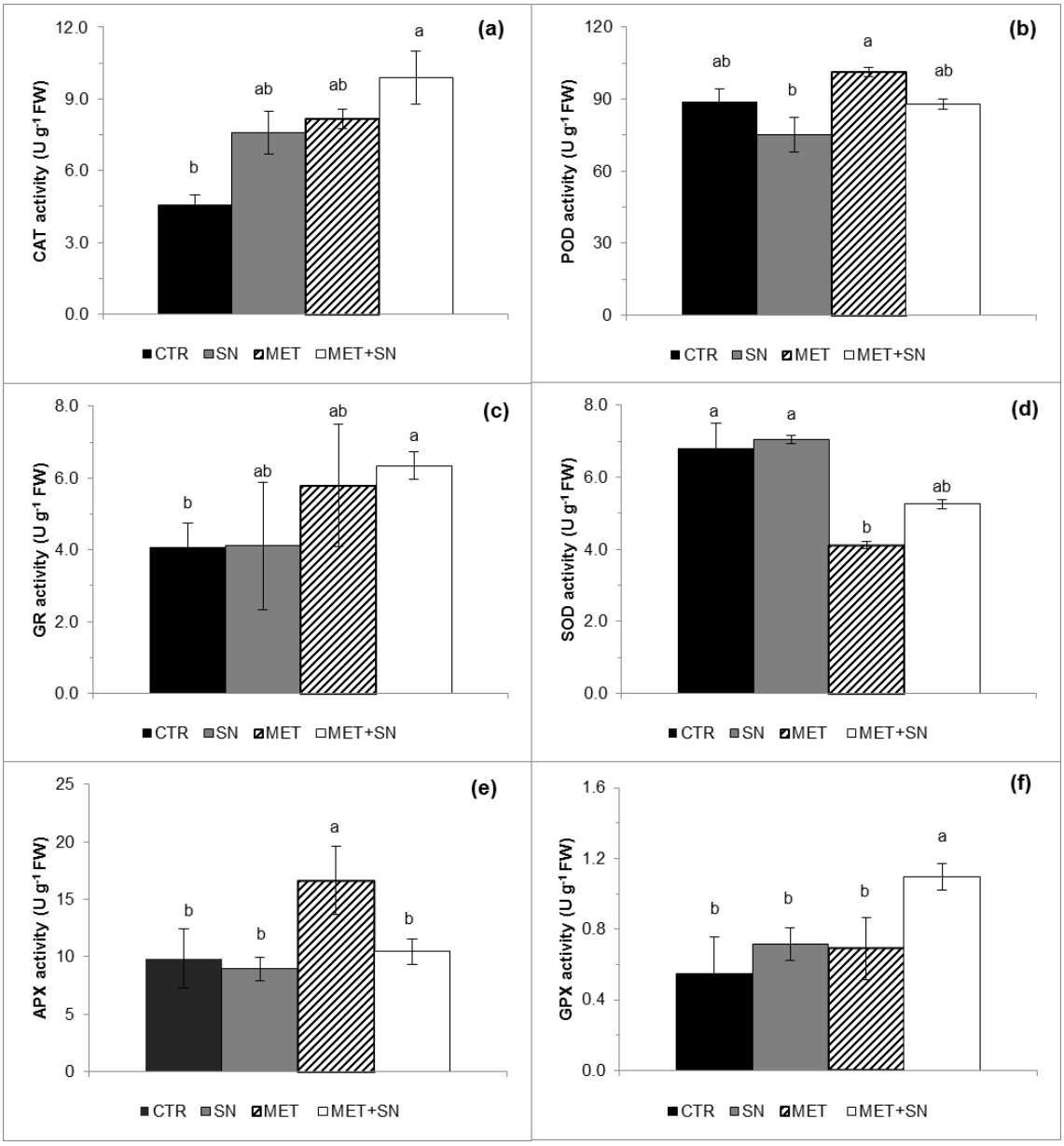

Figure 3.6 - Catalase (CAT) (a), guaiacol peroxidase (POD) (b), glutathione reductase (GR) (c), superoxide dismutase (SOD) (d), ascorbate peroxidase (APX) (e) and glutathione peroxidase (GPX) (f) activities (U g^{-1} FW) evaluated 5 DAS in the trial of 2018, in Sint-Truiden ('Golden Delicious'). For each parameter, the mean values ± SE (n = 4) followed by different letters express significant differences between treatments within each cultivar after a Tukey's HSD test (p-value ≤ 0.05). SN – Shading net; MET – Metamitron.

3.3.6.3 Ascorbate and Glutathione Content

More than 90% of the total glutathione (GSH + GSSG) was in the reduced form (GSH) in all treatments (Figure 3.7a). All treatments promoted the increase of GSH + GSSG and GSH contents, significantly in MET, and, especially, MET + SN with maximal values (3 fold higher than CTR content).

Ascorbate showed a somewhat inverse pattern of that displayed by glutathione. All treatments reduced AsA and AsA + DHA contents, with minimal values observed under SN and MET + SN (Figure 3.7b).

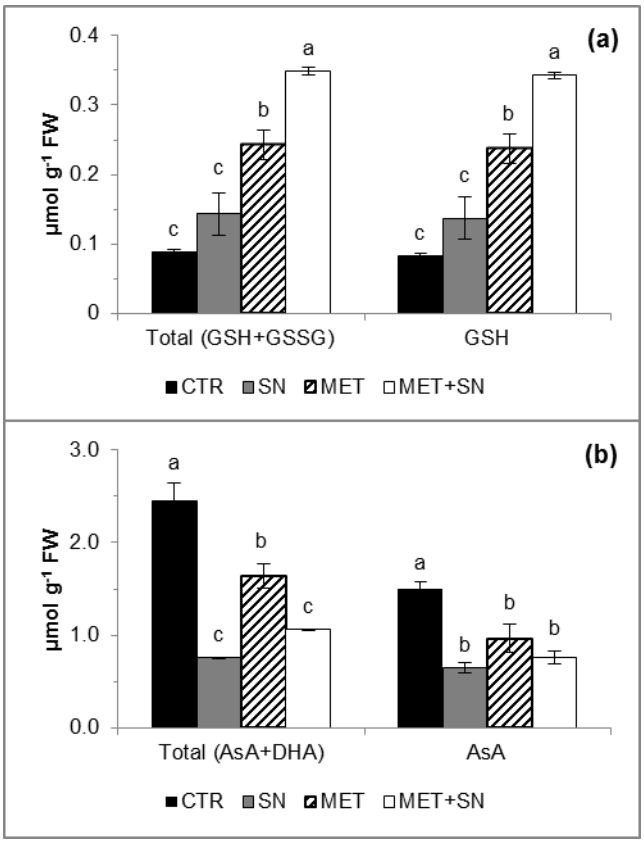

Figure 3.7 **-** Total glutathione (GSH + GSSG) and reduced glutathione (GSH) (a) and total ascorbate (AsA + DHA) and reduced ascorbate (AsA) (b) (µmol g^{-1} FW) evaluated 5 DAS in the trial of 2018, in Sint-Truiden ('Golden Delicious'). For each parameter, the mean values ± SE (*n* = 4) followed by different letters express significant differences between treatments within each cultivar after a Tukey's HSD test (*p*-value ≤ 0.05). SN – Shading net; MET – Metamitron.

3.3.7 Yield Parameters

The MET treatment caused a consistent reduction in the number of fruits per 100/flowers clusters, although always non-significantly, whereas fruit weight and the percentage fruits > 70 mm followed an opposite tendency (the latter significantly only in Girona, 2018 with a 2.7 fold increase (Table 3.5). Despite these changes, yield per tree was barely affected by MET.

The SN treatment induced the exact same pattern as MET, although with strong (significant) variations in fruits per 100/flowers (Girona and Sint-Truiden in 2018), fruit weight (Lleida 2017; Sint-Truiden, 2018), percentage of larger fruits (Lleida, 2017), and a somewhat stronger negative impact on yield (significantly in Sint-Truiden in 2018).

Table 3.5 **-** Number of fruits per 100 flower clusters, fruit weight (g), yield per tree (kg) and percentage of fruits in fruit size class >70 mm at harvest in the trials of 2017, in Lleida ('Gala Brookfield') and Girona ('Golden Reinders') and in the trials of 2018, in Lleida ('Gala Brookfield'), Girona ('Golden Reinders') and Sint-Truiden ('Golden Delicious'). Shading nets were removed 5 DAS. In 2017 ± SE (*n* = 16) represent the average of 9–10 and 13–14 mm trials and in 2018 values ± SE (*n* = 8) represent each trial. Values followed by different letters express significant differences between treatments for each trial independently after a Tukey's HSD test (*p*-value ≤ 0.05). No letters indicate *p*-value > 0.05. SN – Shading net; MET – Metamitron.

		Fruits/100 Flower Clusters		**Average Fruit Weight (g)**		**Yield/Tree (kg)**		**% fruits > 70 mm**	
				2017					
Lleida	CTR	108.0 ± 9.8		135.5 ± 3.5	b	2.0 ± 3.4		40.5 ± 3.0	b
	SN	87.1 ± 6.0		152.9 ± 3.6	a	37.5 ± 2.6		56.0 ± 2.7	a
	MET	97.3 ± 5.0		145.9 ± 4.6	ab	39.9 ± 1.7		50.0 ± 3.5	ab
	MET + SN	84.9 ± 7.0		155.6 ± 4.3	a	36.3 ± 3.3		50.0 ± 4.1	a
Girona	CTR	184.3 ± 13.4		103.8 ± 1.4	b	21.5 ± 0.6		12.8 ± 2.0	b
	SN	173.3 ± 10.0		111.5 ± 4.4	b	19.3 ± 0.9		27.6 ± 4.3	a
	MET	169.0 ± 21.7		116.6 ± 4.2	ab	21.6 ± 0.9		27.4 ± 5.2	a
	MET + SN	163.0 ± 24.3		134.1 ± 7.9	a	20.4 ± 0.8		27.5 ± 7.1	a
		Fruits/100 flower clusters		**Average fruit weight (g)**		**Yield/tree (kg)**		**% fruits > 70 mm**	
				2018					
Lleida	CTR	71.8 ± 11.4		125.8 ± 2.4	b	48.0 ± 6.1		48.3 ± 8.4	b
	SN	63.6 ± 5.7		133.5 ± 6.9	b	44.5 ± 3.8		73.4 ± 12.7	b
	MET	68.5 ± 4.1		132.8 ± 4.0	b	47.8 ± 2.8		58.0 ± 10.8	b
	MET + SN	47.3 ± 6.4		156.0 ± 2.3	a	37.8 ± 3.8		114.8 ± 9.8	a
Girona	CTR	121.5 ± 13.9	a	125.0 ± 2.7	b	36.5 ± 2.4	a	34.0 ± 3.3	b
	SN	49.8 ± 12.7	b	199.5 ± 18.8	ab	23.3 ± 5.8	ab	83.0 ± 6.5	a
	MET	79.5 ± 13.9	ab	197.8 ± 9.5	ab	32.5 ± 1.6	a	90.3 ± 2.2	a
	MET + SN	33.0 ± 11.7	b	234.3 ± 28.5	a	15.5 ± 4.7	b	89.0 ± 9.0	a
Sint-Truiden	CTR	99.0 ± 6.2	a	141.0 ± 6.3	c	27.5 ± 1.3	a	51.8 ± 9.3	b
	SN	57.5 ± 8.9	bc	176.8 ± 7.2	ab	20.0 ± 2.5	b	51.8 ± 2.5	b
	MET	81.3 ± 8.4	ab	154.3 ± 8.5	bc	24.5 ± 1.8	ab	63.0 ± 8.2	ab
	MET + SN	41.5 ± 4.2	c	191.8 ± 6.1	a	24.5± 1.1	ab	81.0 ± 4.0	a

Among treatments, the MET + SN combination resulted in the greatest impacts in the studied parameters. That was the case of the reduction in fruits per 100/flowers in all trials, although significant only in Girona and Sint-Truiden in 2018. Consequently, there was a maximal significant increase in fruit weight, ranging from 20.1 g (Lleida 2017) to 109.3 g (Girona 2018),

and fruit size, between 23% (Lleida, 2017), to more than double (Girona, 2017; Lleida and Girona, 2018) in all trials.

A significant reduction the yield per tree was registered only in Girona in 2018, with a decline of 58% as compared to the respective control.

3.4 Discussion

3.4.1 Influence of Irradiance on Metamitron Absorption

Herbicide absorption and susceptibility is highly dependent on climatic conditions, namely radiation (Varanasi et al., 2016), which can also inactivate growth regulators such as 2.4-D and indol-3-acetic acid by photolysis (Hollósy, 2002). Metamitron is a selective herbicide which can be inactivated by a deamination reaction (Schmidt and Fedtke, 1977). This reaction consists in slight modifications in the compound, associated with the rupture of the $N\text{-}NH_2$, bond, which occurs in the presence of light, oxygen, and water. This forms deaminated compounds as main degradation metabolites, mainly desamino-metamitron, which is no longer capable of inhibiting the photosystem activity (Palm et al., 1997, Kouras, 2012). Since metamitron maximum absorption wavelength is 306 nm, direct photodegradation reaction can occur in the field (Cox et al., 1996), in accordance with our findings that showed a higher metamitron content (Figure 3.2) and lower (and more stable) degradation (assessed by desamino-metamitron content) (Figure 3.3) in the leaves under shade (MET + SN) than under full sun exposure (MET).

3.4.2 Effect on Gas Exchanges and RuBisCO Activity

The P_n of leaves of trees under control conditions were in line with the findings of Avery (1977), which reported values within the range between 15 and 25 μmol CO_2 m^{-2} s^{-1} in apple tree leaves. PPFD is crucial to photosynthetic performance and, consequently, to photo-regulation of plant growth and development. If on one side MET disrupts the photosynthetic apparatus functioning, ultimately interrupting CO_2 fixation, on the other side light deprivation (SN) would reduce light energy availability and, therefore, ATP and NADPH production.

Considerable thinning results can be obtained by shading the trees during a specific post-bloom period (Zibordi et al., 2009; Peifer et al., 2020; Basak, 2011), likely resulting from carbon starvation caused by the reduction in light availability. A significantly lower total hourly net carbon exchange rate was recorded previously (Zibordi et al., 2009) in shaded apple trees during the 8^{th} day shading period and observed an almost complete recovery after the nets were removed, which agrees with our results that demonstrated a strong P_n decline in SN, and a prompt recovery of C-assimilation at 6 DAS (just one day after shade removal), and an absence of effects at 10 DAS. Moreover, RuBisCO V_t did not show differences from CTR values at 5 DAS, even with the shading nets in place, what would help to support the quick P_n recovery at 6 DAS.

Metamitron can reduce P_n with a linear dose response in 'Golden Delicious' trees (the higher the concentration the greater the inhibition and the longer period for recover) (Brunner, 2014). In a study conducted previously by Gabardo et al. (2017), in 'Maxi Gala' and 'Fuji Suprema', at 3 DAS, P_n decreased 19.5% and 45.7% with very high metamitron doses of 800 and 1050 ppm, respectively. In our study, using a commercial dose of 247.5 ppm, P_n was reduced between 25 and 79% from 2 to 6 DAS (Figure 3.4), when P_n was still greatly suppressed. In fact, in our study, MET treated plants showed a P_n fully recovery only 10 DAS, while in a previous trial Gabardo et al. (2017), that happened already 8 DAS. However, g_s was reduced to ca. half 4 DAS using the 247.5 ppm dose, whereas an impact was observed only with 1050 ppm in 1000 L ha^{-1} in Gabardo et al. (2017). Furthermore, RuBisCO inhibition of to less than half of the control value (5 DAS) would have also contributed to the strong impact in P_n by MET.

MET + SN treatment led to minimal P_n and g_s values 2 DAS, and to an incomplete recovery 10 DAS. This treatment imposed a restriction of light energy availability, while reduced g_s and inhibited RuBisCO activity. RuBisCO activity together with a reduction of thylakoid electron transport (Cheng et al., 2001), contributed to the observed P_n limitation.

3.4.3 Effect on Non-Structural Carbohydrates

Fruit abscission is triggered by a shortage in carbohydrate content. In this context, attempts to develop models aiming to support the grower in the thinning decision have been made (Lakso and Johnson, 1990; Lakso et al., 2001; Doerflinger et al., 2015; Lordan et al., 2019). Variability in thinner efficacy is related to both the stage of fruit development, and the availability of carbohydrates to support fruit growth (Lordan et al., 2019). Metamitron application was made between 9–14 mm in fruit diameter, which has been proved to be the most critical and sensitive time (Gonzalez et al.a, 2019). In fact, fruit growth is mostly dependent on tree reserves in their initial growth stage, but from the 10-mm stage onwards, become dependent on shoot photosynthesis (Lakso et al., 2001; Byers et al., 1985; Byers et al., 1990, Corelli Grapadelli et al., 1994). In this way, the reduced P_n, caused either by metamitron and/or by shade, would have reduced the carbohydrate availability needed for fruit growth, justifying variations in non-structural sugar content (Robinson, 2016).

A study conducted by Klages et al. (2001) showed leaf sucrose and sorbitol contents by 105 to 112 DAFB, close to our values under control. In addition, taking into account the differences that result from sampling in different periods within the season (cultivar, weather, soil, and the tree conditions), our quantitative results of glucose and sorbitol (around 30 DAFB) are consistent with the work developed by other researchers (Naschitz et al., 2010).

Notably, with a few exceptions, glucose and fructose remained usually stable, even with the lower P_n induced by MET + SN, irrespective of location or day. These reducing sugars are involved in primary metabolism, but they did not respond to any source and sink manipulations, such as girdling and defoliation (Loescher et al., 1982; Naschitz et al., 2010; Wang et al., 1999; McQueen et al., 2004). By contrast, leaf sucrose and sorbitol were mostly reduced in all

treatments. Sucrose is formed in the cytoplasm and is then exported from source leaves to sink tissues (Zhou and Quebedeaux, 1995). Sorbitol is the most abundant non-structural sugar in the Rosaceae family and is a major phloem-transported sugar (Klages et al., 2001; McQueen et al., 2004; Zhou and Quebedeaux, 2003; Wang et al., 1995). The single, and particularly, the combined imposition of shade and metamitron, led to reductions of sucrose and sorbitol content. In agreement, Polomski (1986) reported a reduction in fruit carbohydrates between 15 and 35% as compared to CTR, by shading limbs or whole trees, with a PPFD reduction of 92% for a range between 5 to 10 days.

Starch can be formed as an end product of photosynthesis in chloroplasts, being a primary storage form that can be mobilized in case of need (Zhou and Quebedeaux, 2003; Breen et al., 2020). In the present work, starch content was reduced 2 and 4 (and partially by 6) DAS in all treatments, likely associated with the lowered P_n values, and to a remobilization of the available starch molecules to the global metabolism.

3.4.4 Oxidative Stress and Antioxidative Response

By interrupting thylakoid electron transport, metamitron might promote the transfer of electrons to alternative acceptors, as molecular oxygen (Foyer and Noctor, 2000; Noctor et al., 2002). Hydroxyl radicals generated from H_2O_2 have been shown to be potent inhibitors of PSII function (Asada, 1994; Jakob and Heber, 1996; Grace, 2005; Logan, 2005). However, shade would reduce the flux of photons reaching the antenna, which can decrease the oxidative conditions, as reflected in the absence of MDA and H_2O_2 variation of all treatments as compared to the control (Figure 3.5). In apple trees subjected to abiotic stresses, as progressing drought, the increased enzymatic activity and more reduced redox state of glutathione during the acclimation period were considered an initial stress response due to changes in the redox state (Sircelj et al., 2015). Here, MDA and H_2O_2 values did not showed significant changes in comparison with control regardless of treatment (Figure 3.5), and only the MET (APX) and MET + SN (CAT and GPX) promoted moderate activity increases (Figure 3.6). APX was slightly more active in MET treatment, what might have conferred some protection, as observed by Pandey et al. (2017), when abiotic stresses were imposed to trees. However, APX and CAT rises were not reflected in the H_2O_2 levels, even considering that SOD activity and AsA contents were decreased by 5 DAS (Figure 3.7). Furthermore, the values of APX and GR activity obtained in this work, along with total AsA and GSH, are generally significantly lower than the ones that Li et al. (2009) obtained and lower than MDA and SOD results of another study (Wei et al., 2018), both obtained in apple leaves. On the other side, our H_2O_2 and POD values are higher than the ones obtained previously (Wei et al., 2018). The triggering of these antioxidative components is often observed under oxidative stress conditions. Therefore, overall, our findings pointed that increased oxidative stress conditions were not present in neither of the applied treatments.

3.4.5 Environmental Conditions and Metamitron Thinning Efficacy

In general, the results of 2017 and 2018 showed that the SN treatment has a stronger impact in yield related parameters (fruits/100 flower clusters, average fruit weight, yield/tree and % fruits > 70 mm) than the single metamitron application. The number of fruits per 100 clusters was reduced by MET and/or SN (reducing radiation by 50% in the whole tree canopy for 5 days) treatments, between 6 and 42% in fruit drop, depending on year, location and treatment (stronger in MET + SN). These results in SN treatment were in close agreement with the strong fruit abscission of 90% induced by shading of the whole tree to 50% of normal light for 4 days from 20 to 41 DAFB (Kondo and Takahashi, 1987). Decreases of 35% in the number of fruits after reducing radiation by 40% during 12 days at 12-mm fruit diameter in 'Golden' trees (Brunner, 2014) or 23% more fruit abscission using a 90% radiation reduction for 8 days (Zibordi et al., 2009) were also found. Furthermore, it was registered a reduction of 50% of the crop load in 'Gala Must' trees with a 70% radiation reduction for a larger period of 14 days at the stage of 14- to 26-mm fruit diameter (Basak, 2011), and heavy abscission rates with radiation decrease to 4% during three days (Lakso et al., 2001). Finally, after reducing 90% of irradiance for 77 daylight hours in cv. 'Gala Mondial', Peifer et al. (2020) observed a 27% reduction in the number of fruits as compared with control.

Metamitron application (247.5 ppm) on 'Golden' trees, at 12-mm fruit diameter, led to crop load reductions between 12 and 41% in trials performed previously (Brunner, 2014), while the combination of shading nets and metamitron led to the highest crop load reduction (40% less fruits than in control). Our results show that metamitron, shade, and their combination increasingly reduced the number of fruits. However, since these treatments concomitantly promoted an increase in the number (and their %) of larger size fruits (>70 mm), that resulted in the absence of significant yield reductions in most trials, except in Girona (SN) and Sint-Truiden (MET + SN), both in 2018. Polomski (1986) sprayed terbacil, a photosynthesis inhibitor, and concluded that shading leads to more fruit drop comparing to the compound application, although, in the present study, the SN and MET treatments did not show significant differences in any of the trials.

In 2017, initial fruit set was low resulting in a higher difficulty to create a negative carbohydrate balance and finally, low thinning efficacies, even in MET + SN. However, in 2018, initial fruit set was very high and higher night-time temperature during the 5 nights before application (in Girona) and some cloudy days during the 3 days after (in the tree locations) might have potentiated the reduction in photoassimilates needed for fruit growth (Gonzalez et al., 2019b). This might have contributed to some over-thinning with MET + SN treatment, which promoted abscission rates of 58% in Sint-Truiden and above 70% in Girona, responsible by a 58% cut in yield in the latter. Taking these observations together, it was clear that the extent of metamitron effect can be amplified by a lowered PPFD reaching the leaf surface. However, by comparing 2017 to 2018, other factors than low radiation that also reduce carbohydrates availability (Gonzalez et al., 2019a), such as other meteorological conditions like high night-time

temperature, might contribute to increase metamitron thinning efficacy. This highlights the importance of weather conditions at the moment of imposing the treatments and in the following days, regardless of the use of shade or a chemical agent.

3.5 Conclusions

Shading net and/or metamitron application significantly limited C-assimilation, (associated with reduced g_s and RuBisCO V_t), with after effects observed until 10 DAS.

Low radiation seems to increase metamitron absorption and/or reduce its degradation, likely resulting in a stronger and longer effect, associated to sucrose and sorbitol decreases, leading to a negative carbohydrate balance. Thus, low radiation and/or metamitron created a transient carbohydrate stress in the tree that resulted in activation of the fruit abscission zone, with a stronger abscission effect under their combined imposition (MET + SN).

Only moderate increases were observed as regards the antioxidant enzymes in MET (APX) or MET + SN (CAT, GR, GPX), accompanied by increased GSH content. Additionally, leaf lipoperoxidation and H_2O_2 content remained unaltered, indicating that these metabolic defense mechanisms were able to keep oxidative stress conditions controlled.

The single use of these thinning agents can restrict the photosynthetic metabolism and sugar content, promoting thinning and the increase in fruit weight and size (fruits > 70 mm), without significant negative implications to fruit yield. However, their combination can promote an over-thinning (in Girona and Sint-Truiden, 2018), due to a further reduction of light irradiance due to cloudy days, what may lead to significant yield losses (in Girona, 2018). Therefore, thinning efficacy is also clearly dependent of environmental conditions at the time of the treatment's implementation which are of extreme importance for the achievement of an optimal thinning goal.

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CHAPTER 4

Response of *Malus x domestica* Borkh to metamitron and high nighttime temperature: effects on physiology and fruit abscission

Plastic covers down, in the evening, in Lleida (left side) and plastic covers up, during the day, in Girona (right side) to increase nighttime temperature

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4. Response of *Malus x domestica* Borkh to metamitron and high nighttime temperature: effects on physiology and fruit abscission

Abstract: Periods of high nighttime temperature may induce carbohydrate (CH) shortage by increased dark respiration. Metamitron is a thinning agent that inhibits photosynthesis and enhances fruit abscission due to a reduction in CH production. To clarify how both interact in apple tree physiologic mechanisms and on fruit abscission, five field trials were carried out in Lleida, Girona and Sint-Truiden (2017+2018), using orchards of 'Golden' apple trees. At the stage of 12-14 mm fruit diameter, four treatments were established: (A) CTR – control, trees under natural environmental conditions; (B) HNT – high nighttime temperature, trees exposed to artificially increased nighttime temperature during 5 nights after the day of spraying, without metamitron application; (C) MET - 247.5 ppm of metamitron application and (D) MET+HNT - trees submitted to the combined exposure to metamitron application (MET) and to artificially increased nighttime temperature (HNT). HNT did not affect metamitron absorption, net photosynthesis (P_n) and stomatal conductance however, promoted significant reductions in leaf CH content mainly before sunrise, especially in sucrose (18-45%) and in sorbitol (19-26%). Metamitron significantly reduced P_n to about 50% of CTR, which resulted in decreases in leaf sucrose and sorbitol, reaching minimum values 5 days after spraying, between 21-57% and 19-26%, respectively. Fruit growth rate of both treatments was retarded by 30%, 2 days after either metamitron application or HNT. Both treatments originated a similar reduction in the number of fruits and size improvement. The combined exposure (MET+HNT) promoted similar P_n reductions as MET, but was the treatment that showed greatest sucrose (44-60%) and sorbitol (73-84%) decreases comparing to CTR that resulted in the strongest thinning efficacies. Lipid peroxidation was not affected by the treatments however, antioxidant enzyme activity showed moderate changes with activity increases mainly under MET and MET+HNT, accompanied by a rise in glutathione content and reduction in ascorbate. This work shows that the overlap of photosynthesis inhibition (reducing CH production) by means of metamitron spraying, and likely greater respiration (increased CH consumption), by HNT imposition, translates less CH production than the growing fruits demand (negative CH balance) leading to a metamitron thinning effect enhancement. Periods of high nighttime temperature must be considered when deciding the best metamitron rate to achieve an optimal crop load result.

Keywords: carbohydrate balance, photosynthesis, reactive oxygen species, sucrose, sorbitol, thinning efficacy

4.1 Introduction

Apple (*Malus domestica* Borkh.) is one of the most economically important deciduous tree fruits worldwide. Every year the apple tree sets too many fruitlets that if not reduced will origin poor size fruits and stimulate biennial bearing. The difference between the optimum crop load and over or under thinning can translate in losses for growers (Robinson et al., 2013) and situations of lack of thinning precision keep happening. Hence, crop load management is one of the most important, yet difficult, strategies that will determine the annual profit of an orchard and establish a regular production.

Despite being a technique used already for decades, chemical thinning remains one of the more unpredictable practices of apple production with great disparities in results obtained within years and orchards. Chemical dose, uptake, crop load, fruitlet sensitivity and environmental conditions are some of the many factors that affect abscission response to chemical thinners and contribute to this variability (Jones et al., 2000; Robinson et al., 2013; Doerflinger et al., 2015; Lakso and Robinson, 2013). Carbohydrate (CH) balance seems to be the major reason for the vast variability since it is the support for fruitlet development and integrates both environment, namely nighttime temperature and radiation, and crop demands. It is difficult for the grower to control and integrate all these factors, thus several models have been developed to assist on the decision of when and at what concentration to spray thinning agents (Robinson and Lakso, 2011; Greene et al., 2013; Clever, 2018; Gonzalez et al., 2019; Lordan et al., 2019). Therefore, the output of these models integrates the weather variables along with tree requirements to provide a baseline for tree sensitivity to chemical thinners. Briefly, clear sky with good irradiation values and cold nights are the perfect combination for excellent CH production and maintenance, especially together with initial low crop load which requires less CH consumption (Robinson and Lakso, 2011; Clever, 2018; Gonzalez et al., 2019; Lordan et al., 2019). Under these weather conditions and crop load there will be a CH surplus that will reduce the thinner efficacy. Under cloudy weather and warm nights that reduce CH production and stimulate the consumption by enhanced dark respiration (Jing et al., 2016), the result can be an enhancement of fruit abscission or even over thinning, as it was demonstrated by Kondo and Takahashi (1987) and Stern al., (2014). This is the baseline for the information provided to growers to help predict thinning efficacy and give them the tools to adjust dosage to achieve the optimum crop load.

In fruit species of the Rosaceae family, sorbitol is a primary end product of photosynthesis, accounting for 60–80% of the photosynthates produced in apple leaves (Bieleski, 1969; Cheng et al., 2005). Sucrose is a disaccharide and is the main form of transport of assimilated carbon within the plant, from source sites to the sink or storage sites (Rees, 1984). Studies in apple (Kondo and Takahashi, 1987; Yoon et al., 2011; Stern, 2014, 2015) and other crops such as citrus (Stander et al., 2018), cotton (Turnbull et al., 2002; Arevalo et al., 2008; Loka and Oosterhuis, 2010), wheat (Prasad et al., 2008) and rice (Mohammed and Tarpley, 2009; Peraudeau et al., 2015) reported an enhancement in fruit abscission rate after exposure to high

nighttime temperature. Studies to evaluate the effect of high nighttime temperature in cotton (Turnbull et al., 2002) and wheat (Mohammed and Tarpley, 2009) have shown an increase in dark respiration and a shortage in soluble sugars content in apple (Kondo and Takahashi, 1987), cotton (Turnbull et al., 2002; Loka and Oosterhuis, 2010), wheat (Mohammed and Tarpley, 2009), rice (Peraudeau et al., 2015) and citrus (Stander et al., 2018).

Metamitron is a triazinone herbicide that inhibits photosystem (PS) II and disrupts thylakoid electron transport by blocking the electron transfer between the primary and secondary quinones of PSII (Abbaspoor et al., 2006; Guidi and Degl'Innocenti, 2011). It reduces net photosynthesis and induces a soluble sugar shortage due to a limited carbon fixation (Stander et al., 2018; Rosa et al., 2020), causing an enhancement in fruit abscission (Basak, 2011; Brunner, 2014; Gabardo et al., 2017). The electron transport impairment caused by metamitron, leads to an excess of excited energy that cannot be consumed via CO_2 assimilation (Foyer and Noctor, 2000). Although the excess energy can be partially dissipated through non-photochemical quenching, photorespiration, and other processes, plant leaves often undergo photo-oxidative stress caused by a greater reactive oxygen species (ROS) production in the chloroplast (Foyer and Noctor, 2000; Noctor et al., 2002). If these accumulated ROS cannot be quickly eliminated by the enzymatic and non-enzymatic antioxidant mechanisms, cell damage might occur (Sharma et al., 2012). The activation of such mechanisms, important to maintain the oxidative plant status during the permanence of metamitron effect, is still not well understood for apple trees.

The effect of high nighttime temperature and metamitron, and also of the combined metamitron application followed by a period of high nighttime temperatures, need further investigation in order to understand how it affects metamitron leaf absorption, photosynthesis, fruit growth and the sugar metabolic processes and, ultimately, fruit abscission. By understanding these parameters, it will be possible to advise with precision the rate of application to achieve an optimal crop load and increase grower's profit.

4.2 Materials and Methods

4.2.1 Plant Material and Experimental Design

4.2.1.1 Plant Material

The trials were performed in experimental orchards of *Malus x domestica* in Lleida and Girona (Spain) in 2017, and in Lleida, Girona and Sint-Truiden (Belgium) in 2018. In Lleida, the trials were carried out in the experimental orchards of IRTA – Lleida research station, in Mollerussa, northeast of Spain (41◦ 61' 96. 37" N / 0◦ 87' 06. 66" E, 245 m altitude) and in Girona, in IRTA Más Badia research station, in the province of Girona, northeast of Spain (42°03'12. 97'' N / 3°03'46. 13'' E, 12 m altitude). In both locations, 'Golden Reinders' apple trees were used, both planted in 2003, in Lleida grafted in M9, spaced 4 x 1.4 m, with a canopy height of 3 m and 'Gala Brookfield' as pollinator, and in Girona, grafted on M9 NAKB, planting distance was 3.8 x

1.1 m with a canopy height of 2.5 m and with 'Granny Smith' as pollinator. Both orchards are trained in a central leader system. In Sint-Truiden, the trials were performed in the orchards of PCFruit Research Station – Proefcentrum Fruittteelt vzw, Belgium (50° 45' 49" N / 05° 09' 26" E, 96 m altitude), using 'Golden Delicious' apple trees, grafted on M9, spaced 3.5 x 1.5 m, with a canopy height of 3 m, planted in 2005, without pollinator.

In 2017, biochemical and physiological measurements and determinations were performed only in Lleida. The yield parameters were assessed in Lleida and Girona. In 2018, all determinations and yield assessments were performed in Lleida, Girona and Sint-Truiden.

For biochemical evaluations, the leaves were cleaned with a water-wet tissue before being frozen in liquid N_2. All leaves were finely powdered with a mortar and pestle in liquid N_2 and kept at -80 °C until analysis.

4.2.1.2 Treatment Implementation

Four treatments were established: (A) CTR – control, corresponding to trees under natural environmental conditions; (B) HNT – high nighttime temperature, trees exposed to artificially increased nighttime temperature during 5 nights after the day of spraying, without application of metamitron; (C) MET - trees sprayed with 247.5 ppm of metamitron and (D) MET+HNT - trees submitted to the combined exposure to metamitron application (MET) and to artificially increased nighttime temperature during 5 nights after the day of spraying (HNT). Metamitron and/or artificially increased nighttime temperature treatments were imposed between the 7th and the 18th of May, in the five performed trials.

To increase nighttime temperature, a structure able to hold a plastic cover was installed along with three 3.3 kW heaters (in Lleida and Girona) and one diesel heater ITA30 (Thermobile Industries B.V, Breda, Netherlands) (in Sint-Truiden) was used in each block. A thermostat regulated to keep the inside temperature at 16 ºC was installed in all trials. The plastic cover placed from 20:00 h to 8:00 h.

Spraying of metamitron, the active ingredient of Brevis® (ADAMA, Telaviv, Israel), was carried out always in the early morning with the recommended dose of 247.5 ppm per 1000 L ha^{-1}, using a hand-gun sprayer. The moment of application was determined by fruit diameter: 12-14 mm, the fruit size at which metamitron is more efficient (Gonzalez et al., 2019).

To monitor the environmental conditions in each trial, sensors for temperature and relative humidity record were installed inside and outside of the structures on both sides and in the middle (with and without HNT); in each case in the upper (2 m) and lower (1 m) level of the trees. In Girona, six EasyLog USB Data Loggers (Lascar Electronics, Wiltshire, UK) were used; in Lleida, six Testo 177-h1 sensors were used (Testo, Titisee-Neustadt, Germany); and six Testo 174H sensors (Testo, Titisee-Neustadt, Germany USA) were used in Sint-Truiden.

The initial number of flower clusters per tree was homogeneous among treatments in each orchard. The experimental design in each orchard was a randomized complete block, with four

blocks each with four trees per treatment in each block, in which the two central trees of each set of four were measured, for a total of eight measured trees per treatment.

4.2.2 Metamitron Leaf Analysis

In 2017, leaf samples for metamitron concentration were collected only in Lleida, 1 and 3 days after spraying (DAS) whereas in 2018 the samples were taken 2 DAS in Lleida, Girona and Sint-Truiden. Each sample was a pool of three shoot leaves from the top, middle and bottom part of each tree, with four samples being taken from the Eastern and four from Western side of the canopy, for a total of eight repetitions per treatment. All leaves were clean with a water-wet tissue before frozen in N_2 for further analysis.

Metamitron extraction was conducted according to the QuEChERS method (Lesueur et al., 2008) using 500 mg fresh weight (FW) of frozen leaf powder and 3 mL of acetonitrile. The samples were shaken manually for 1 min, after which, 1.95 g of extraction Supel™ QuE Citrate Extraction Tube (Sigma, USA) was added, containing 1.2 g of magnesium sulfate, 0.3 g of sodium chloride, 0.15 g of sodium citrate dibasic sesquihydrate, and 0.3 g of sodium citrate tribasic dehydrate. The samples were further shaken manually for 1 min and centrifuged (6000 ×*g*, 5 min, 4 °C). An aliquot of 1.2 mL of the supernatant was transferred to a 2 mL Supel™ QuE Verde clean-up tube (Sigma, USA), vortexed, and further centrifuged (6000 ×*g*, 5 min, 4 °C). The obtained supernatant was filtered with a polytetrafluoroethylene (PTFE) 0.45 µm filter, and injected. Standard curves were used for the quantification of metamitron (Sigma, USA) and desamino-metamitron-desamino (LGC Standards, USA).

4.2.3 Leaf Gas Exchanges

Leaf gas exchanges measurements included net photosynthesis rate (P_n) and stomatal conductance to water vapor (g_s), and were obtained using a portable Infra-Red Gas Analyzer (IRGA) LCi Ultra Compact Photosynthesis System (ADC BioScientific, Hoddesdon, UK), under ambient conditions of irradiance, temperature, humidity and CO_2 supply, in recently fully developed shoot leaves at *ca*. 1.5 m height, between 10-12:00 h. In each of the four blocks, two evaluations in the Eastern and two in the Western side of the canopy were performed, totaling eight leaves per treatment. In 2017, measurements were taken 1, 3, 5 and 10 DAS, in Lleida, and, in 2018, measurements were taken 2, 5 and 10 DAS, in Sint-Truiden.

4.2.4 Leaf Soluble Sugars

In 2017, leaf sampling for non-structural sugar quantification was performed 1, 3, 5 and 10 DAS, before sunrise (around 6:00 h) and at midday (between 11:00-12:30 h), in Lleida. In 2018, leaf sampling was performed 5 DAS, before sunrise (6:00 h), in Lleida and Sint-Truiden, and 2, 5 and 10 DAS, at midday (between 11:00-12:30 h), in Lleida, Girona and Sint-Truiden. Samples were constituted by 2 shoot leaves and 2 cluster leaves, 4 and 8 repetitions per treatment, in 2017 and 2018, respectively.

Quantification of sucrose, fructose, glucose and sorbitol was based on the method described by Ramalho et al. (2013) using *ca*. 150 mg FW frozen leaf material. The separation of sugars was performed using a Sugarpak1 column (300 x 6.5 mm, Waters) at 90 °C, using H_2O (containing 50 mg EDTA-Ca L^{-1}) as eluent, at a flow rate of 0.5 mL min^{-1} in an HPLC system equipped with a refractive index detector (Model 2414,Waters, Milford, USA). Standard curves of each sugar were used for quantification.

4.2.5 Leaf Oxidative Status Evaluation

Sampling was performed 5 DAS in Sint-Truiden, in 2018, between 10-12:00 h. Each sample was a pool of three shoot leaves (one sample per block, totaling four samples per treatment) that was frozen in liquid N_2 and stored at - 80 °C until analysis.

4.2.5.1 Lipoperoxidation and H_2O_2 Content

Sample extraction was performed using 200 mg FW frozen material, homogenized with 2.0 mL of 0.1% trichloroacetic acid (TCA), and centrifuged (12000 *g*, 15 min, 2 ºC). Lipid peroxidation was estimated by measuring malondialdehyde (MDA) content, using the thiobarbituric acid (TBA) method, as described by Demiral and Turkan (2005). After extraction, 4 mL of 20% TCA containing 0.5% thiobarbituric acid TBA was added to a 1 mL aliquot of the supernatant. This mixture was heated (95 ºC, 30 min) followed by quick cooling in an ice bath and centrifugation (10000 *g*, 15 min, 2 ºC). The amount of MDA was calculated from the coefficient of absorbance at 532 nm after subtracting the non-specific absorption at 600 nm. The extinction coefficient 155 mM^{-1} cm^{-1} for MDA was used. Hydrogen peroxide (H_2O_2) content was measured using the method described by Singh et al. (2006). To a 50 µL aliquot of the supernatant obtained in the extraction, 959 µL 100 mM phosphate buffer, pH 7.6, and 1 mL 1 M potassium iodide were added. The absorbance of the supernatant was measured at 390 nm and for quantification was used a standard curve of hydrogen peroxide (0, 1.1, 2.2, 3.3, 4.4 and 5.5 µg mL^{-1}).

4.2.5.2 Antioxidative Enzyme Assays

For catalase (CAT), guaiacol peroxidase (GPOD), superoxide dismutase (SOD) and glutathione redutase (GR) 200 mg FW frozen material were homogenized in 2 mL of cold 100 mM Tris-hydrochloric acid (HCl) buffer, pH 7.8, containing 3 mM dithiothreitol, 1 mM EDTA, 2% (w/w) insoluble PVPP and centrifuged (12000 *g*, 20 min, 4 ºC). For ascorbate peroxidase (APX) activity determinations, 10 mM of ascorbate was added to the previously described solution. For glutathione peroxidase (GPX) activity determinations, 0.1% (w/v) Triton X-100, 5 mM cysteine, and 0.1 mM Phenylmethanesulfonyl fluoride were added to the solution described for CAT, SOD, GPOD and GR. The resulting supernatant was used for determination of enzymatic activity (four replicates were used for each determination). Absorbance was measured in a Hitachi (U-2000 UV/Vis, Hitachi, Japan) spectrophotometer, at *ca*. 25 ºC. The enzyme activity was expressed as unit g^{-1} FW.

Catalase

CAT activity (EC 1.11.1.6) was evaluated as described earlier Aebi (1983), with some changes, by following the decrease in absorbance at 240 nm for 2 min in a solution containing 10 mM of H_2O_2 in 50 mM phosphate buffer, pH 7.0. Enzymatic activity was defined as the consumption of 1 µmol H_2O_2 per min and per cm^3 using a coefficient of absorbance of 39.4 mM^{-1} cm^{-1}.

Guaiacol Peroxidase

Guaiacol peroxidase (GPOD) activity (EC 1.11.1.7) was determined following the increase of absorbance at 470 nm, according to a modification of methodology described in Gajewska et al. (2006), using a reaction mixture containing 30 mM 2-methoxyphenol (guaiacol) and 4 mM H_2O_2 in 0.2 M sodium acetate buffer, pH 6.0. Enzymatic activity was defined as the consumption of 1 µmol of guaiacol per min and per mL using a coefficient of absorbance for tetraguaiacol of 26.6 mM^{-1} cm^{-1}.

Glutathione Reductase

Glutathione reductase (GR) activity (EC 1.8.1.7) was determined using a modified method (Shanker et al. 2004), measuring the increase in absorbance at 412 nm, using a reaction mixture containing 3 mM 5,5'-dithio-bis(2-nitrobenzoicacid) (DTNB), 2 mM nicotinamide adenine dinucleotide phosphate (NADPH) and 20 mM oxidized glutathione (GSSG) in 100 mM phosphate- ethylenediaminetetraacetic acid (EDTA) buffer, pH 7.6, and 1mM EDTA. Enzymatic activity was defined as the consumption of 1 µmol of GSSG per min and per mL using a coefficient of absorbance of 6.2 mM^{-1} cm^{-1}.

Superoxide Dismutase

Superoxide dismutase (SOD) activity (EC 1.15.1.1) was determined using a modified method (Rubio et al., 2002), following the variation of absorbance at 550 nm, using a reaction mixture with 0.1 mM EDTA, 0.5 mM Xantine and 0.05 mM of ferricytochrome c in 100 mM phosphate buffer, pH 7.6, and 1 U mL^{-1} xantine-oxidase. Enzymatic activity was defined as µmol of ferricytochrome c reduction by superoxide radical min^{-1}.

Ascorbate Peroxidase

Ascorbate peroxidase (APX) activity (EC 1.11.1.11) was determined according to Sharma and Dubey (2004), in a reaction mixture containing 0.25 mM ascorbate and 0.3 mM hydrogen peroxide in 50 mM phosphate buffer, pH 7.0, following the decrease in absorbance at 290 nm. Enzymatic activity was defined as the consumption of 1 µmol ascorbate per min and per mL using a coefficient of absorbance of 2.8 mM^{-1} cm^{-1}.

Glutathione Peroxidase

Glutathione peroxidase (GPX) activity (EC 1.11.1.9) was determined according to Aravind and Prasad (2005), in a reaction mixture containing 1.14 mM sodium chloride, 2 mM reduced glutathione, 2.5 mM hydrogen peroxide, 2 mM NADPH in 50 mM Tris-HCl buffer, pH 7.9. Enzymatic activity was defined as the glutathione-peroxidase necessary to reduce 1 µmol

NADPH per min and per mL at room temperature using a coefficient of absorbance of 6.2 mM^{-1} cm^{-1}.

4.2.5.3 Non-enzyme Antioxidants Quantification

For glutathione and ascorbate evaluations, samples of 100 mg FW frozen leaf were homogenized in 0.5 mL of ice-cold 6% meta-phosphoric acid, pH 2.8, containing 1 mM EDTA and 1% activated charcoal powder for chlorophyll removal. Homogenates were centrifuged (27000 *g*, 15 min, 4 ºC), and the obtained supernatant was stored at -80 ºC prior to glutathione and ascorbate analysis.

Glutathione

The quantification of reduced (GSH) and oxidized (GSSG) glutathione was based on the method described by Anderson et al. (1992). Total glutathione was measured spectrophotometrically at 412 nm in a microplate reader (Synergy HT, BioTek Instruments, Vermont, USA). Oxidized glutathione (GSSG) was measured by incubating the diluted sample in 0.5% 2-vinylpyridine for 1 h at 25 ºC and then proceeding as described above. Reduced glutathione (GSH) was determined as the difference between total glutathione and GSSG.

Ascorbate

The quantification of ascorbic (AsA) and dehydroascorbic (DAsA) acids was based on a method adapted from Okamura (1980), as described in Carvalho and Amâncio (2002). Absorbance was recorded at 525 nm in a microplate reader (Synergy HT, BioTek Instruments, Vermont, USA). Concentration of AsA was determined using a calibration curve of AsA in the range of 10–60 mM prepared in 5% metaphosphoric acid. The concentration of DAsA was calculated by subtracting the AsA concentration measured from the total ascorbate assayed.

4.2.6 Fruit Growth Rate

The fruit growth rate was registered in Lleida, in three fruits from control trees (CTR), artificially increased nighttime temperature (HNT) and metamitron (MET) treatments, using type DF fruit dendrometers (Ecomatik, Dachau, Germany). The devices were installed 2 days before spraying and kept registering the data until 7 days after. The data was registered with a data logger DL2 (Delta-T Devices, Cambridge, UK). Growth rate was calculated for each hour of the day.

4.2.7 Yield Parameters

All fruits were picked from each observed tree at harvest, on one time. The number of fruits per tree, yield, fruit weight and distribution per fruit size was determined using a commercial sort machine (Maf Roda Agrobotic, Montauban Cedex, France).

4.2.8 Statistical Analysis

The data was subjected to an analysis of variance, through a one-way ANOVA, to evaluate the differences between treatments on one single day after spraying, or a two-way ANOVA to evaluate the differences between the four treatments, across the several days after spraying. Means were compared by Tukey's Honestly Significant Difference (HSD) test at $\alpha = 0.05$. Each ANOVA was performed independently for each trial. A 95% confidence level was adopted for all tests. The statistical analysis was performed using Statistix 9 (Analytical Software, Tallahassee, Florida).

4.3 Results

4.3.1 Environmental Conditions

A brief characterization of the environmental conditions in the five performed trials is shown in Table 4.1. Global irradiance values were quite homogenous within all trials, representing days of clear sky. The 2017 trial in Girona stands out due to the higher relative humidity, *ca.* 25% higher than the average of the other 4 trials. The nighttime temperature after the spraying date was very homogeneous among trials, although in Girona (2018), the average nighttime temperature during the 5 nights prior spraying is higher than the other four trials. The difference between environmental nighttime temperature and the artificially increased nighttime temperature varied between 2.9 to 6.7 ºC.

Table 4.1 – Summary of meteorological conditions ± SE in trials performed in each year and location and fruit diameter at the time of metamitron application: average of daily irradiance 5 DAS (MJ m^{-2}), average nighttime temperature from 20:00-8:00 h (ºC), 5 nights before and after spraying, and average air relative humidity during the 3 h prior to spraying, in natural environmental conditions (Control) and in artificially increased nighttime temperature conditions (HNT).

Location	Fruit Diameter (mm)	Global Irradiance MJ m^{-2} - 5 days after	Night Temperature ºC-5 nights before	Night Temperature ºC - 5 nights after		Relative Humidity %
		Control	Control	Control	HNT	Control
			2017			
Lleida	14 ± 0.2	21.7 ± 0.6	8.7 ± 0.7	11.9 ± 0.4	15.6 ± 0.1	67.3 ± 3.7
Girona	12 ±0.4	16.1 ± 0.8	9.4 ± 0.4	10.0 ± 0.3	14.3 ± 0.2	93.3 ± 3.9
			2018			
Lleida	13 ± 0.2	17.5 ± 2.4	10.2 ± 0.5	11.8 ± 0.4	17.0 ± 0.1	61.5 ± 4.5
Girona	14 ± 0.1	19.3 ± 2.9	15.5 ± 0.9	11.8 ±0.6	18.5 ± 0.4	69.1 ± 5.1
Sint-Truiden	14 ±0.2	22.1 ± 1.2	11.5 ± 0.3	11.6 ± 0.7	14.5 ± 0.3	60.5 ± 3.9

4.3.2 Metamitron Concentration in Leaves

To evaluate metamitron impacts it is important to determine leaf absorption however, in 2017, the differences in metamitron absorption were not statistically different (*p*-value > 0.05) with an average of 2 mg g^{-1} dry weight (DW) (data not shown). In contrast, in Sint-Truiden (2018) increased nighttime temperature promoted a significant increment in metamitron absorption of about 1/3 as compared with MET (Fig. 4.1).

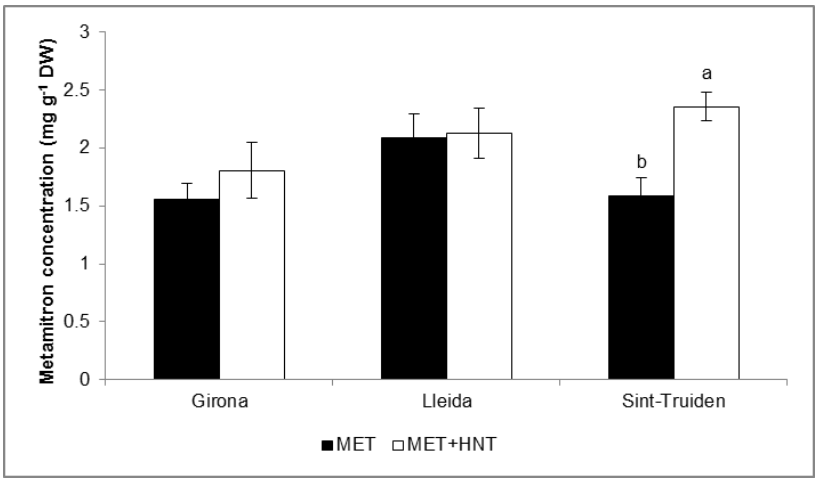

Figure 4.1 - Metamitron content (mg g^{-1} DW) evaluated 2 DAS, in the trials of 2018 in Girona, Lleida and Sint-Truiden. Trees were exposed to artificially increased nighttime temperature for 5 nights after spraying, from 20:00-8:00 h. For each parameter, the mean values ± SE (*n*=8) followed by different letters express significant differences between treatments within each cultivar/location using Tukey's HSD test (*α*-value ≤ 0.05). No letters indicate no significant difference between means. MET – Metamitron; HNT – High nighttime temperature

4.3.3 Leaf Gas Exchanges

High nighttime temperature, did not promote changes in P_n, in comparison with CTR along the entire trial (Fig. 4.2A). Additionally, higher nighttime temperature did not significantly affect the metamitron impact, since no differences were observed between MET and MET+HNT treatments. In contrast, in Sint-Truiden (2018) there was an interaction effect already at 2 DAS in MET+HNT, with P_n 40% lower than CTR (Fig. 4.3). Still, both MET and MET+HNT significantly reduced P_n at 3 and 5 DAS, to about half of the CTR, although by 10 DAS no differences were found anymore among all treatments in Lleida. Notably, a somewhat different pattern of recovery was observed in Sint-Truiden (2018), since by 10 DAS only the MET+HNT maintained a reduced P_n value, 52 % lower than CTR (Fig. 4.3)

Generally, g_s rate was not affected by treatments as compared to the control in the same day, except for MET at 5 DAS when a 50% reduction was observed (Fig. 4.2B). During the experiment, g_s was not a limiting factor of P_n, since its value remained stable and similar within treatments.

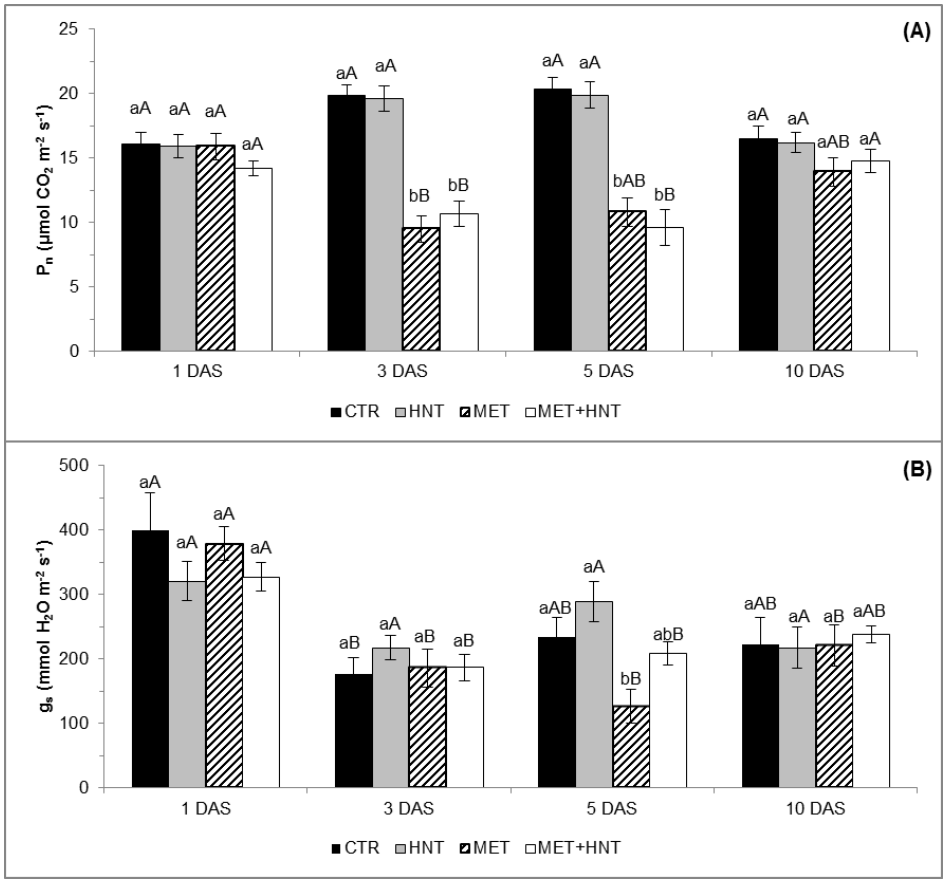

Figure 4.2 – Leaf net CO_2 gas exchange (P_n) (µmol CO_2 m^{-2} s^{-1}) (A) and stomatal conductance to water vapor rate (g_s) (mmol H_2O m^{-2} s^{-1}) (B) evaluated 1, 3, 5 and 10 days after spraying (DAS), in the trial of 2017, in Lleida. Trees were exposed to artificially increased nighttime temperature for 5 nights after spraying, from 20:00-8:00 h. For each parameter, the mean values ± SE (*n*=8) followed by different letters express significant differences between treatments within each day (a and b), or between days within each treatment (A and B), using Tukey's HSD test (α-value ≤ 0.05). MET – Metamitron; HNT – High nighttime temperature; DAS – Days after spraying

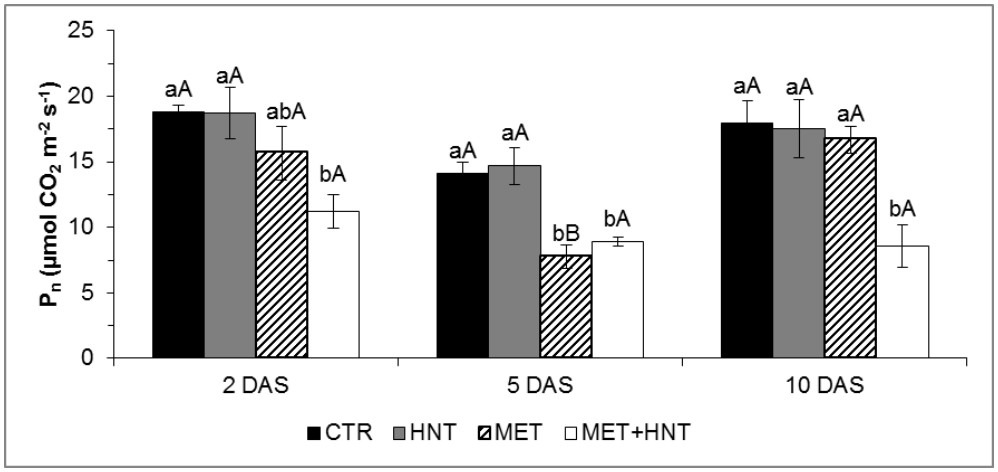

Figure 4.3 – Leaf net CO_2 gas exchange (P_n) (µmol CO_2 m^{-2} s^{-1}) evaluated 2, 5 and 10 DAS, in the trial of 2018, in Sint-Truiden. Trees were exposed to artificially increased nighttime temperature for 5 nights after spraying, from 20:00-8:00 h. For each parameter, the mean values ± SE (*n*=8) followed by different letters express significant differences between treatments within each day (a and b), or between days within each treatment (A and B), using Tukey's HSD test (*α*-value ≤ 0.05). HNT – High nighttime temperature; MET – Metamitron, DAS – Days after spraying

3.4 Leaf Soluble Sugars

In 2017, there were significant changes in total sugars (sum of sucrose, glucose, fructose and sorbitol) before sunrise and at midday (Fig. 4.4). Generally, all treatments presented lower sucrose, sorbitol and total sugar levels before sunrise as compared to midday. The lowest sugar content levels in treated trees were usually observed 5 DAS, when greater differences between treatments were also observed, particularly at midday. In fact, by 5 DAS before sunrise, only MET+HNT showed significantly lower levels of total sugars (65%), whereas at midday, all treatments showed reduced total sugar content as compared to CTR, with the greater reduction (70%) found in MET+HNT. By 10 DAS, MET and MET+HNT continued to present significantly lower sugar content.

The patterns in total sugar content resulted mostly from the similar patterns found in the more represented soluble sugars, particularly sucrose and sorbitol. In fact, both of these sugars usually showed lower contents before sunrise than at midday (especially by 1 and 5 DAS), greater differences between treatments at midday, particularly by 5 DAS, when MET+HNT consistently presented the lower values. By 10 DAS some effects associated to MET+HNT persisted, only for sucrose.

Similarly to 2017, in 2018 there were no variations in glucose and fructose (data not shown). Sucrose and sorbitol followed the same trends as in 2017, being sucrose the sugar that varies the most, generally reaching minimum values 5 DAS. HNT decreased sucrose content (significantly at 2 DAS in Lleida and 5 DAS in Girona) and sorbitol (always non-significantly) that

ranged between 18-45% and 19-28%, respectively. By 10 DAS, there were no differences from CTR.

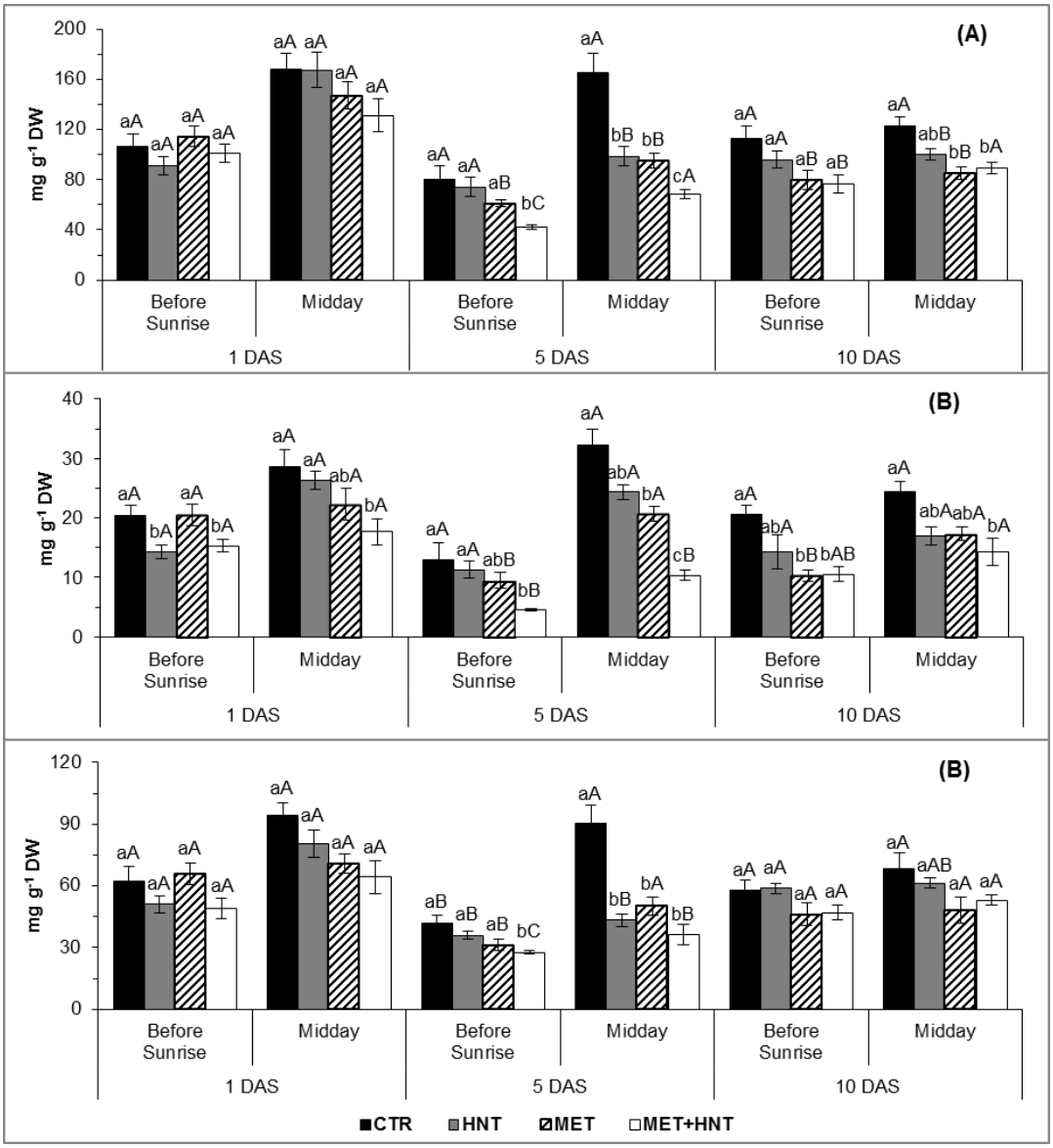

Figure 4.4 – Total sugars (A), sucrose (B) and sorbitol (C) concentration in the leaves (mg g^{-1} DW) evaluated 1, 5 and 10 days after spraying (DAS), before sunrise and at midday, in the trial of 2017, in Lleida. Trees were exposed to artificially increased nighttime temperature for 5 nights after spraying, from 20:00-8:00 h. For each parameter, the mean values ± SE (*n*=4) followed by different letters express significant differences between treatments within each day (a, b, and c), or between days within each treatment (A, B, and C), using Tukey's HSD test (*α*-value ≤ 0.05). HNT – High nighttime temperature; MET – Metamitron, DAS – Days after spraying

Metamitron induced decreases in sucrose, significant at 2 or 5 DAS in all locations, promoting reductions between 21 and 57%, while sorbitol decreased significantly 5 DAS in Girona and in

Sint-Truiden between 19 and 26%, always as compared to CTR. The combination of metamitron spraying with artificially increase night temperature (MET+HNT) resulted in the lowest sucrose and sorbitol contents observed within all treatments. These sugars reached minimum levels at 5 DAS, when it represented between 44 and 60% for sucrose, and between 73 and 84% for sorbitol, as compared to their CTR values. In addition, 10 DAS these two sugars still presented reduced contents in Lleida under MET and MET+HNT.

Table 4.2 – Sucrose and sorbitol concentration in the leaves (mg g^{-1} DW) evaluated 2, 5 and 10 days after spraying (DAS), at midday, in the trial of 2018, in Lleida, Girona and Sint-Truiden. Trees were exposed to artificially increased nighttime temperature for 5 nights after spraying, from 20:00-8:00 h. For each parameter, the mean values ± SE (*n*=8) followed by different letters express significant differences between treatments within each day (a, b, and c), or between days within each treatment (A, B, and C), using Tukey's HSD test (*α*-value ≤ 0.05). HNT – High nighttime temperature; MET – Metamitron, DAS – Days after spraying

		DAS	CTR		HNT		MET		MET+HNT	
Lleida	**Sucrose**	**2**	14.7±1.0	aA	8.2±1.3	bB	9.2±1.7	bB	6.2±0.7	cAB
		5	9.5±0.7	aB	7.9±0.5	aB	6.4±0.8	abB	4.9±0.9	bB
		10	17.0±1.9	aA	16.4±1.3	aA	15.7±1.7	aA	9.9±0.7	bA
	Sorbitol	**2**	62.9±3.5	aB	64.1±2.0	aB	64.1±3.4	aB	56.1±2.2	aB
		5	52.7±2.5	aB	42.3±2.4	abC	44.1±2.0	abC	38.8±3.3	bC
		10	119.1±9.0	aA	120.7±5.1	aA	115.1±3.9	aA	103.3±5.5	bA
Girona	**Sucrose**	**2**	19.6±1.9	aA	15.6±2.9	abA	13.6±1.7	abA	10.6±0.3	bA
		5	15.5±1.4	aA	10.6±1.1	bB	8.7±1.4	bB	9.3±0.7	bA
	Sorbitol	**2**	109.0±7.9	aA	78.7±8.3	abA	88.7±9.6	aA	58.5±6.2	bA
		5	72.5±3.7	aB	67.9±3.5	aA	53.8±2.7	bB	52.8±2.2	bA
Sint-Truiden	**Sucrose**	**2**	25.0±0.9	aA	20.6±1.1	aA	19.7±1.3	aA	20.0±2.5	aA
		5	18.2±2.6	aAB	16.2±0.8	aAB	11.7±1.5	bB	8.0±0.3	cC
		10	13.9±2.2	aB	12.5±0.8	aB	13.0±0.9	aB	10.4±0.6	aB
	Sorbitol	**2**	119.8±6.9	aA	97.8±9.5	aA	105.2±4.4	aA	97.6±4.9	aA
		5	80.1±6.0	aB	76.8±2.7	aB	65.6±4.5	aB	67.0±3.8	aB
		10	102.9±7.0	aA	91.4±4.3	aA	91.4±9.7	aA	96.0±4.8	aA

4.3.5 Leaf Oxidative Status

4.3.5.1 Lipid Peroxidation

None of the treatments induced significant changes in MDA however, HNT and all metamitron treatments significantly increased H_2O_2 leaf content as compared to CTR (Fig. 4.5).

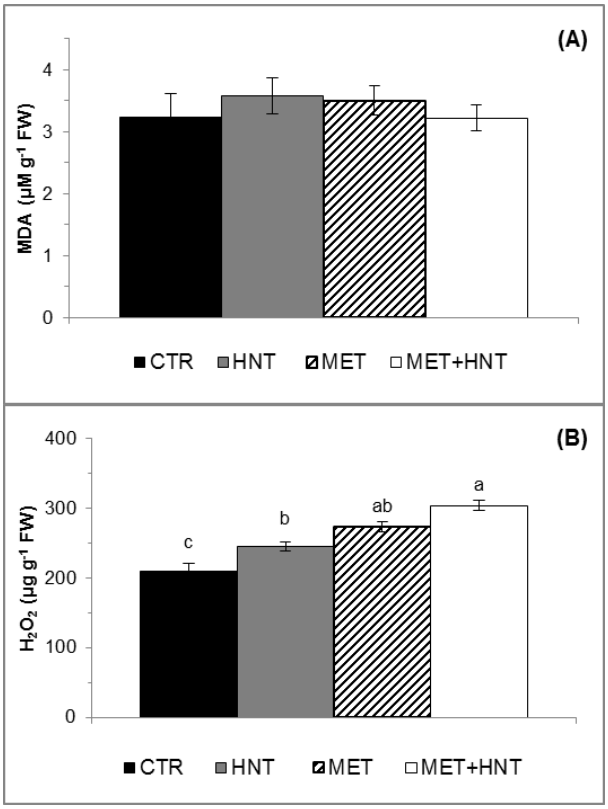

Figure 4.5 – Leaf average contents of malondialdehyde (MDA) (µM g^{-1} FW) (A) and hydrogen peroxide (H_2O_2) (µg g^{-1} FW) (B) evaluated 5 DAS, in the trial of 2018, in Sint-Truiden. Trees were exposed to artificially increased nighttime temperature for 5 nights after spraying, from 20:00-8:00 h. The mean values ± SE (*n*=4) followed by different letters express significant differences between treatments using Tukey's HSD test (*α*-value ≤ 0.05). No letters indicate no significant difference between means. HNT – High nighttime temperature; MET – Metamitron, DAS – Days after spraying

4.3.5.2 Anti-oxidative enzyme activity

High nighttime temperature (HNT) increased the activity of POD, APX and GR, although only significantly in the latter, by 52, 55 and 110%, respectively, as compared to CTR (Fig. 4.6). The MET treatment promoted a significantly higher activity of CAT, POD, GR and APX, generally to double the activity as CTR. The sharpest activity increases were observed in MET+HNT, with activity rises of 88, 142, 187 and 258% in CAT, GR, SOD and APX, respectively, comparing to CTR.

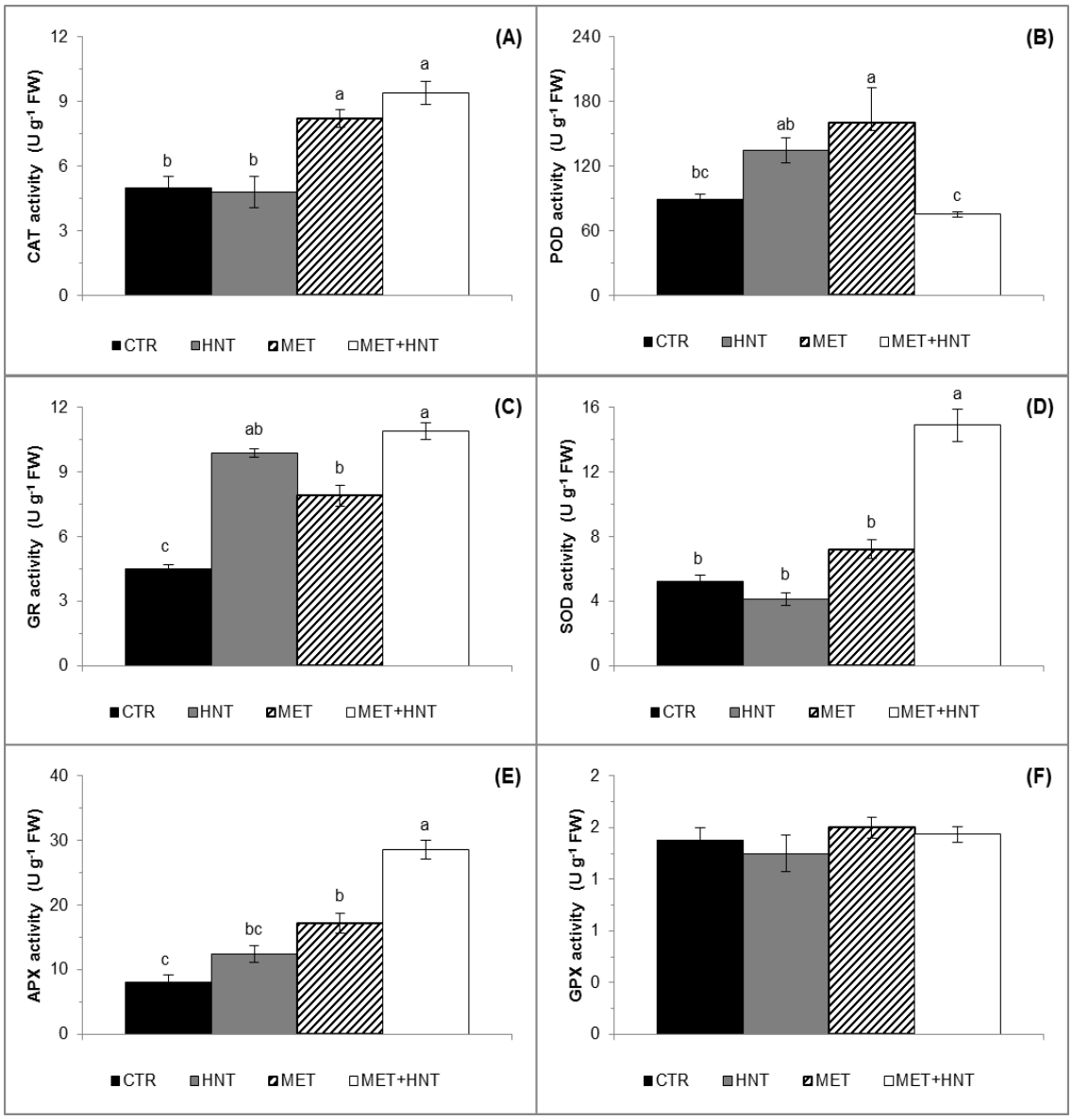

Figure 4.6 – Catalase (CAT) (A), guaiacol peroxidase (POD) (B), glutathione reductase (GR) (C), superoxide dismutase (SOD) (D), ascorbate peroxidase (APX) (E) and glutathione peroxidase (GPX) (F) activities (U g^{-1} FW) evaluated 5 DAS, in the trials of 2018, in Sint-Truiden. Trees were exposed to artificially increased nighttime temperature for 5 nights after spraying, from 20:00-8:00 h. For each parameter, the mean values ± SE (*n*=4) followed by different letters express significant differences between treatments using Tukey's HSD test (*α*-value ≤ 0.05). No letters indicate no significant difference between means. HNT – High nighttime temperature; MET – Metamitron, DAS – Days after spraying

4.3.5.3 Ascorbate and Glutathione Content

More than 90% of the total glutathione (GSH+GSSG) and total ascorbate (AsA+DHA) were in the reduced form (GSH, AsA) in all treatments (data not shown). All treatments significantly

promoted the increase of GSH+GSSG contents, by 3 fold as compared to CTR (Fig. 4.7). Ascorbate showed an inverse pattern of that displayed by glutathione. All treatments promoted the reduction in total (AsA+DHA) ascorbate (Fig. 4.7). Concerning total ascorbate, the sharpest decrease was promoted by MET+HNT, reducing to values 34% lower than CTR.

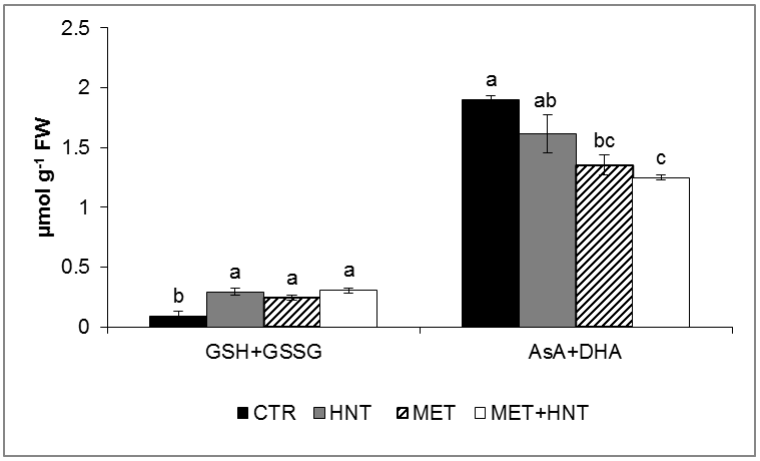

Figure 4.7 - Total glutathione (GSH+GSSG) (A) and total ascorbate (AsA+DHA) (B) (µmol g^{-1} FW) evaluated 5 DAS, in the trials of 2018, in Sint-Truiden. Trees were exposed to artificially increased nighttime temperature for 5 nights after spraying, from 20:00-8:00 h. For each parameter, the mean values ± SE (*n*=4) followed by different letters express significant differences between treatments using Tukey's HSD test (*α*-value ≤ 0.05). HNT – High nighttime temperature; MET – Metamitron, DAS – Days after spraying

4.3.6 Fruit Growth

Fruit growth rate showed no differences between treatments until the application day (0 DAS) however, differences started to arise between 0 and 5 DAS, during the high nighttime temperature period and after metamitron application (Fig. 4.8).

HNT and MET significantly retarded fruit growth from 2 DAS on (until 7 DAS), period in which remained with a strong fruit growth rate reduction of *ca.* 30% as compared to CTR.

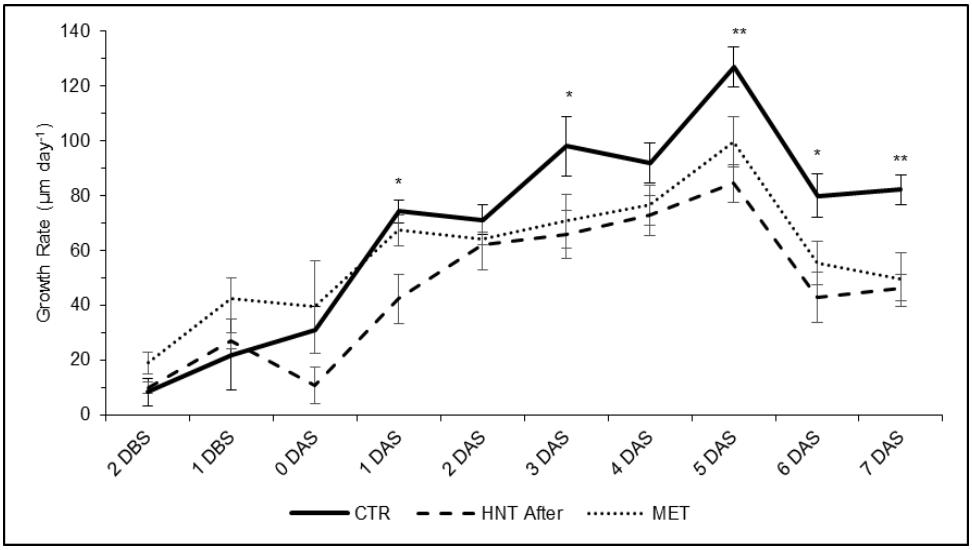

Figure 4.8 – Fruit growth rate (µm day^{-1}) evaluated from 2 days before spraying (DBS) to 7 DAS in CTR, HNT and MET, in the trial of 2018, in Sint-Truiden. Metamitron was sprayed at 0 DAS. Trees were exposed to artificially increased nighttime temperature for 5 nights after spraying (0 to 5 DAS), from 20:00-8:00 h. The mean values ± SE (n=4) followed by *, ** or *** express significant differences between treatments within each day using Tukey's HSD test (α-value ≤ 0.05).

4.3.7 Yield Parameters

Generally, average fruit weight and percentage of fruits in fruit size class greater than 70 mm were the harvest parameters more affected by the treatments applied (Table 3).

The treatment HNT promoted a significant reduction in the number of fruits per 100 flower clusters in both years in Lleida (30 and 23%). There was a tendency to improve average fruit weight in all trials and, consequently, the percentage of fruits greater than 70 mm, although only significant in Lleida 2018, with a 16 and 20% increment as compared to CTR. Increasing nighttime temperature did not affect the tree yield.

Metamitron significantly reduced the number of fruits per 100 flower clusters, in both years in Lleida (2017 and 2018), 40 and 26%, and in Girona (2018) and Sint-Truiden, both with 40% less fruits comparing to CTR. Consequently, there was a significant improvement on average fruit weight in both years in Lleida, 29 and 22% (2017 and 2018), and in Girona (2018), to double the weight of fruits in CTR. Metamitron application improved fruit size, with a significant increase registered in Girona 2018 (91%), without losses in yield per tree.

The combined exposure to metamitron application and high nighttime temperatures (MET+HNT) promoted the strongest reduction in fruits per 100 flower clusters among all treatments in all trials. The strongest and significant fruit reductions per 100 flower clusters were observed in Lleida 54% and 41% in 2017 and 2018, respectively, and in Sint-Truiden, 61% less fruits compared to CTR. Consequently, this treatment resulted in the highest improvements in average fruit weight and fruit size, although with a significant yield reduction of 50% in Sint-

Truiden. MET+HNT was the treatment that caused the greatest fruit reductions, although not significantly different from MET alone. Nevertheless, MET+HNT was for several times the only treatment significantly improving average fruit weight and/or increasing fruit size (Girona 2017 and Sint-Truiden 2018). In addition, it was the only treatment that caused a yield reduction of more than 50% in yield per tree (Sint-Truiden).

Table 4.3 – Number of fruits per 100 flower clusters, fruit weight (g), yield per tree (kg) and percentage of fruits in fruit size class > 70 mm at harvest in the trials of 2017, in Lleida and Girona and in the trials of 2018, in Lleida, Girona and Sint-Truiden. Trees were exposed to artificially increased nighttime temperature for 5 nights after spraying, from 20:00-8:00 h. The mean values ± SE (*n*=8) followed by different letters express significant differences between treatments using Tukey's HSD test (*α*-value ≤ 0.05). NS indicates no significant difference among values. HNT – High nighttime temperature; MET – Metamitron

		Fruits/100 flower clusters		**Average fruit weight (g)**		**Yield/tree (kg)**		**% fruits > 70 mm**	
					2017				
Lleida	**CTR**	213.1±29.6	a	129.2±7.6	c	33.1±1.7	NS	38.2±4.7	NS
	HNT	148.1±13.7	b	140.9±5.7	bc	30.8±3.6		40.3±3.6	
	MET	128.3±11.2	b	157.0±5.7	ab	33.6±1.7		50.0±3.4	
	MET+HNT	97.4±8.3	b	165.8±4.1	a	27.9±2.1		51.9±2.5	
Girona	**CTR**	203.1±12.7		104.2±2.1	b	22.1±0.7	NS	10.7±2.0	b
	HNT	156.8±16.2		113.4±2.4	b	21.5±1.5		20.5±1.0	b
	MET	161.8±32.2		110.1±1.9	b	20.0±0.4		17.5±2.4	b
	MET+HNT	177.8±30.0		136.4±5.0	a	21.6±0.9		51.2±5.8	a
					2018				
Lleida	**CTR**	73.8±3.6	a	121.2±2.2	c	42.3±1.3	NS	57.6±1.7	b
	HNT	57.0±2.2	b	140.7±3.3	b	36.7±1.4		69.0±2.6	a
	MET	54.6±4.1	bc	147.3±7.9	b	37.9±2.2		67.7±3.4	ab
	MET+HNT	43.8±3.3	c	177.7±3.6	a	40.3±2.8		75.5±2.6	a
Girona	**CTR**	132.8±11.9	a	125.5±4.1	b	37.9±3.5	NS	33.6±6.7	b
	HNT	125.8±12.1	a	131.2±7.9	b	38.1±3.7		40.4±8.2	b
	MET	79.3±9.3	b	183.2±9.5	a	32.7±1.6		82.3±3.5	a
	MET+HNT	70.8±6.3	b	178.4±9.8	a	28.6±2.5		82.6±9.0	a
Sint-Truiden	**CTR**	73.9±7.9	a	152.4±6.0	b	22.8±3.1	a	51.7±6.3	b
	HNT	55.2±1.4	ab	168.7±6.5	ab	20.3±1.8	a	69.8±5.8	ab
	MET	43.9±2.3	bc	173.7±5.8	ab	16.3±1.4	ab	69.0±5.8	ab
	MET+HNT	28.9±3.6	c	191.9±2.3	a	11.3±0.8	b	82.2±1.2	a

4.4. Discussion

4.4.1 Metamitron Concentration in the Leaves and Effect on Gas Exchanges

There are many meteorological parameters that affect chemical absorption and uptake such as radiation, humidity and temperature (Orbovic et al., 2001; Robinson et al., 2013). Higher diurnal temperature can increase chemical absorption, as observed by Orbovic et al. (2001) after spraying urea when temperature was 28ºC instead of 19ºC. Usually, chemical applications are made during the day nevertheless; nighttime temperature increases might result in an uptake increment as observed in one out of four trials distributed over two years (Sint-Truiden). Despite this one result out of four trials, we can conclude that in general nighttime temperature does not affect metamitron absorption by the leaves.

The control values of P_n observed agree with Zhou and Quebedeaux (2003), who observed an average P_n in control trees that varied between 12 and 22 µmol CO_2 m^{-2} s^{-1}. High night temperatures did not have an effect on P_n and g_s. This result is similar with those obtained by Moura et al. (2017) that observed no differences in P_n and g_s by increasing night temperature from 22 to 28 ºC in two cultivars of *Oryza sativa* L.. The application of metamitron resulted in a P_n reduction of 50%, stronger than the 19% reduction observed by Brunner (2014) in 'Golden Delicious' also with 247.5 ppm and than the 30% reduction observed by Gabardo et al. (2017), three days after spraying 350 ppm metamitron in 'Fuji Suprema' trees. In Sint-Truiden, MET+HNT showed a significantly lower P_n at 2 DAS and incomplete recovery at 10 DAS, explained by the increase in metamitron absorption verified under these conditions. Metamitron application also reduced g_s which is in line with a study developed by Rosa et al. (2020) in 'Golden' and 'Gala' trees, after the application of 247.5 ppm of metamitron.

4.4.2 Effect on Leaf Soluble Sugars

Apple leaf sugar content varies significantly depending on the time of the day, with peak concentrations for sucrose at midday, and for sorbitol later in the afternoon, both sugars comprising about 70% of total soluble sugars (Chong and Taper, 1970; Chong, 1971; Wang et al., 1999; Klages et al., 2001). Glucose and fructose not only represent a small percentage of total soluble sugars but also showed small fluctuations between night and day, and between treatments, like Klages et al. (2001) observations of diurnal changes of non-structural sugar in leaves of 'Braeburn'. The sugar alcohol sorbitol, and the disaccharide sucrose, are synthesized in source leaves and transported to fruit for supporting fruit growth in tree fruit species of the Rosaceae family (Li et al., 2018). The diurnal fluctuation of carbohydrate content is related to the temporary storage and accumulation in mesophyll tissues, whilst the decrease observed before sunrise is related to the translocation to sinks that occurs during the night (Moing, 2000). In this study, sorbitol and sucrose accumulated during the day and declined at night. There was an increase of 52% in total sugar content, more specifically, 60 and 53% in sucrose and sorbitol, respectively, from samples taken in CTR before sunrise and at midday at the 5th day after spraying, according with Klages et al. (2001) results who observed 71% and 40% more

sucrose and sorbitol content at midday, respectively. Moreover, sugars are respiratory substrates for the generation of energy and metabolic intermediates, necessary during the night to maintain the Krebs cycle. An increased respiration rate in high nighttime temperature conditions has been reported in many crops with consequent soluble sugar decreases (Kondo and Takahashi, 1987; Turnbull et al., 2002; Arevalo et al., 2008; Prasad et al., 2008; Mohammed and Tarpley, 2009; Loka and Oosterhuis, 2010; Peraudeau et al., 2015). In line with the significant decrease in sucrose and sorbitol observed in leaves sampled before sunrise from the treatments subjected to increased nighttime temperature (HNT and MET+HNT).

The values of sucrose (± 20 mg g^{-1} DW), glucose (± 25 mg g^{-1} DW), fructose (± 5 mg g^{-1} DW), and sorbitol (± 100 mg g^{-1} DW) obtained by Wünsche et al. (2005) in leaves sampled at midday, 40 days after full bloom (DAFB), are consistent with our study. In *Rosaceae* species, the immediate end products of leaf photosynthesis are sorbitol, sucrose and starch (Loescher et al., 1982), justifying the greatest differences between treatments at midday, when the trees are photosynthetically active, in some cases, attenuating the effect of high nighttime temperature and every time highlighting the CH production decrease in metamitron treatments. Our trials implemented a nighttime temperature increase that despite the efforts, was not the exact same in each trial. Moreover, meteorological conditions among the two years and three locations along with orchard characteristics have a strong influence on sugar fluctuation, which translates in responses among the trials that have the same trend, although not always showing statistical differences. Despite this, in 2018 there was significant decrease in sucrose and sorbitol caused by high nighttime temperature (HNT) which was likely caused by an increase in respiration induced by HNT. Increased nighttime temperature in cotton, resulted in a respiration rate increase finally translating in a consistent decrease of sucrose, between 64 and 80%, as compared to cotton plants exposed to low nighttime temperatures (Loka and Oosterhuis, 2010).

The strong P_n reduction caused by metamitron would likely limit photoassimilates production, which is in concordance with the lower sugar content observed in MET treatment in both years. A study developed by Rosa et al. (2020) in three apple cultivars shows significant decreases in sucrose, sorbitol and total sugars between 20-50% five days after the application of 247.5 ppm of metamitron. In mandarin, Stander et al. (2018) reported a 12% shortage in total sugars one day after spraying 300 ppm of metamitron, which persisted until 7 DAS. In this study, in 2017, we observed a 37% reduction in total sugars 5 days after spraying 247.5 ppm of metamitron, with effects lasting until 10 DAS.

The combination of MET with high nighttime temperature (MET+HNT) promoted the greatest sugar reductions. These results are likely due to a double stress effect imposed at the same time, by the high nighttime temperature that is likely increasing respiration and consuming photoassimilates, and by metamitron that is limiting the tree's P_n e restricting carbohydrate production.

4.4.3 Oxidative Stress and Antioxidative Response

Due to the interruption in the electron transport chain, metamitron may promote the transfer of electrons to alternative donors such as molecular oxygen, leading to an oxidative status (Foyer and Noctor, 2000; Noctor et al., 2002). There was an absence of MDA variation however, HNT and MET treatments increased H_2O_2 content. Nighttime temperature is one of the major environmental factors influencing plant metabolic processes, namely increase total antioxidant capacity, as observed Mohammed and Tarpley (2009) after increasing nighttime temperature from 27 ºC to 32 ºC in *Oryza Sativa* L.. The glutathione-ascorbate cycle, or Asada Halliwell pathway, is a pathway that detoxifies H_2O_2 involving a series of antioxidant metabolites such as ascorbate, glutathione and NADPH and also enzymes such as APX, GR and others (Tausz et al., 2004). Increased antioxidant levels can detoxify superoxide radicals, thereby preventing oxidative damage, which is in agreement with the high levels of glutathione observed in HNT and MET treatments and justify the lack of lipid peroxidation. In addition, HNT promoted an increase in GR activity and MET treatments (single and in combination with HNT) promoted an increase in CAT, GR, SOD and APX. Kumar et al (2012), set an experiment conducted in *Oryza Sativa* L. with increased nighttime temperature and observed an increment in H_2O_2, the higher the nighttime temperature, and in enzymatic activity of CAT, SOD, GR and APX, and also in glutathione content. APX was significantly more active in MET+HNT treatment, what might have conferred some protection, as observed by Pandey et al. (2017) when abiotic stresses were imposed to trees. Moreover, APX and CAT rises are reflected in the increased H_2O_2 levels, in pair with enhanced SOD activity and decreased ascorbate contents. Likewise, GR activity increase promoted by these treatments supports the intensified glutathione content. Tausz et al. (2004) also observed a more reduced redox state of glutathione during the acclimation period to progressing drought and considered as overcompensation that led to enhanced regeneration of glutathione. The study developed by Ma et al. (2008) in apple leaves of 2 year old potted trees showed increase in H_2O_2 concentrations and total GSH after an increase from 28 ºC to 40 ºC diurnal temperature, in agreement with our study however, Ma et al., (2008) reports that the high temperature promoted high MDA levels and increased ascorbate concentration, while in this study the results are the opposite.

The triggering of these antioxidative components is often observed under oxidative stress conditions. Therefore, overall, our findings pointed that increased oxidative stress conditions were present in HNT and all MET applied treatments but controlled by all the cell antioxidant products and by enhanced enzyme activity.

4.4.4 Effect of Environmental Conditions on Fruit Growth and Metamitron Thinning Efficacy

Chemical fruit thinning strategies are generally applied during the 2nd phase of fruit growth, the cell division and expansion period, in which the fruit grows at an exponential rate, requiring a big

demand for carbohydrates (Gillapsy et al., 1993). However, there are many physiological factors, such as spur position, crop load, seed number (Denne et al., 1963; Lakso and Goffinet, 2013) and environmental factors including diurnal and nighttime temperature, radiation (Corelli-Grappadelli and Lakso, 2004) that affect fruit growth rate, the latters, by limiting carbohydrate availability (Lakso and Goffinet, 2013).

After increasing nighttime temperature 27 and 34 DAFB, Kondo and Takahashi (1987) observed a reduction in apple fruit growth rate on the 4th day after the beginning of increased nighttime temperature, as compared with fruits exposed to natural environmental conditions. Gabardo et al. (2017) refer to a decrease in 'Maxi Gala' growth rate 7 days after a 350 ppm metamitron application. In opposition, Rosa et al. (2018) observed no changes in growth rate of 'Gala' and an increase in 'Red Delicious' and 'Pink Lady' a few days after a two time 165 ppm spraying of metamitron. In our study, both metamitron and high nighttime temperature significantly reduced fruit growth rate (Fig. 4.8). The metabolism of sorbitol and sucrose fuels fruit growth (Li et al., 2012) and as discussed in 4.4.2, leaves of both treatments experienced a sugar shortage, which finally resulted in a fruit growth rate limitation. A decrease in growth rate usually leads to fruit drop, since abscising fruits stop growing several days before (Greene et al., 2013; Lakso and Goffinet, 2013), like has observed by Kondo and Takahashi (1987).

This work showed significantly higher abscission after tree exposure to high nighttime temperatures (HNT), however only significant in both years in Lleida and in Sint-Truiden, always enhancing abscission by 30%, as compared to CTR. In addition, fruit number reduction translated in significant improvements in fruit weight and average fruit size only in Lleida (2018). Kondo and Takahashi (1987) observed an 34% increase in abscission after a nighttime temperature increase of 4.0-5.6 ºC than natural environmental conditions, 27 DAFB, in 8 year old 'Starking Delicious' apple trees. Moreover, a study with potted 'Empire' trees in which nighttime temperature was increased from 13 ºC to 18 ºC and 21 ºC, during 5 nights, promoted a reduction in fruit set (more fruit abscission) from 39.2% in CTR, to 17.8% and 19.3% (Yoon et al., 2011).

Metamitron significantly reduced the number of fruits per 100 flower clusters in all trials except in Girona (2017), between 26 and 40%, usually with significant improvements in fruit quality and without yield losses. However, except for Girona (2018), the thinning and fruit quality improvements caused by 247.5 ppm of metamitron was similar to the effect cause by 5 nights of increased nighttime temperature.

Since every orchard is a unique combination of tree vigor, environment and management, the response to environmental changes such as high nighttime temperature or chemical thinners as metamitron is not always linear between years and locations. When metamitron was combined with high nighttime temperatures, in some cases there was not an extra thinning effect as compared with MET, whereas in both years in Lleida and in Sint-Truiden, the combination of MET+HNT consistently promoted tendencies for stronger fruit abscission as compared with MET alone. In some trials, MET+HNT was the only treatment that promoted a significantly

increased fruit weight and fruit size. Moreover, the only yield reduction (over-thinning) observed within the 5 performed trials was caused by MET+HNT, in Sint-Truiden. The effect of environmental conditions, namely nighttime temperature, on the efficacy of chemical thinners has been described by several authors (Lakso et al., 1999; Byers et al., 2002) and included in models, either to estimate carbohydrate balance (Lordan et al., 2019) or to accurately predict the thinning effect of metamitron based on irradiance and nighttime temperature (Clever, 2018). Stern (2015) sprayed 190 ppm of metamitron in 'Golden Delicious' trees in the warm climate of Israel, in which nighttime temperature during the 3 weeks after spraying varied between 12.8 and 14.6 ºC in the three trials, and observed a strong fruit abscission (with a 10 fold increase in kg in fruit size class > 70 mm). Metamitron efficacy in 'Golden Delicious' trials set up by Stern (2015) were higher than the ones obtained by Brunner (2014), using 247.5 ppm, by Gabardo et al. (2017) using higher metamitron dosages in 'Fuji', or than the results here obtained. All the previously mentioned studies were performed in regions in which average nighttime temperature is about 10 ºC lower than in Israel by the time application was made. Similar strong abscission results were obtained in 'Gala' by Stern (2014). This author attributes the efficacy of the relatively low dose compared to those used in Europe and the USA, to the higher night temperatures for 3 weeks after application, which increased dark respiration at a critical point of fruitlet growth and caused assimilation deficiencies that triggered the abscission process. The same explanation applies to our results.

4.5 Conclusions

It is more and more accepted that thinning is highly depend on carbon hydrate balance being the nighttime temperature an environmental factor that has a great impact on carbohydrate content. Consequently, nighttime temperature after metamitron application has an influence on fruit abscission and on the chemical thinning response. High nighttime temperature did not affect metamitron absorption neither stomatal conductance, however it was observed a faster consumption of the carbohydrates synthesized during the daytime likely because of enhanced leaf respiration. Metamitron and warm nighttime temperatures intensify competition for carbohydrates at a time when metabolic demand is highest in the tree, the first by reducing P_n and consequently, sucrose and sorbitol production decline, and the second, through a respiratory-driven reduction in leaf carbohydrate concentration, in both cases finishing in fruit growth rate decline and increased abscission.

No changes in MDA indicate inexistence of lipid peroxidation however, an increase in the H_2O_2 content was observed in apple leaves in response to high nighttime temperature and metamitron application in the present study, which indicates that oxidative stress has occurred under these conditions. The results suggest that the ascorbate–glutathione cycle is up-regulated in response to high nighttime temperature that together with the increased activity of CAT, GR, SOD and APX contributed to the maintenance of the oxidative status and avoided cell damage.

Thus, weather monitoring, namely nighttime temperature, during the days after the spraying date, allow the prediction of periods that origin negative carbohydrate balance situations, when fruit is most susceptible to thinning, allowing to determine the best timing and rate of chemical application. Nevertheless, there are other factors affecting the tree susceptibility that need further research, namely the effect of nighttime temperature before metamitron application, which likely might also cause changes in carbohydrate balance and enhance the thinning efficacy.

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CHAPTER 5

Implications of nighttime temperature on metamitron impacts on the photosynthetic machinery functioning of *Malus* x *domestica* Borkh.

Potted trees in the growth chamber in Oeiras

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5. Implications of nighttime temperature on metamitron impacts on the photosynthetic machinery functioning of Malus x domestica Borkh.

Abstract

Metamitron (MET) is a fruitlet thinning compound for apple trees, needing better understanding of its action on leaf energy metabolism, depending on nighttime temperature. A trial under environmental controlled conditions was set with 'Golden Reinders' potted trees, under 25/7.5 and 25/15 ºC (diurnal/nighttime temperature), with (MET, 247.5 ppm) or without (CTR) application, and considering the monitoring of photosynthetic and respiration components from day 1 (D1) to 14 (D14). Net photosynthesis (P_n) decline promoted by MET after D1 was not stomatal related. Instead, non-stomatal constraints, reflected on the photosynthetic capacity (A_{max}), included a clear photosystem (PS) II inhibition (but barely of PSI), as shown by severe reductions in thylakoid electron transport at PSII level, maximal (F_v/F_m) and actual (F_v'/F_m') PSII photochemical efficiencies, estimate of quantum yield of linear electron transport ($Y_{(II)}$), and the rise in PSII photoinhibition status (F_s/F_m' and PI_{Chr}) and uncontrolled energy dissipation ($Y_{(NO)}$). To P_n inhibition also contributed the impact in RuBisCO along the entire experiment, regardless of night temperature, here reported for the first time. Globally, MET impact on the photosynthetic parameters was usually greater under 7.5 ºC, with maximal impacts between D4 and D7, probably associated to a less active metabolism at lower temperature. Cellular energy metabolism was further impaired under 7.5 ºC, through moderate inhibition of NADH-dependent malate dehydrogenase (MDH) and pyruvate kinase (PK) enzymes involved in respiration, in contrast with the increase of dark respiration in MET 7.5 until D7. The lower impact on PK and MDH under 15 ºC and a likely global higher active metabolism at that temperature would agree with the lowest sucrose levels in MET 15 at D4 and D7. Our findings showed that MET alters the cell energy machinery in a temperature dependent manner, affecting the sucrose balance mainly at 15 ºC, justifying the observed greater thinning potential.

Keywords: carbon assimilation, fruit thinning, photosynthesis inhibition, PSII functioning, soluble sugars

Abbreviations

A_{max} - Photosynthetic capacity (µmol O_2 m^{-2} s^{-1}); CH – Carbohydrate; CTR – Control; DW – Dry weight; F_0 – Minimal fluorescence from the antennae; F_s/F_m' – Predictor of the rate constant of PSII inactivation; F_v/F_m – Photochemical efficiency of PSII; F_v'/F_m' – PSII photochemical efficiency under light exposure; FW – Fresh weight; g_s – Stomatal conductance do water (mmol H_2O m^{-2} s^{-1}); MDH - malate dehydrogenase; MET – Metamitron; OEC – Oxygen-evolving complex; PI – Photoinhibition Index; PK – Pyruvate kinase; P_n – Net photosynthetic rate (µmol CO_2 m^{-2} s^{-1}) ; PS – Photosystem; Q_A – Plastoquinone A; Q_B – Plastoquinone B; q_L –

Photochemical quenching based on the concept of interconnected PSII antennae; R_d – Dark respiration (µmol O_2 m^{-2} s^{-1}); RuBisCO – Ribulose-1,5-bisphosphate carboxylase/oxygenase; RWC – Relative water content (%); V_i – RuBisCO initial activity (µmol m^{-2} s^{-1}); V_t – RuBisCO total activity (µmol m^{-2} s^{-1}); $Y_{(II)}$, $Y_{(NPQ)}$, $Y_{(NO)}$ – Estimates of the quantum yields of non-cyclic electron transfer, photoprotective regulated energy dissipation, and non-regulated energy dissipation (heat and fluorescence) of PSII, respectively.

5.1 Introduction

Climate change and global warming is a reality the world is facing (IPCC, 2015), carrying along unpredicted impacts to natural ecosystems and agriculture and a serious threat to agricultural sustainability, both regarding the amount and quality of agricultural products (Beach et al., 2015; Tack et al., 2015; Pais et al., 2020), among them fruit crops such as apple trees (*Malus* x *domestica* Borkh.). Understanding how these conditions affect the orchard management is one step further to solve practical problems, such as yield variability, and improve the crop economic and environmental sustainability.

Studies have shown that increases in nighttime temperature during the crucial period of rapid apple fruit growth, in which the fruit is highly dependent on photoassimilate production, enhance the formation of the fruit abscission zone and increase fruit drop (Kondo and Takahashi, 1987; Stern, 2014; Clever, 2018; Rosa et al., 2020b). This is likely associated with an increase in dark respiration and consequent increase in this metabolic pathway, leading to the increase of respiratory enzymes activity, as previously reported in other plants, such as *Oryza sativa* L. (Mohammed and Tarpley, 2009; Peraudeau et al., 2015), and *Populus deltoides* W. Bartram ex Marshall (Turnbull et al., 2002; Loka and Oosterhuis, 2010). However, in apple tree extensive studies comprising this subject were still not developed.

Thinning is a common practice among growers to reduce crop load while improve fruit size and quality parameters. The thinning strategy needs to be adjusted every year, depending on the fruit set, and desired crop load, and considering environmental conditions, the latter responsible which can for strong differences of the product efficacy between years and regions (Lakso et al., 2001a; Robinson and Lakso, 2004), frequently in an unpredictably manner.

Metamitron is a triazinone herbicide, a systemic xylem-translocated compound photosynthesis inhibitor, which can be used as chemical thinner. It can be absorbed by roots and directly by leaves (Aper et al., 2012), and acts as a photosystem II (PSII) inhibitor (Corbet, 1974), binding on protein D1, interrupting the electron transport chain between Q_A and Q_B (Horovitz et al., 1988; Guidi and Degl'Innocenti 2011). By disrupting the thylakoid electron transport, photosynthesis is decreased, and excess excited energy cannot be consumed via CO_2 assimilation, activating mechanisms of energy dissipation. Therefore, metamitron can strongly reduce photosynthetic carbon fixation and may contribute to a negative carbohydrate balance, and enhance fruit drop (Stander et al., 2018; Rosa et al., 2020 a, b). In fact, it has been reported

that a shortage in carbohydrate availability can trigger fruit abscission (Byers et al., 1990, 1991; Lakso and Corelli Grapadelli, 1992; Lordan et al., 2019). Consequently, the photosynthetic metabolism that provides photoassimilates and energy (ATP), and the respiratory pathway that uses sugar to produce energy to cellular metabolic needs, can regulate the tree carbohydrate balance, both of which depending on the meteorological conditions. This justifies the need to deepen the knowledge regarding the action mode of single and combined metamitron and nighttime temperature on the photosynthetic and respiration pathways.

A previous study under field conditions with apple trees allowed to conclude that metamitron application followed by higher nighttime temperatures strongly decreased leaf sucrose and sorbitol content, what was associated with an increased fruit drop (Rosa et al., 2020 b). Our hypothesis is that night temperature will affect the thinning extent of MET, what need that the mechanisms underlying for such impact on fruit thinning must be unveiled. Therefore, the objective of this study was to better identify the physiological and biochemical mechanisms behind the metamitron action and the impact of nighttime temperature. For that, it was analyzed the absorption of metamitron (and its degradation) at leaf level, and its impact in several parameters related with the photosynthesis (*e.g.*, gas exchanges, PSII photochemical efficiency, thylakoid electron transport rates), and respiration, including the impact of key enzymes of both pathways, as well as the resulting impact on non-structural carbohydrates.

5.2 Material and Methods

5.2.1 Plant Material and Experimental Design

A total of 32 potted four years old apple trees *Malus* x *domestica* Borkh cv. Golden Reinders grown in 50 L pots, grafted in M9, were transferred from a greenhouse (ambient [CO_2]) into two walk-in growth chambers (EHHF10000, ARALAB, Oeiras, Portugal) at BBCH 01 stage. The plants were then grown under controlled environmental conditions of temperature (25/7.5 ºC, day/night), RH (75%), irradiance (600-700 µmol m^{-2} s^{-1}), photoperiod (14 hours) and CO_2 concentration (400 µL L^{-1}) for 45 days to acclimate. After this period, at fruit stage 10-12 mm fruit diameter, nighttime temperature was increased in one of the growth chambers and four treatments were established: CTR 7.5 (7.5/25 ºC, no metamitron), MET 7.5 (7.5/25 ºC, 247.5 ppm metamitron), CTR 15 (15/25 ºC, no metamitron) and MET 15 (7.5/25 ºC, 247.5 ppm metamitron) with eight trees per treatment.

On the application day, half of the plants were taken out of the chamber and sprayed with 247.5 ppm (1.65 kg ha^{-1}) of metamitron (Brevis®, ADAMA, Tel Aviv, Israel) with a hand sprayer (Vito, Portugal) and brought inside (4 h later) when the leaves were dry.

Each of the 8 plants per treatment used to perform measurements and collect material for further determinations was fertilized and irrigated according to the good agricultural practices. A large set of photosynthetic and respiratory related parameters was evaluated 1, 4, 7 and 14

days after metamitron application, in recently mature shoot leaves. from the top branches (light exposed). For eco-physiological (non-destructive) evaluations it were used two leaves per each of the four plants/replicates, whereas for biochemical (destructive) analysis, four leaves (approximately 2 g of leaf material) per plant was collected after approximately 2 h of illumination, frozen in liquid nitrogen and stored at -80 °C until analysis. Whenever possible, all analyses were performed on the same leaves.

5.2.2 Metamitron Leaf Analysis

Metamitron extraction was conducted as described in detail in Rosa et al. (2020 a) based in Lesueur et al., 2008. Briefly 500 mg fresh weight (FW) of frozen leaf powder was used and extraction made using the QuEChERS method. Quantification was made with resource to a LC-MS/MS (Waters, Milford, USA). Standard curves were used for the quantification of metamitron (Sigma, St. Louis, USA) and desamino-metamitron-desamino (LGC Standards, Teddington, UK). Besides the already described moments of sampling, for metamitron determination sampling was also performed 2 days after spraying metamitron.

5.2.3 Relative Water Content

For leaf relative water content (RWC) was evaluated as in Ramalho et al. (2014). Briefly, eight leaf discs (0.5 cm2 each) were cut from two leaves per plant. The fresh weight (FW) was determined immediately after cutting the discs, the turgid weight (TW) after overnight rehydration of the discs in a humid chamber at ca. 20 °C, and the dry weight (DW) after drying the discs at 80 ºC for 24 h. The RWC, expressing the water content at a given time in relation to full turgor, was calculated as: RWC (%) = (FW – DW/TW – DW) x 100.

5.2.4 Membrane Impact

The impact of metamitron on cell membrane was evaluated through electrolyte leakage, as described elsewhere (Dias et al., 2010). Briefly, 10 leaf discs (0.5 cm2 each) from two leaves per plant were cut and immediately rinsed 3 times (approximately 1 min) with demineralized water, and subsequently floated on 10 mL of demineralized water at 20 ºC. The electrolyte leakage readings were taken after 22 h of floating, using a conductometer (Crison GLP31, Crison Instruments, S.A., Barcelona, Spain). Total conductivity was obtained exposing the sample flasks to 90 ºC for 2 h and after cooling. Leakage results were expressed as percentage of total conductivity.

5.2.5 Leaf Gas Exchanges Analysis

Leaf net photosynthetic rate (Pn) stomatal conductance to water vapour (gs) were assessed under steady-state photosynthetic conditions under the growth chamber conditions, from four trees after 1:30-2:00 h of illumination, using a portable open-system infrared gas analyzer (CIRAS 3, PP Systems, Amesbury, USA).

The photosynthetic capacity (Amax) measurements were performed as previously described (Ramalho et al., 2018). Briefly, Amax was measured through O2 evolution in a Clark-type leaf disc O2 electrode (LD2/2; Hansatech, UK) in leaf discs (1.86 cm2) under saturating conditions of CO2 (ca. 7%), and irradiance (PPFD ca. 900 µmol m-2 s-1, provided by a Björkman lamp, Hansatech, Norfolk, UK), at 25 ºC.

Dark respiration rate (Rd) representing the consumption of O2 was measured through O2 evolution in a Clarktype O2 electrode (LD2/2, Hansatech, Norfolk, UK) using leaf discs (1.86 cm2), in the dark, at either 7.5 and 15 ºC.

5.2.6 Chlorophyll *a* Fluorescence Parameters

Chlorophyll (Chl) a fluorescence parameters were determined in the same type of same leaves used for the gas exchange measurements, using a PAM-2500 system (H. Walz, Effeltrich, Germany), following the formulae and meaning of each parameter discussed elsewhere (Kramer et al., 2004; Krause and Jahns, 2004; Schreiber, 2004; Klughammer and Schreiber, 2008; Huang et al., 2011; Stirbet and Govindjee, 2011). Briefly, measurements of the minimal fluorescence from the antennae, F0, and photochemical efficiency of PSII, Fv/Fm, were performed on overnight dark-adapted leaves. F0 denotes the fluorescence emission by the excited Chl a molecules before excitation energy migrate to the reaction centers and was determined using a weak light (<0.5 μmol m−2 s−1). Fv/Fm reflects the maximal PSII photochemical efficiency and was obtained using a 0.8 s saturating pulse of ca. 6500 μmol m−2 s−1 of actinic light.

A second set of parameters were assessed under photosynthetic steady-state conditions, under approximately 650 μmol m-2 s-1 of actinic light and superimposed saturating flashes. This included the calculation of qL, qN, Y(II), Y(NPQ), Y(NO) and Fv´/Fm´ (Kramer et al., 2004; Klughammer & Schreiber, 2008) and Fs/Fm' (Stirbet and Govindjee, 2011). The F0', necessary for the quenching calculations was obtained in the dark immediately after actinic light was switched off and before the first fast phase of fluorescence relaxation kinetics. Fv'/Fm' expresses the PSII efficiency of energy conversion under light exposure. qL is the photochemical quenching based on the concept of interconnected PSII antennae and represents the proportion of energy captured by open PSII centers and driven to photochemical events, and Fs/Fm' is a predictor of the rate constant of PSII inactivation (Stirbet and Govindjee, 2011).

Estimates of photosynthetic quantum yields of non-cyclic electron transfer (Y(II)), photoprotective regulated energy dissipation of PSII (Y(NPQ)), and non-regulated energy dissipation (heat and fluorescence) of PSII (Y(NO)), were also obtained (Kramer et al., 2004; Huang et al., 2011), where (Y(II)+Y(NPQ)+Y(NO)=1).

Additionally, it were estimated the PSII photoinhibition indexes of: A) chronic photoinhibition (PIChr), representing the percent reduction in Fv/Fm at each temperature relative to the maximal Fv/Fm obtained during the entire experiment; B) dynamic photoinhibition (PIDyn), representing the decline in Fv/Fm that is fully reversible overnight, being measured as the percent reduction in midday Fv'/Fm' relative to Fv/Fm at each temperature, relative to the maximal Fv/Fm from the entire experiment; C) total photoinhibition (PITotal = PIChr + PIDyn), following Werner et al. (2002).

5.2.7 Thylakoid Electron Transport Rates

Pools of leaves (ca. 5 g FW) from four plants were used to obtain sub-chloroplast membrane fractions, following the procedures of Droppa et al. (1987), with minor modifications, which excluded ascorbate from the homogenization buffer since this antioxidant partly removes the metamitron action, confirming previous reports of Fedtke and Schmidt (1983) likely associated with ascorbate role in metamitron deamination (Fedtke and Schmidt, 1979).

The assays were performed using 1 mL of the reaction mixture (containing ± 100 mg Chl) and measured polarographically using a Clark-type O2 electrode (LW2, Hansatech, Norfolk, UK) at 25 ºC, under a PPFD of approximately 3000 µmol m-2 s-1 given by a Björkman lamp (Hansatech, Norfolk, UK).

For the in vivo electron transport rates associated with PSII activity including the oxygen evolving complex, OEC (H2O→DCPIP) 400 µL of 1 mmol L-1 DCPIP was used as electron acceptor from the quinone pool. For the PSII activity without the OEC complex (DPC→DCPIP) 25 µL of 25 mmol L-1 DPC (as electron donor to PSII) and 400 µL of 1 mmol L-1 DCPIP were used. The electron transport associated to PSI (DCPIPH2 →MV) was measured using 200 µL of 250 mmol L-1 DCMU (to inhibit the electron transport before the PQ-9), 400 µL of 1 mmol L-1 DCPIP, 25 µL of 50 mmol L-1 ascorbate (for reduction of DCPIP which in turn will act as electron donor to cyt f), and 200 µL of 10 mmol L-1 MV as electron acceptor were used. Sodium azide (400 µL from a 6 mol L-1 solution) was used in all the reactions to inhibit peroxidase activity.

Total chlorophylls were extracted from four freshly cut leaf discs (0.5 cm2 each) using 80% (v/v) aqueous acetone and quantified according to procedures described elsewhere (Lichtenthaler, 1987).

5.2.8 Photosynthetic and Respiratory Enzymes Activity

Samples of 100 mg FW of powdered frozen leaf material were used to evaluate the activity of several enzymes involved in carbon metabolism. Samples were processed as described in Semedo et al. (2020), being homogenized in a cooled mortar using 100 mg insoluble PVPP and 1 mL of the extraction buffer 100 mM Tris-HCl (pH 8), which contained 10 mM MgCl2, 10 mM ß-mercaptoethanol, 2 mM DTT, 1% (v/v) Triton X-100, 10% (v/v) glycerol and a “complete-protease inhibitor cocktail” 2% (v/v) designed to protect the enzymes from protease action (Roche, ref. 04693159001). The extracts were centrifuged (16,000 g, 20 min, 4 °C) and the clean supernatant was used for the enzyme assays, all of which were based on NADH oxidation at 340 nm, at 25 °C, in 1 mL final volume in the cuvette.

The initial and total carboxylation activities of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO: EC 4.1.1.39) were assayed by using 20 µL of the supernatant, exactly as described in Semedo et al. (2020), based in Tazoe et al. (2008).

The activities of pyruvate kinase (PK: EC 2.7.1.40) and NADH-dependent malate dehydrogenase (MDH: EC 1.1.1.37), which are enzymes involved in the respiratory pathway, were assayed by using 20 µL of the supernatant exactly as described in Ramalho et al. (2013), based in the methods of Diaz et al. (1996) and Lopez-Millan et al. (2000), respectively.

5.2.9 Non-structural Carbohydrates Evaluation

Samples for leaf soluble sugars assessment were collected at two moments: after a 10 h dark period, immediately before turning on the lights, and after 2 h of illumination. Soluble sugars were determined in ± 150 mg FW per plant of powdered frozen material, based on Damesin and Lelarge (2003). Briefly, the samples were homogenized in 2 mL of cold H2O, left to extract for 20 min on ice and centrifuged (12,000x g, 5 min, 4 °C). The supernatant was boiled to denature the proteins (3 min), placed on ice (6 min) and centrifuged again. The obtained clear solution was then filtered (0.45 µm, nylon) before the injection of a 50 µL aliquot into an HPLC system equipped with a refractive index detector (Model 2414, Waters, Milford, USA). The separation of sugars was performed using a Sugar- Pak 1 column (300 x 6.5 mm, Waters, Milford, USA) at 90 ºC, with H2O as the eluent (containing 50 mg EDTA-Ca L-1 H2O), at a flow rate of 0.5 mL min-1. Standard curves were used for the quantification of each sugar.

5.2.10 Statistical Analysis

The various measured and calculated parameters were analyzed using a one-way ANOVA to evaluate the differences between treatments on one single day, and a two-way ANOVA to evaluate the differences between the treatments, across the several days after (with and without metamitron spraying), followed by a Tukey's test for mean comparisons. A 95% confidence level was adopted for all tests. The statistical analysis was performed using Statistix 9 (Analytical Software, Tallahassee, Florida).

5.3 Results

5.3.1 Leaf Metamitron Absorption and Degradation

A consistent tendency for greater metamitron (MET) absorption was observed under higher (15 ºC) than at lower (7.5 ºC) nighttime temperature, although significantly only by D2 when approximately doubled its content (Fig. 5.1A). Although not significant variation was found along the experiment within each nighttime temperature treatment, MET tended to decline after D4 (MET 15) or D7 (MET 7.5), thus indicating a quite long persistence inside the leaves. These findings were somewhat consistent with significant increase of desamino-metamitron (the main degradation metabolite of MET) only by D14 in both nighttime temperature treatments, despite the gradual rising trend from D1 onwards (Fig. 5.1B). Notably, MET degradation metabolite become significantly greater under 7.5 ºC than 15 ºC from one week onwards after MET application.

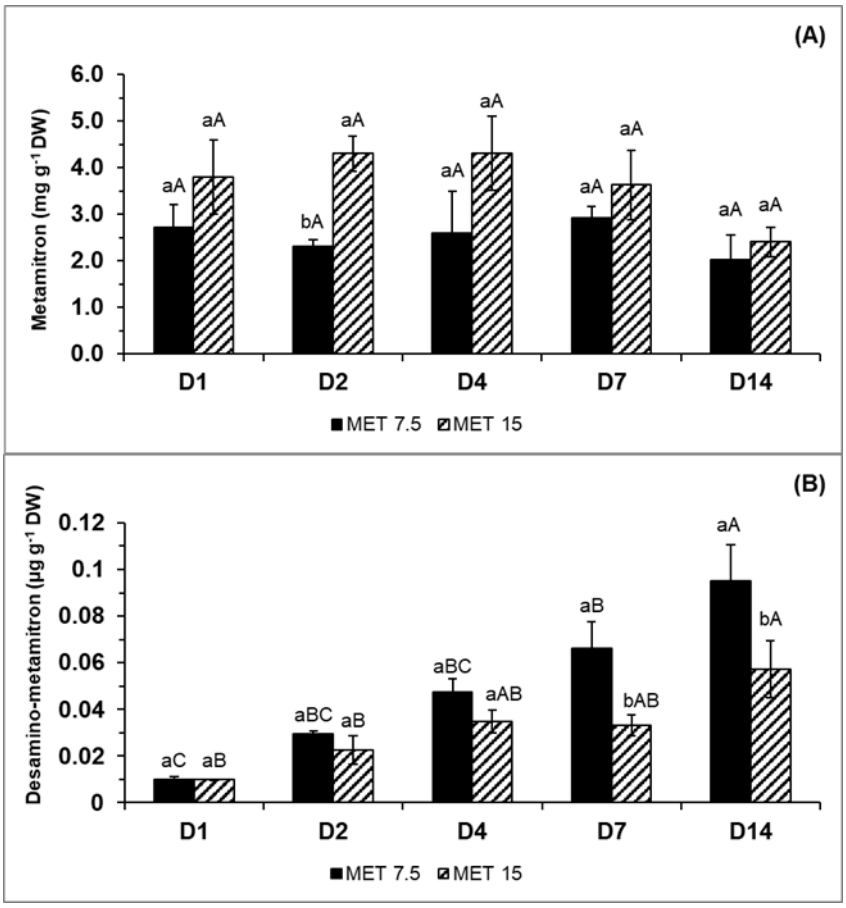

Figure 5.1 - Variation of metamitron (A) and desamino-metamitron (B) in Malus x domestica Borkh cv. Golden Reinders plants submitted to nighttime temperature of 7.5 ºC or 15 ºC, under control (CTR) and metamitron sprayed (MET) conditions 1, 2, 4, 7 and 14 days after metamitron application. For each parameter, the mean values ± SE (n = 4) followed by different letters express significant differences between treatments for the same day (a and b) or between days within each treatment (A, B and C).

5.3.2 Leaf Relative Water Content and Membrane Impacts

Among treatments, leaf hydration, here evaluated by means of RWC, significantly increased in all treatments when compared with CTR 7.5, only by D4 (Fig. 5.2A), what might be related, at least partly, with a g_s decline of those treatments (see Fig 5.2B).

Electrolyte leakage values were not significantly altered by either MET or nighttime temperature along the entire experiment, thus reflecting an absence of important impacts at membrane selectivity level (Fig. 5.2B).

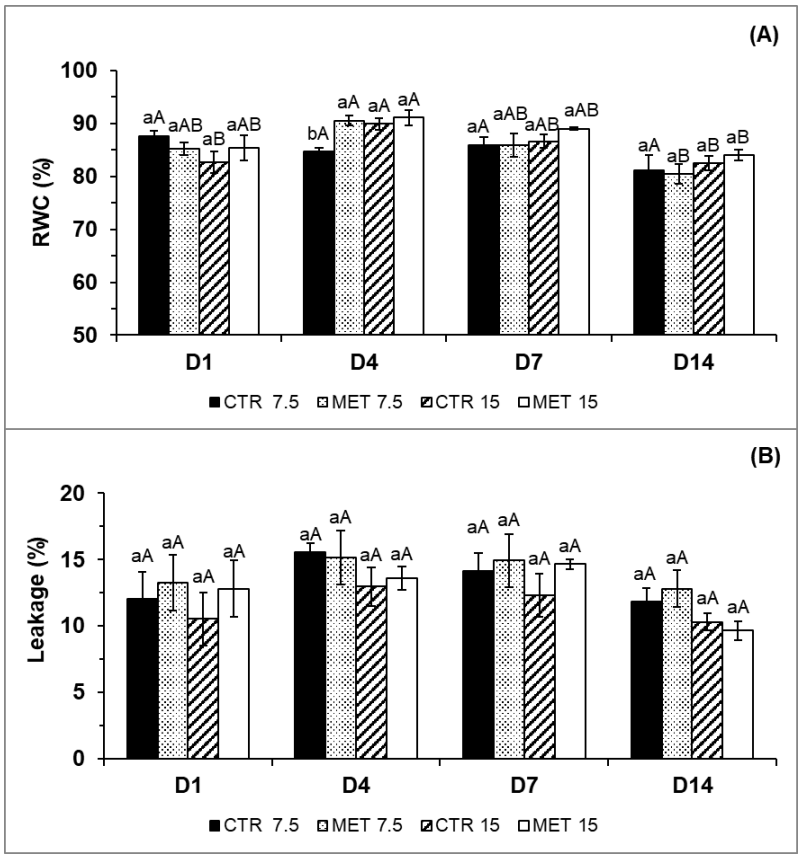

Figure 5.2 - Changes in relative water content (A) and leakage (B) in Malus x domestica Borkh cv. Golden Reinders plants submitted to nighttime temperature of 7.5 ºC or 15 ºC, under control (CTR) and metamitron sprayed (MET) conditions 1, 4, 7 and 14 days after metamitron application. For each parameter, the mean values ± SE (n = 4) followed by different letters express significant differences between treatments within each day (a and b), or between days within each treatment (A and B), after a Tukey's HSD test (p-value ≤ 0.05).

5.3.3 Leaf Gas Exchanges Analysis

Nighttime temperature did not affect net photosynthetic rate (Pn) along the experiment under control conditions. Metamitron effect was observed since D1, although significantly only after D4 and with a recovery by D14, with greater impact at 7.5 ºC than 15 ºC by D4 and D7, when MET 7.5 when reached minimum values representing 42 and 36% of their respective CTR 7.5 values (Fig 5.3A). However, due to a significant recovery of MET 7.5, no significant differences were observed among all treatments by D14. The lower impacts in MET 15 by D4 and D7 were not related with stomatal conductance to water vapor (gs), since at 15 ºC gs values with or without MET where lower (D4) or similar (D7) than those at 7.5 ºC. Additionally, MET impact on P_n at 7.5 ºC from D4 onwards was also independent of gs, since no significant differences were observed between MET and CTR plants in those days (Fig. 5.3B). This pointed to lower non-stomatal impacts under 15 ºC, as suggested by the slight, but systematic, greater photosynthetic capacity values (Amax) in MET 15 along the entire experiment, as compared to MET 7.5 (Fig. 5.3C). Additionally, greater nighttime temperature reduced gs until D4 in both

CTR and MET conditions, but as for P_n, the gs values did not differ among treatments by D14, whereas Amax still showed an incomplete recovery in MET plants, with a greater impact persisting in MET 7.5.

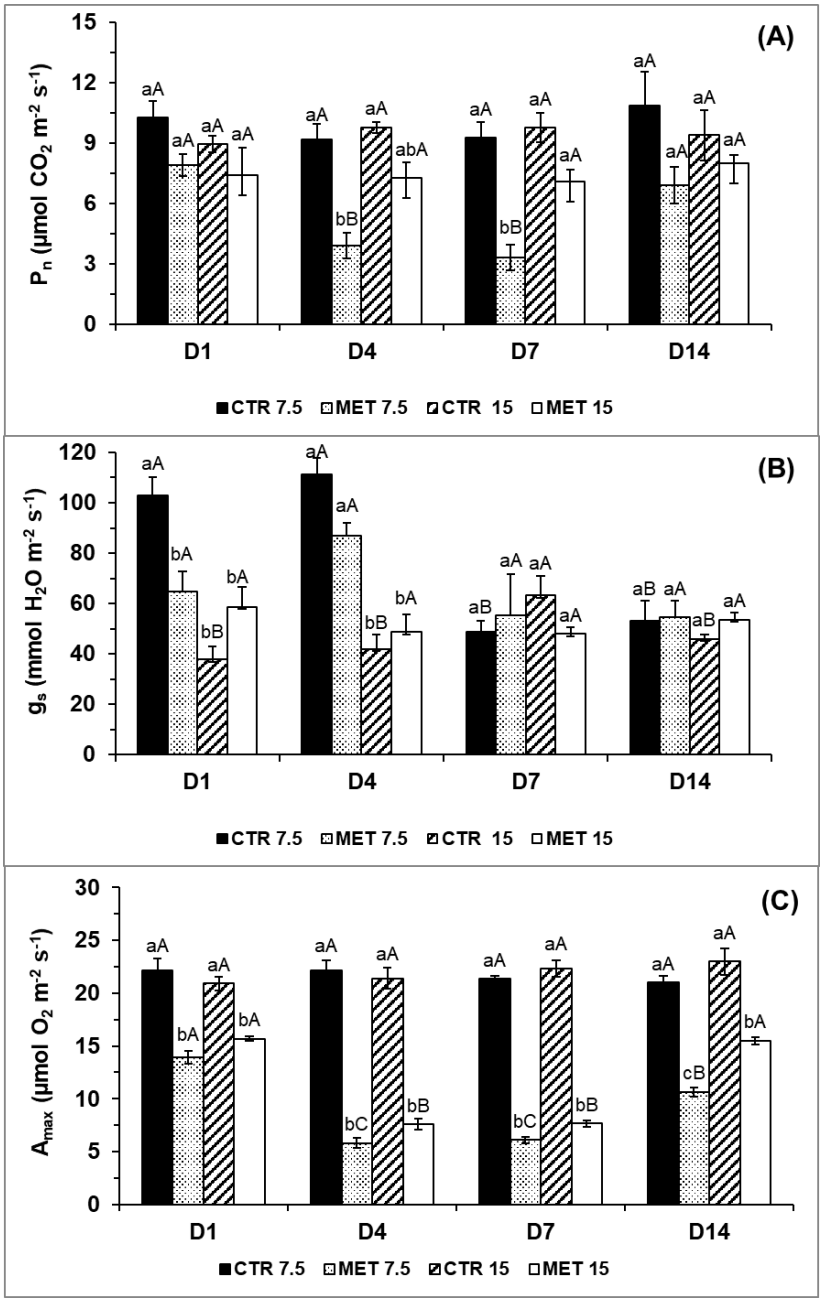

Figure 5.3 - Changes in leaf net photosynthesis (P_n) (A), stomatal conductance to water vapor (g_s) (B) and photosynthetic capacity (A_{max}) (C) in *Malus* x *domestica* Borkh cv. Golden Reinders plants submitted to nighttime temperature of 7.5 ºC or 15 ºC, under control (CTR) and metamitron sprayed (MET) conditions 1, 4, 7 and 14 days after metamitron application. For each parameter, the mean values ± SE (n = 4) followed by different letters express significant differences between treatments within each day (a, b, and c), or between days within each treatment (A, B, and C), after a Tukey's HSD test (p-value ≤ 0.05).

5.3.4 Dark Respiration

In CTR plants the nighttime temperature rise from 7.5 to 15 ºC enhanced dark respiration (Rd) until D7, although significantly only on D1, when the value reached 4-fold as compared to CTR 7.5 (Fig. 5.4). Notably, Rd was also increased by MET, but only in the plants under 7.5 ºC (significantly also only on D1). At the end of the experiment (D14) similar Rd values were observed across all treatments.

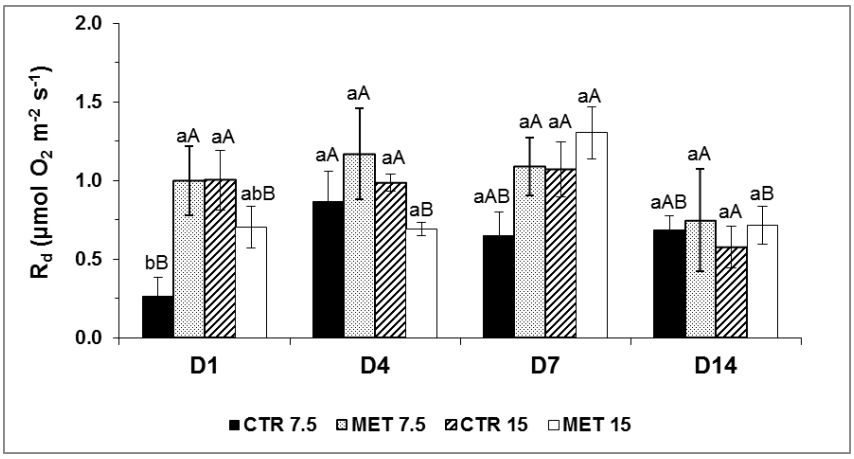

Figure 5.4 - Changes in dark respiration (R_d) in *Malus* x *domestica* Borkh cv. Golden Reinders plants submitted to nighttime temperature of 7.5 ºC or 15 ºC, under control (CTR) and metamitron sprayed (MET) conditions 1, 4, 7 and 14 days after metamitron application. For each parameter, the mean values ± SE (n = 4) followed by different letters express significant differences between treatments within each day (a and b), or between days within each treatment (A and B), after a Tukey's HSD test (p-value ≤ 0.05).

5.3.5 Chlorophyll a Fluorescence Parameters

The increase in nighttime temperature from 7.5 to 15 ºC (CTR) did not usually significantly changed most evaluated chlorophyll a fluorescence parameters, except for a reduction in Y_{NPQ} (although significantly only on D7).

In sharp contrast, MET application largely changed most parameters, usually with maximal impacts in D4 and D7, and a recovery thereafter. This was observed in the parameters obtained under dark adapted conditions (F_0, F_v/F_m) and under photosynthetic steady-state conditions, regarding both the ones associated with the photosynthetic performance (e.g., $Y_{(II)}$, F_v'/F_m'), and with uncontrolled energy dissipation processes ($Y_{(NO)}$) and inactivation of PSII (e.g., F_s/F_m', PI_{Chr}, PI_{Total}), thus denoting a worse use of energy through photochemical processes and the presence of impairments (Table 5.1). Although this was found for the plants under both temperatures, a tendency to greater and longer impact persistence was usually observed at MET 7.5 than MET 15 (except for q_L).

5.3.6 Thylakoid Electron Transport Rates

The thylakoid electron transport rates involving the PSII followed a similar pattern regardless of the presence (PSII+OEC) or absence (PSII-OEC) of the oxygen evolving complex (Fig. 5.5A and B). The single nighttime temperature rise promoted a significant increase in the potential activity of PSII with and without the presence of OEC, between 16 and 43% on D1 and D4, what was reverted afterwards with CTR 15 showing reductions between 9 and 22%, as compared to CTR 7.5 in D7 and D14, respectively.

Metamitron promoted severe PSII activity reductions along the entire experiment under either 7.5 or 15 ºC, with maximal impacts on D4 of 83 and 84% (PSII+OEC) and 80 and 80% (PSII-OEC) in MET 7.5 and MET 15, respectively, as compared to their respective controls. After D4 a gradual recovery was observed, but large significant impacts persisted by D14, with values well below half of their CTR plants. Additionally, despite the similar trend under both temperatures, the MET 15 plants showed somewhat greater PSII activity values than their MET 7.5 counterparts after D4.

The electron transport rates associated with PSI also increased under the single increased nighttime temperature in D1 and D4, by 23 and 55%, respectively (Fig. 5.5C). On the other hand, MET had a much lower impact in PSI than that observed in PSII, with a reduction of about 20% in MET 7.5 by D7 and D14. Under 15 ºC, MET led to a reduction 24% by D4, but no other effect was found including the absence of persisting impacts by the end of the experiment.

Table 5.1 - Evaluation of leaf chlorophyll *a* fluorescence parameters in *Malus* x *domestica* Borkh cv. Golden Reinders plants submitted to nighttime temperature of 7.5 ºC or 15 ºC, under control (CTR) and metamitron sprayed (MET) conditions, 1, 4, 7 and 14 days after metamitron application. Parameters include: initial fluorescence (F_0), maximum *PSII* photochemical efficiency (F_v/F_m), photochemical quenching coefficient (q_L), actual PSII photochemical efficiency of energy conversion (F_v'/F_m'); and the rate constant of PSII inactivation (F_s/F_m'), as well as the estimate of quantum yields of non-cyclic electron transport ($Y_{(II)}$), of regulated energy dissipation in PSII ($Y_{(NPQ)}$), and of non-regulated energy dissipation in PSII ($Y_{(NO)}$), and the indexes for chronic (PI_{Chr}), dynamic (PI_{Dyn}), and total (PI_{Total}) photoinhibition. For each parameter, the mean values ± SE (n = 4) followed by different letters express significant differences between treatments within each day (a, b, and c), or between days within each treatment (A, B, and C), after a Tukey's HSD test (p-value ≤ 0.05).

		D1				D4				D7				D14			
F_o	**CTR 7.5**	0.260	±	0.002	abA	0.281	±	0.006	bA	0.292	±	0.016	bcA	0.232	±	0.006	bB
	MET 7.5	0.313	±	0.016	aB	0.523	±	0.064	aA	0.526	±	0.037	aA	0.388	±	0.025	aB
	CTR 15	0.237	±	0.004	bA	0.239	±	0.004	bA	0.235	±	0.003	cA	0.208	±	0.013	bA
	MET 15	0.300	±	0.014	abB	0.485	±	0.041	aA	0.425	±	0.049	abAB	0.309	±	0.019	abB
F_v/F_m	**CTR 7.5**	0.831	±	0.007	aA	0.803	±	0.011	aA	0.806	±	0.009	aA	0.819	±	0.008	aA
	MET 7.5	0.768	±	0.028	abA	0.667	±	0.034	bAB	0.632	±	0.033	bB	0.751	±	0.018	bA
	CTR 15	0.820	±	0.008	aA	0.831	±	0.005	aA	0.831	±	0.006	aA	0.834	±	0.003	aA
	MET 15	0.745	±	0.020	bAB	0.697	±	0.037	bB	0.769	±	0.019	aAB	0.797	±	0.015	aA
F_v'/F_m'	**CTR 7.5**	0.692	±	0.019	abA	0.661	±	0.024	aA	0.652	±	0.016	aA	0.698	±	0.007	aA
	MET 7.5	0.573	±	0.049	bcA	0.382	±	0.034	bA	0.369	±	0.052	bA	0.579	±	0.045	bA
	CTR 15	0.754	±	0.011	aA	0.736	±	0.006	aA	0.737	±	0.005	aA	0.751	±	0.021	aA
	MET 15	0.488	±	0.043	cB	0.673	±	0.028	aAB	0.629	±	0.072	aAB	0.753	±	0.013	aA

Table 5.1 – *Cont.*

q_L	**CTR 7.5**	0.515	±	0.019	aA	0.549	±	0.010	aA	0.531	±	0.001	aA	0.515	±	0.019	aA
	MET 7.5	0.395	±	0.027	aA	0.369	±	0.038	abA	0.347	±	0.001	abA	0.398	±	0.038	abA
	CTR 15	0.434	±	0.052	aA	0.505	±	0.036	aA	0.498	±	0.019	aA	0.415	±	0.022	abA
	MET 15	0.419	±	0.035	aA	0.262	±	0.054	bA	0.268	±	0.038	bA	0.271	±	0.048	bA
$Y_{(II)}$	**CTR 7.5**	0.538	±	0.020	aA	0.520	±	0.026	aA	0.499	±	0.016	aA	0.542	±	0.013	abA
	MET 7.5	0.357	±	0.041	bA	0.199	±	0.035	cA	0.188	±	0.046	bA	0.363	±	0.052	bA
	CTR 15	0.559	±	0.018	aA	0.580	±	0.017	aA	0.580	±	0.011	aA	0.558	±	0.020	aA
	MET 15	0.286	±	0.029	bA	0.335	±	0.032	bA	0.312	±	0.057	bA	0.425	±	0.046	abA
$Y_{(NPQ)}$	**CTR 7.5**	0.193	±	0.021	aA	0.207	±	0.031	aA	0.243	±	0.022	aA	0.154	±	0.014	aA
	MET 7.5	0.158	±	0.027	aA	0.180	±	0.028	aA	0.164	±	0.030	abA	0.126	±	0.019	aA
	CTR 15	0.107	±	0.009	aA	0.104	±	0.008	aA	0.100	±	0.007	bA	0.129	±	0.025	aA
	MET 15	0.103	±	0.027	aA	0.142	±	0.022	aA	0.156	±	0.023	abA	0.128	±	0.016	aA
$Y_{(NO)}$	**CTR 7.5**	0.269	±	0.009	cA	0.273	±	0.011	bA	0.259	±	0.008	bA	0.304	±	0.007	bA
	MET 7.5	0.485	±	0.043	abA	0.621	±	0.035	aA	0.648	±	0.039	aA	0.511	±	0.063	aA
	CTR 15	0.333	±	0.018	bcA	0.316	±	0.018	bA	0.320	±	0.012	bA	0.313	±	0.013	bA
	MET 15	0.612	±	0.042	aA	0.522	±	0.028	aA	0.532	±	0.050	aA	0.447	±	0.037	abA
F_s/F_m'	**CTR 7.5**	0.462	±	0.018	bA	0.480	±	0.017	cA	0.501	±	0.016	bA	0.458	±	0.020	abA
	MET 7.5	0.643	±	0.029	aA	0.801	±	0.032	aA	0.812	±	0.046	aA	0.637	±	0.046	aA
	CTR 15	0.441	±	0.018	bA	0.420	±	0.017	cA	0.420	±	0.011	bA	0.442	±	0.020	bA
	MET 15	0.714	±	0.029	aA	0.665	±	0.032	bA	0.688	±	0.057	aA	0.575	±	0.046	abA

Table 5.1 – *Cont.*

PI_{Chr}	**CTR 7.5**	1.44	±	0.59	bA	5.22	±	1.29	bcA	4.64	±	0.70	bA	2.55	±	0.50	bA
	MET 7.5	9.06	±	2.12	aB	19.31	±	1.64	aAB	27.52	±	3.69	aA	11.28	±	0.93	aB
	CTR 15	2.92	±	0.81	bA	1.68	±	0.35	cA	1.61	±	0.43	bA	1.34	±	0.29	bA
	MET 15	11.86	±	1.22	aA	14.08	±	2.31	abA	7.91	±	2.68	bA	4.67	±	0.98	bA
PI_{Dyn}	**CTR 7.5**	16.64	±	2.42	abA	16.56	±	2.89	bA	18.19	±	1.70	aA	14.87	±	1.02	abA
	MET 7.5	23.13	±	5.98	abA	35.48	±	3.09	aA	28.84	±	6.99	aA	23.71	±	5.21	aA
	CTR 15	7.86	±	1.88	bA	11.22	±	0.92	bA	11.14	±	0.65	aA	9.78	±	2.37	bA
	MET 15	30.42	±	4.41	aA	5.73	±	2.61	bB	20.36	±	7.86	aAB	5.52	±	1.82	bB
PI_{Total}	**CTR 7.5**	17.42	±	2.30	bcA	21.57	±	2.84	bA	22.82	±	1.85	bA	17.42	±	0.80	bA
	MET 7.5	32.18	±	5.84	abA	54.79	±	4.07	aA	56.36	±	6.18	aA	35.28	±	5.57	aA
	CTR 15	10.78	±	1.25	cA	12.91	±	0.72	bA	12.75	±	0.54	bA	11.12	±	2.48	bA
	MET 15	42.28	±	5.04	aA	20.41	±	3.29	bAB	25.58	±	8.54	bAB	10.94	±	1.51	bB

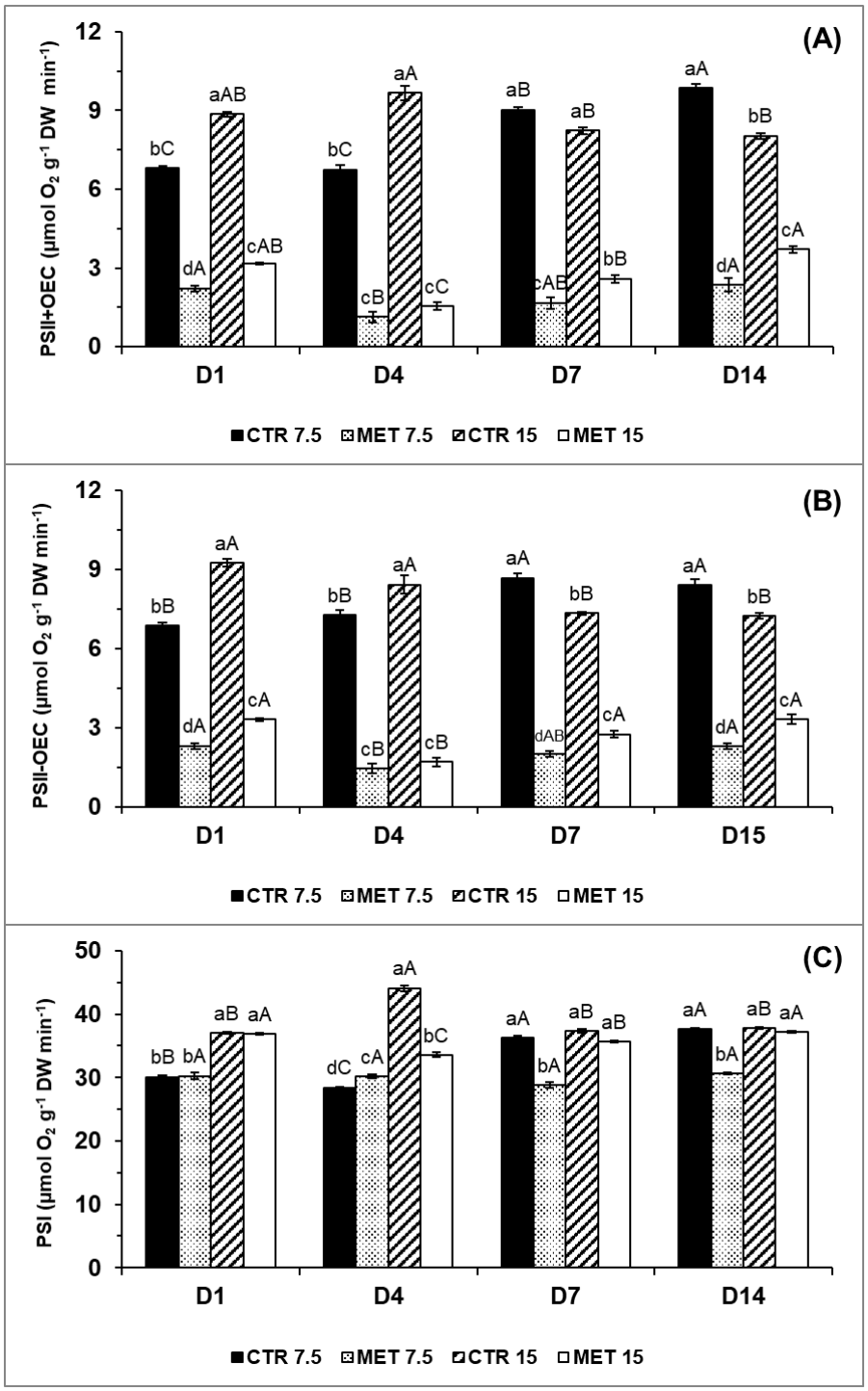

Figure 5.5 - Changes in the thylakoid electron transport rates associated with PSII (with and without the inclusion of OEC) and PSI in *Malus* x *domestica* Borkh cv. Golden Reinders plants submitted to nighttime temperature of 7.5 ºC or 15 ºC, under control (CTR) and metamitron sprayed (MET) conditions, 1, 4, 7 and 14 days after metamitron application. For each parameter, the mean values ± SE (n = 3) followed by different letters express significant differences between treatments within each day (a, b, and c), or between days within each treatment (A, B, and C), after a Tukey's HSD test (p-value ≤ 0.05).

5.3.7 Photosynthetic and Respiratory Enzymes Activity

In CTR plants, the nighttime temperature increase showed no significant impact in the in the initial RuBisCO activity (except by D1), and activation state, but a consistent increase of total activity was observed along the entire experiment (Table 5.2).

The single MET application strongly reduced RuBisCO activities after D1, regardless of nighttime temperature, and without differences between MET 7.5 and MET 15 plants. From D4 to D14, the initial activity showed declines between *ca*. 30 and 60%, maintaining significantly reduced values after two weeks of MET application. Total RuBisCO activity was somewhat less affected in MET 7.5 than in MET 15, recovering to values that did not differ from their respective CTR plants by D14. The activation state was significantly reduced by metamitron, in MET 7.5 to 44% than CTR 7.5 only on D4, and in MET 15 to 49% than CTR 15 only on D14.

Concerning two enzymes from the respiratory pathway (Table 5.2), the temperature rise significantly reduced pyruvate kinase (PK) activity in all days, whereas MDH (malate dehydrogenase) activity was mostly irresponsive, except by D14 when CTR 15 plants showed a significant decrease (20%), as compared with CTR 7.5. On the other hand, MET showed a temperature dependent impact. In fact, activity decreases were observed from D1 onwards only in MET 7.5 in both PK (between 60 and 76%) and MDH (between 9 and 27%). By opposition, MET 15 plants showed a consistent tendency to greater activity values for PK (all days) and MDH (D4 and D14) in comparison to their CTR 15 plants. These opposite trends resulted in greater activity values in MET 15 than in MET 7.5 in PK significantly in D4 and D7) and, especially in MDH (significantly in all days), with MET impact persisting by D14 in both PK and MDH only in the plants under 7.5 ºC.

Table 5.2 - Variation in the initial, total carboxylation activities and activation state of the ribulose-1,5 bisphosphate carboxylase/oxygenase (RuBisCO),as well as total activity of NADH-dependent malate dehydrogenase (MDH), and pyruvate kinase (PK) in *Malus* x *domestica* Borkh cv. Golden Reinders plants submitted to nighttime temperature of 7.5 ºC or 15 ºC, under control (CTR) and metamitron sprayed (MET) conditions, 1, 4, 7 and 14 days after metamitron application. For each parameter, the mean values ± SE (n = 4) followed by different letters express significant differences between treatments within each day (a, b, and c), or between days within each treatment (A, B, and C), after a Tukey's HSD test (p-value ≤ 0.05).

		Days after metamitron application			
		D1	**D4**	**D7**	**D14**
Initial	**CTR 7.5**	27.4 ± 1.4 bAB	33.4 ± 2.0 aA	30.8 ± 1.4 aAB	24.9 ± 2.1 aB
RuBisCO	**MET 7.5**	23.0 ± 1.6 bA	13.2 ± 1.0 bA	21.9 ± 5.2 abA	16.0 ± 1.9 bA
Activity$_i$	**CTR 15**	42.6 ± 2.7 aA	33.8 ± 3.6 aB	31.1 ± 2.3 aB	28.7 ± 1.3 aB
(μmol m^{-2} s^{-1})	**MET 15**	36.2 ± 6.4 abA	16.4 ± 1.1 bB	17.2 ± 2.7 bB	11.8 ± 1.7 bB
Total	**CTR 7.5**	65.1 ± 3.5 bA	84.5 ± 0.6 bA	86.6 ± 10.2 abA	69.5 ± 4.9 abA
RuBisCO	**MET 7.5**	68.9 ± 5.4 bA	62.1 ± 6.9 cA	57.9 ± 9.6 bA	66.1 ± 1.6 bA
Activity	**CTR 15**	116.4 ± 9.8 aA	108.0 ± 5.5 aA	101.6 ± 10.3 aA	95.3 ± 3.0 aA
(μmol m^{-2} s^{-1})	**MET 15**	100.1 ± 7.0 aA	54.2 ± 2.9 cC	48.8 ± 6.0 bC	77.1 ± 3.6 abB
RuBisCO	**CTR 7.5**	42.2 ± 1.5 aA	39.5 ± 2.2 aA	37.0 ± 4.8 aA	36.0 ± 2.5 aA
Activation	**MET 7.5**	33.6 ± 1.7 aA	22.2 ± 3.1 bA	38.1 ± 5.6 aA	25.8 ± 4.2 abA
State	**CTR 15**	37.1 ± 2.6 aA	30.7 ± 3.3 abA	31.0 ± 1.6 aA	30.1 ± 1.4 aA
(%)	**MET 15**	35.5 ± 3.9 aA	30.4 ± 2.1 abA	35.0 ± 3.7 aA	15.4 ± 2.3 bB
	CTR 7.5	113.9 ± 1.8 aB	146.9 ± 9.6 aA	129.6 ± 9.4 aAB	129.6 ± 7.2 aAB
PK	**MET 7.5**	47.0 ± 10.9 bA	35.2 ± 3.8 cA	40.5 ± 2.2 cA	42.9 ± 7.0 bA
(μmol m^{-2} s^{-1})	**CTR 15**	67.4 ± 6.1 bA	66.7 ± 7.0 bA	67.2 ± 8.2 bcA	49.0 ± 4.5 bB
	MET 15	72.5 ± 3.7 bA	90.0 ± 4.6 bA	80.1 ± 8.0 bA	52.9 ± 4.4 bB
	CTR 7.5	195.6 ± 3.0 abC	228.9 ± 8.2 bB	221.6 ± 8.8 aBC	281.3 ± 10.7 aA
MDH	**MET 7.5**	157.8 ± 9.0 bA	208.2 ± 21.3 bA	176.3 ± 13.3 bA	204.5 ± 9.6 cA
(μmol m^{-2} s^{-1})	**CTR 15**	233.3 ± 34.8 aA	255.4 ± 21.9 abA	256.7 ± 8.0 aA	223.2 ± 8.7 bcA
	MET 15	235.5 ± 14.8 aB	301.9 ± 15.7 aA	239.1 ± 9.1 aB	256.1 ± 10.1 abB

5.3.8 Non-structural Carbohydrates During Nighttime and Diurnal Periods

The fructose content in leaves collected in the night or diurnal periods was residual (< 0.1 mg g^{-1} DW) (data not shown), reason why it was not presented.

Table 5.3 - Variation in concentrations of soluble sugars in the leaves sampled after a 10 h dark period in Malus x domestica Borkh cv. Golden Reinders plants submitted to nighttime temperature of 7.5 ºC or 15 ºC, under control (CTR) and metamitron sprayed (MET) conditions, 1, 4, 7 and 14 days after metamitron application. For each parameter, the mean values ± SE (n = 4) followed by different letters express significant differences between treatments within each day (a, b, and c), or between days within each treatment (A, B, and C), after a Tukey's HSD test (p-value ≤ 0.05).

		Days after metamitron application			
Parameter	**Treatment**	**D1**	**D4**	**D7**	**D14**
Sucrose (mg g^{-1} DW)	**CTR 7.5**	25.9 ± 1.5 aA	21.7 ± 2.6 aA	24.0 ± 2.5 aA	26.3 ± 2.3 aA
	MET 7.5	19.6 ± 1.7 abA	14.6 ± 2.3 bAB	10.9 ± 1.4 bB	9.2 ± 1.3 bB
	CTR 15	14.3 ± 1.5 bA	10.5 ± 0.9 bcAB	8.9 ± 0.6 bcB	10.5 ± 0.8 bB
	MET 15	13.3 ± 3.4 bA	3.6 ± 0.4 cB	4.9 ± 0.7 cB	7.8 ± 0.9 bAB
Glucose (mg g^{-1} DW)	**CTR 7.5**	25.3 ± 2.0 aA	22.3 ± 1.7 aA	22.3 ± 1.5 aA	20.7 ± 1.3 aA
	MET 7.5	21.8 ± 3.2 aA	22.2 ± 2.5 aA	19.7 ± 1.1 abA	23.5 ± 2.1 aA
	CTR 15	20.4 ± 1.8 aA	18.2 ± 1.8 aA	17.8 ± 1.2 abA	25.2 ± 1.4 aA
	MET 15	20.3 ± 2.2 aA	17.2 ± 1.6 aA	16.9 ± 1.6 bA	20.6 ± 2.0 aA
Sorbitol (mg g^{-1} DW)	**CTR 7.5**	45.1 ± 1.7 aA	47.9 ± 2.7 aA	42.4 ± 2.2 aA	45.5 ± 3.3 aA
	MET 7.5	43.4 ± 1.7 aA	41.5 ± 2.2 abAB	36.1 ± 2.4 aB	36.2 ± 1.9 aB
	CTR 15	47.3 ± 2.3 aA	43.8 ± 2.3 abA	39.9 ± 1.6 aA	41.4 ± 1.6 aA
	MET 15	40.6 ± 3.9 aA	37.1 ± 1.3 bA	35.3 ± 2.2 aA	40.3 ± 2.2 aA
Total Soluble (mg g^{-1} DW)	**CTR 7.5**	96.5 ± 3.7 aA	91.9 ± 5.0 aA	88.8 ± 4.3 aA	92.5 ± 4.6 aA
	MET 7.5	84.8 ± 5.5 abA	78.3 ± 6.1 abA	66.7 ± 4.0 bA	72.6 ± 3.1 bA
	CTR 15	81.9 ± 3.9 abA	72.5 ± 3.9 bcA	66.6 ± 2.8 bA	73.0 ± 2.8 bA
	MET 15	74.2 ± 6.3 bA	57.9 ± 2.6 cA	56.4 ± 3.3 bA	68.4 ± 3.4 bA

In the leaves obtained at the end of the night period, only a few changes were noted in glucose and sorbitol content (Table 5.3). The temperature rise or MET per se, did not promote significant differences, and only in MET 15 there were significant reductions of 25% of glucose by D7, and of 23% in sorbitol by D4, both as compared with CTR 7.5, but without differences to CTR 15. In contrast, higher nighttime temperature promoted a decline in the content of sucrose to values below half, and total soluble sugars in CTR 15 plants, as compared with CTR 7.5, along the entire experiment, particularly from D4 to D14.

Table 5.4 - Variation in concentrations of soluble sugars in the leaves sampled after 2 hours of light in Malus x domestica Borkh cv. Golden Reinders plants submitted to nighttime temperature of 7.5 ºC or 15 ºC, under control (CTR) and metamitron sprayed (MET) conditions, 1, 4, 7 and 14 days after metamitron application. For each parameter, the mean values ± SE (n = 4) followed by different letters express significant differences between treatments within each day (a, b, and c), or between days within each treatment (A, B, and C), after a Tukey's HSD test (p-value ≤ 0.05).

		Days after metamitron application			
Parameter	**Treatment**	**D1**	**D4**	**D7**	**D14**
Sucrose (mg g^{-1} DW)	**CTR 7.5**	21.6 ± 4.1 aA	24.0 ± 2.2 aA	25.4 ± 2.9 aA	29.4 ± 2.9 aA
	MET 7.5	19.2 ± 3.8 aA	19.0 ± 3.5 abA	14.6 ± 1.6 bA	12.7 ± 3.1 bA
	CTR 15	11.8 ± 1.5 aA	10.8 ± 2.4 bcA	11.7 ± 2.2 bA	13.5 ± 5.2 bA
	MET 15	12.2 ± 0.5 aA	6.7 ± 0.9 cB	7.7 ± 1.7 bAB	10.6 ± 1.4 bAB
Glucose (mg g^{-1} DW)	**CTR 7.5**	22.1 ± 1.6 aA	17.4 ± 1.4 bA	21.1 ± 4.8 aA	18.5 ± 1.7 bA
	MET 7.5	29.2 ± 3.8 aA	28.4 ± 1.9 aA	22.4 ± 0.5 aA	27.3 ± 1.8 aA
	CTR 15	25.0 ± 5.3 aA	24.9 ± 1.9 abA	21.1 ± 2.7 aA	20.5 ± 3.4 abA
	MET 15	20.4 ± 2.6 aA	26.6 ± 3.2 aA	22.1 ± 2.1 aA	20.2 ± 2.1 abA
Sorbitol (mg g^{-1} DW)	**CTR 7.5**	41.5 ± 6.8 aA	50.6 ± 2.4 aA	55.0 ± 7.6 aA	61.2 ± 4.3 aA
	MET 7.5	50.7 ± 6.0 aA	51.2 ± 2.7 aA	37.3 ± 3.1 aA	41.2 ± 3.6 bA
	CTR 15	46.2 ± 9.4 aA	59.0 ± 3.4 aA	50.1 ± 5.0 aA	54.6 ± 2.7 abA
	MET 15	51.9 ± 9.2 aA	52.0 ± 3.7 aA	48.9 ± 3.8 aA	52.4 ± 2.3 abA
Total Soluble (mg g^{-1} DW)	**CTR 7.5**	85.2 ± 9.1 aA	91.9 ± 5.3 aA	91.9 ± 5.5 aA	109.7 ± 8.2 aA
	MET 7.5	97.4 ± 9.9 aA	99.9 ± 3.0 aA	74.3 ± 4.5 aA	82.5 ± 4.6 bA
	CTR 15	82.3 ± 9.8 aA	94.1 ± 3.4 aA	82.9 ± 5.7 aA	72.1 ± 2.7 bA
	MET 15	84.5 ± 8.9 aA	85.3 ± 7.2 aA	78.7 ± 4.2 aA	83.2 ± 2.1 bA

Additionally, MET also strongly reduced sucrose values, especially under 15 ºC in D4 and D7. Still, MET 15 plants showed a recovery in comparison to their CTR 15 plants by D14, whereas in MET 7.5 plants no recovery was observed, maintaining approximately 65% less than in CTR 7.5 counterparts. These sucrose changes implicated a quite similar pattern in total soluble sugar content, including the persistence of a significant MET effect only in under 7.5 ºC. However, it seems noteworthy that the lowest absolute sucrose (and total soluble sugar) contents were observed in MET 15 in all days, but with minimum values by D4 e D7 that represented ca. 80 and 84% less than CTR 7.5, and less than half than in MET 7.5.

Regarding illuminated samples, soluble sugar changes were not as strong as in the night sampled leaves (Table 5.4). Still, greater nighttime temperature (CTR 15) strongly reduced sucrose content, with a maximal decline of 65% on D7, as compared to CTR 7.5. MET promoted a sucrose reduction only under 7.5 ºC, significantly by D7 and D14, when minimum values were observed, with 65% less sucrose than CTR 7.5. In contrast, MET 7.5 presented increases of glucose content by D4 (63%) and D14 (48%), as compared to CTR 7.5. Values of sorbitol and total soluble sugars were maintained until D7, but in D14 were reduced by 33% and 30% on MET 7.5.

Under higher nighttime temperature, MET did not significant alter none of the studied sugars along the trail, as compared with CTR 15 ºC. Nevertheless, this treatment resulted in the lowest observed absolute sucrose (and total soluble sugar) values on D4 and D7, promoting decreases of 72 and 77%, respectively, when compared with CTR 7.5.

5.4 Discussion

Previous studies in apple orchards involving MET application and nighttime temperature has shown strong effects on photosynthesis, soluble sugars and final yield (Rosa et al., 2020 b). Here, we aim at going deeper, to unveil key points of the MET action in the leaf energy balance, with the benefit of controlled conditions, without environmental cross-changes frequently observed under field trials.

5.4.1 Metamitron Absorption and Degradation

Temperature may affect chemical absorption and uptake (Orbovic et al., 2001), what was globally in line with the tendency to greater absorption at night temperature of 15 ºC than at 7.5 ºC (Fig. 5.1).

Metamitron degradation (MET) happens by a deamination reaction (Schmidt and Fedtke, 1977) which occurs in the presence of light, oxygen and water. After the rupture of the N-NH_2 several degradation metabolites which lack the capacity of inhibiting the photosystem activity are formed, being the main one desamino-metamitron (Palm et al., 1997; Kouras, 2012). Metamitron degradation was found to be light promoted (Cox et al., 1996; Palm et al., 1997). This might justify the very low levels of desamino-metamitron under the growth chamber moderate irradiance conditions, even after two weeks after application, well below the values observed in field trials with ‘Gala’ (Rosa et al., 2020 a).

5.4.2 Impacts of Temperature and Metamitron on Photosynthesis

Photosynthesis is one of the most heat-sensitive physiological processes in plants, with stomatal limitations usually constituting the main constraint to photosynthesis (Martins et al., 2014). That was not the case here in response to a higher night temperature, since despite stomatal conductance to water vapour (g_s) decline in CTR 15 until D4 (in comparison to CTR

7.5), no corresponding change was observed in net photosynthetic rate (P_n) (Fig. 5.3). Similarly, the large reduction in P_n in MET treated plants was not accompanied by g_s changes between MET and CTR plants in either temperatures.

The single (and moderate) increase in night temperature showed a quite limited impact in the studied parameters (e.g., greater PSI activity in CTR plants until D4), in line with reports showing no impact in spinach by growth temperature (Wijk and Hasselt, 1990), or with the slight changes in F_v/F_m and F_s/F_m' under night temperature of 15 ºC (Table 5.1), reflecting the same stability observed under an increase of the growth temperature by 15 ºC in *Capsicum annuum* L. (Bhandari et al., 2018).

In sharp contrast with night temperature increase, non-stomatal limitations to photosynthesis were shown due to the strong photosynthetic capacity (A_{max}) reduction only by MET (especially by D4 and D7) (Fig. 5.3). Since P_n did not decline more than A_{max}, no mesophyll diffusional constraints to CO_2 flux towards the carboxylation sites would have occurred. In this way, A_{max} decline would be related with photo and/or biochemical limitations (Semedo et al., 2020). That was in fact the case, as shown by multiple photosynthetic related parameters. Impairments can be observed already in the energy capture in the antennae (F_0 rise) (Dubberstein et al., 2020). However, the strongest impacts were pointed by the strong declines in the thylakoid electron transport involving PSII (regardless of OEC participation) (and in PSI at 7.5 ºC by D7 and D14) (Fig. 5.5), in the maximal (F_v/F_m) and actual (F_v'/F_m') photochemical efficiency of PSII, and in the estimate of quantum yield of linear electron transport ($Y_{(II)}$). These impacts were particularly severe by D4 and D7, and were prolonged until the end of the experiment, when only an incomplete recovery was found. This is in agreement with the known action of MET, which belongs to the class of triazinone herbicides, acting as PSII inhibitors, affecting photosynthetic electron transport in chloroplasts (Corbet, 1974), inhibits the electron transport in the Hill reaction due to its binding to the D1 protein in PSII (Oettmeier and Hilp, 1991; Almeida et al. 2019).

Our finding of the impacts on PSII function due to MET application fully agreed with the greater PSII inactivation (F_s/F_m'), as well as chronic (PI_{Chr}) and total (PI_{Total}) photoinhibition status (Table 5.1), possibly related to D1 protein inactivation due to metamitron bind (Horovitz et al., 1988). Moreover, the photoprotective thermal dissipation mechanisms ($Y_{(NPQ)}$) remained mostly unchanged, thus not balancing the decreased use of energy through photochemistry ($Y_{(II)}$). Instead, a significant increase of PSII non-regulated energy dissipation processes ($Y_{(NO)}$) was observed in MET treated plants, what is usually attributable to photoinactivation and uncontrolled energy (heat and fluorescence) dissipation in PSII (Kramer et al. 2004, Busch et al., 2009; Huang et al. 2011).

In addition, with the impairments in the photochemical components, mostly regarding PSII performance, MET implications to P_n and A_{max} declines were beyond those known for PSII functioning. In fact, as we are aware, we report for the first time the implications of MET application in the initial and total RuBisCO activity, which were greatly reduced, although

without a clear impact in their activation state.

Globally, these impacts on the photosynthetic photo and biochemical components were usually observed in a greater extend at 7.5 ºC.

5.4.3 Impact in the Enzymes Involved in the Photosynthetic and Respiratory Pathways and on Soluble Sugars

A shortage of soluble sugars in leaves is a common response to environmental changes, namely nighttime temperature (Rosa et al., 2020b), and to metamitron application (Stander et al. 2018; Rosa et al., 2020b). In this study, differences in sugar content were somewhat more pronounced in dark collected samples than in samples taken after 2 hours of light.

Since P_n, g_s or A_{max} or even the photochemical use efficiency or inhibition were not affected by the increase in nighttime temperature (CTR 15), *per se*, RuBisCO initial (on D1) (Table 5.2) and total activity (during the whole experiment) was in line with our results, with activities enhanced under higher nighttime temperature, allowing a greater photoassimilates production and justifying the tendency for higher glucose and sorbitol content during the day. Respiration generally consumes between 30% and 80% of the CO_2 taken up by photosynthesis per day (Loveys et al., 2002), increasing with temperature as our results pointed out. These results are concomitant with a study developed by Mohammed and Tarpley (2009) that saw an increase in dark respiration (R_d) of *Oryza sativa* L. after an increase in nighttime temperature of 5 ºC. In addition, Turnbull et al. (2002) conducted an experiment in *Populus deltoides* in which an increase of 10 ºC in nighttime temperature resulted in greater R_d (77%) (Fig. 5.4). Sugars are the respiratory substrates for the generation of energy and metabolic intermediates, necessary during the night to maintain the Krebs cycle. An increased respiration rate in high nighttime temperature conditions has been reported in many crops with consequent soluble sugar decreases (Mohammed and Tarpley, 2009; Loka and Oosterhuis, 2010; Peraudeau et al., 2015), such as the decrease in sucrose levels observed in our study (Tables 5.3 and 5.4). On the other side, pyruvate kinase (PK) activity was lower under 15 ºC, and malate dehydrogenase (MDH) activity was similar to CTR 7.5 values (Table 5.2). Since we analyzed the maximum enzyme activity, in addition to R_d, the reason for the sucrose shortage during the night that remained during the day might be explained by a greater activity of MDH, with consequent consumption of sugar molecules as substrate. This is corroborated by a study in cotton, in which nighttime temperature was increased 3 and 7 ºC degrees, and in both cases, there was a significant decrease in the ATP levels per leaf area comparing to lower nighttime temperatures (Loka and Oosterhuis, 2010).

The impact of metamitron on PSII has been long time reported, with consequences to the photochemical functioning, as confirmed by our data. However, an additional impact in the photosynthetic machinery, specifically at the key enzyme RuBisCO was, to our knowledge, reported here for the first time. RuBisCO was strongly affected after metamitron application, although this enzyme was more affected after metamitron application under 7.5 ºC than under

15 ºC (Table 5.2). On the other side, metamitron significantly increased R_d rates regardless of the nighttime temperature, although only significantly on D1, probably due a longer-term thermal acclimation process, as such a response is usually reflected in a reduction in R_d rates (Atkin and Tjoelker, 2003). Also, the acclimation hypothesis is in agreement with the results obtain by Peraudeau et al. (2015) that saw a sharp increase in R_d rates in the first days after an increase in nighttime temperature in *Oryza sativa* L. that reduced to lower values some days after. In contrast, respiration rates were not accompanied by a respiratory enzyme activity, whose activity decayed under 7.5 ºC, during the whole experiment. Under 15 ºC, PK activity was significantly higher than under 7.5 ºC and the same tendency verified in MDH (Table 2).

The application of metamitron caused all these impacts on the energy machinery which together, have changed the soluble carbohydrate balance. However, there were marked differences in the response to metamitron application on the different nighttime temperatures, and the application under 15 ºC promoted the strongest sugar reductions within all treatments, especially on sucrose. The described respiratory activity at moderately higher temperatures (such as 15 ºC) might contribute to sugar consumption as substrate since the respiratory flux is less limited by enzymatic capacity because of increases in the V_{max} of enzymes. This increase in activity likely associated to a global greater metabolic activity, allied to less daily production, originated the extremely low values of sucrose observed in MET 15, justifying a greater thinning potential at 15 ºC than at 7.5 ºC observed in the trials conducted by Rosa et al. (2020b).

5.5 Conclusions

Metamitron has been used for fruitlet thinning, linked to photosynthesis inhibition. Here we reported that MET alters the cell energy machinery in a night temperature dependent manner. Photosynthesis was not affected by stomatal constraints, but instead by non-stomatal limitations. Impacts in the photosynthetic apparatus included strong declines of the photochemical performance and the thylakoid electron transport involving PSII (but barely of that regarding PSI), and of the estimate of quantum yield of linear electron transport ($Y_{(II)}$), with particular severity by D4 and D7, and only a partial recovery thereafter. These impacts were in line with a greater PSII photoinhibition (given by F_s/F_m' and PI_{Chr}), associated with deleterious uncontrolled energy dissipation processes ($Y_{(NO)}$) in MET treated plants. However, MET implications to P_n and A_{max} declines were beyond those known for PSII functioning, since it included inhibition of RuBisCO, here reported for the first time. These impacts on the photosynthetic components were usually observed in a greater extend at 7.5 ºC.

Additionally, MET also inhibit PK and MDH enzymes from the respiratory pathway under 7.5 ºC. Although MDH could be in excess and a partial inhibition might not be strongly relevant for glycolysis performance (Schreier et al., 2018; Yokoshi et al., 2021), MDH is crucial for maintaining redox homeostasis in chloroplasts under these conditions changing light environment. Therefore, altogether, these impacts on the energy machinery could help to explain the observed changes in the soluble carbohydrate balance, clearly reflected in a heavy sucrose decline, particularly under 15 ºC. Such reduction, was likely associated with a lower

impact in the respiration enzymes what would allow a greater photoassimilate use despite the greater MET absorption, thus justifying a greater thinning potential at 15 ºC than at 7.5 ºC. Still, a higher night temperature also supported a greater (although incomplete) recovery by D14 in most parameters, likely associated to a global greater metabolic activity due such higher temperature. Therefore, our findings showed that the action of MET is far more complex than the sole direct inhibition of PSII, since other components of the photosynthetic and respiratory pathways are affected, with implications in leaf sugar balance. Furthermore, the MET effect depends not only from the absorbed amount, but also from the interaction with factors like of night temperature, thus pointing the need of future experiments to fully unveil the complex MET interaction with other environmental conditions that would determine the fruitlet thinning impact.

In times in which climate changes are a constant treat, particularly global warming, this study has a growing interest due to the impact of nocturnal temperature on the (over)efficacy of thinning compounds. By unveiling some of the mechanisms impacted by increased nighttime temperature in combination with metamitron, this work clearly point that MET application should be optimized taking into account regional/local temperature conditions at the time of application in order to achieve an optimal crop load.

5.6 References

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CHAPTER 6

Final Considerations

6. Final Considerations

6.1 General Discussion

During the first weeks after fruit set, the fruitlets are in the cellular division phase, which is highly demanding on carbohydrates (Gillapsy et al., 1993; Lakso et al., 2001). In addition, during this phase, a natural process of crop load control occurs, which may be enhanced by the use of thinners. Therefore, it is a critical period when the number of fruits, final fruit size and quality, and yield are established.

Our data clarified that certain meteorological conditions affect metamitron leaf absorption. Metamitron is a selective herbicide, which can be inactivated by a deamination reaction originating the compound desamino-metamitron, which is not capable of inhibiting PSII activity, namely at photosystem (PS) II level. This deamination reaction occurs in the presence of light, oxygen and water, and since metamitron maximum absorption wavelength is 306 nm, direct photodegradation reaction can occur in the field (Cox et al., 1996). There is evidence in our trials that low radiation not only increases absorption (Fig. 3.1), but also retards metamitron degradation (assessed by desamino-metamitron content) (Fig. 3.2), likely due to the lower photodegradation caused by the lower irradiance reaching the leaves comparing to full sun exposure conditions.

Generally, high relative humidity conditions increase the drying period since a liquid layer remain on the leaf surface for a longer period, promoting a chemical solution to be absorbed over a longer period (Byers, 2002). Our data showed that humidity may increase metamitron absorption in leaves of trees developed in regions with climates with higher humidity levels, such as Sint-Truiden (Fig 2.1). Under these type of climate, leaf morphological structure changes: the thickness of the parenchyma and both the adaxial and abaxial epidermis tend to be reduced, leaving a softer and thinner (likely less waxy and more permeable) cuticle, which will be more prone to chemical absorption (Nemeskéry et al., 2009; Locatelli et al., 2019). No effect on metamitron absorption was observed with nighttime temperature increase (Fig. 4.1).

With the applied rate of 247.5 ppm in all the performed trials, photosynthetic rate was reduced, generally, to at least 50% of the control trees, as already registered in other studies (Brunner, 2014; Gabardo et al., 2017). The metamitron concentration assessment, allowed concluding that the higher the absorption the greater the photosynthetic inhibition, since when metamitron was applied under low radiation (imposed by shading nets) and with high relative humidity conditions in Sint-Truiden, it was observed that P_n significantly decreased between 2 and 5 days after spraying, comparing to metamitron sprayed at non-changed environmental conditions. It is important to underline that in the case of the photosynthetic rate decrease measured under the shading nets, the effect was not only caused by the increase in metamitron absorption but also by the low radiation levels. Concomitantly, in the other cases in which the meteorological change did not enhance absorption, it was not observed an extra reduction in P_n rate, namely high nighttime temperature and high humidity in Spain. The simulation of low radiation by

means of shading nets demonstrated that, *per se*, g_s is significantly reduced, however, that was not the case with high nighttime temperature and high relative humidity. Nevertheless, the general g_s response to metamitron among all trials, alone or in combination with changed meteorological conditions, is a clear reduction, usually, by at least 50%, as compared to the values measured in the control trees.

Shade did not affect RuBisCO total activity (Table 3.2), whereas, higher night temperature (15 ºC) increased both initial and total RuBisCO activities (Table 5.2) comparing to plants exposed to 7.5 ºC, during the first 4 days of experiment, probably until acclimation. The application of metamitron induced a significant reduction of RuBisCO total activity, similar with and without shading nets. The same strong reduction in initial and total activity was observed under 15 ºC nighttime temperature, although the impact was faster under 7.5 ºC (Table 5.2).

The field nighttime temperature trial raised some questions, mainly regarding how nighttime temperature affects photosynthesis and respiration, which revealed the need of setting up a growth chamber trial, with the plants under accurate controlled conditions of temperature, and without other potential varying climate conditions (as is common under field conditions). In terms of the status of the photosynthetic apparatus, P_n and g_s changes are in agreement with field results, further clarifying the impact on RuBisCO activity. These results were complemented with a deeper analysis on the photosynthetic machinery performance. The photosynthetic capacity (A_{max}) was not affected by increased nighttime temperature (15ºC), contrarily to metamitron, which significantly reduced A_{max}, similarly at both temperatures (7.5 and 15 ºC) during the 14 days of the experiment. A_{max} results from both carboxylation and electron transport capacity, and our analysis indicated that metamitron application has negative impacts on both components. According to the literature, metamitron acts by inhibiting photosystem II: disrupting the thylakoid electron transport up to 60% (McArtney et al., 2012), by binding on D1 protein and blocking the normal transfer of electrons between the primary (Q_A) and secondary quinones (Q_B) of PSII (Guidi and Degl'Innocenti, 2011). Our findings confirmed such site of action of metamitron by analyzing the thylakoid electron transport rates, which showed that PSII+OEC and PSII-OEC activity was strongly reduced by 2/3, as compared with control plants (Fig. 5.5). Regarding PSI, values indicate that after the application of metamitron, its activity was up-regulated under high nighttime temperatures (15 ºC) during the first four days of the experiment, likely until acclimation, and afterwards only plants with metamitron and exposed to 7.5 ºC showed significant lower rates than the control, meaning that metamitron had some negative impact in PSI only at lower temperature. In general, with metamitron and 15 ºC nighttime temperature, the thylakoid transport rate of PSI was usually significantly higher than in plants exposed sprayed at 7.5 ºC, in concomitancy with the effect in PSII and P_n. An explanation for the enhanced activity during the first days would be the heat shock/stress caused by nighttime temperature increase, which caused an increase in the tree metabolic processes followed by an acclimation process.

The increase in nighttime temperature *per se*, barely affected photochemical parameters, (Fv'/Fm'), regarding the energy dissipation processes, $Y_{(NPQ)}$ and $Y_{(NO)}$, photoinhibition index estimates, (PI_{Total}, PI_{Dyn} and PI_{Chr}), and just a tendency to an increase in F_v/F_m and F_s/F'_m. (Fv'/Fm') (from D4 onwards).

In contrast, most fluorescence parameters were strongly affected by metamitron application with the higher nighttime temperature attenuating some of those effects. The decrease in F_v/F_m ratio in leaves exposed to metamitron was usually concomitant with altered rates of electron transport in PSII and/or PSI level. Additionally, the increase in F_s/F'_m and the strong decline of the PSII activity (as assessed by the thylakoid electron transport rate), regardless of OEC participation, confirm that metamitron impairs PSII functioning, promoting photoinhibition, possibly related to D1 protein inactivation due to metamitron bind (Horovitz, 1988). Notably, the impact of metamitron was partly mitigated under higher nighttime temperature. This was evidenced by the lower impact (and faster recovery from D4 onwards) in several parameters, such as, P_n, A_{max} and lower rate constant for PSII inactivation (F_s/F'_m) and non-photochemical quenching attributable to photo-inactivation and non-regulated energy dissipation in PSII ($Y_{(NO)}$) observed in trees exposed to 15 ºC nighttime temperature, as compared to those at 7.5 ºC. This mitigation of metamitron impact was further observed in enzymes associated with the respiration pathway (PK and MDH, see below), pointing to a greater potential functioning of the energy metabolism (photosynthesis and respiration) under higher night temperature. Altogether, our findings point to a strong metamitron impact on the photochemical functioning and, in some of the studied parameters a reduction of that impact under higher nighttime temperature.

A shortage of non-structural carbohydrates in leaves is a common response to environmental changes, namely radiation (Polomski et al., 1986), nighttime temperature, (Robinson and Lakso, 2011; Clever, 2018; Gonzalez et al., 2019; Lordan et al., 2019) and to metamitron application (Stander et al. 2018). Fruit abscission is triggered by a shortage in carbohydrate content (Zibordi et al., 2009; Eccher et al., 2013; Sawicki et al., 2015; Ackerman et al., 2015). In our case, clear variations in leaf non-structural sugars content in all trials that was associated with impacts in photosynthetic parameters. Our analysis included sucrose, glucose, fructose and sorbitol as the main soluble sugars. Glucose and fructose remained mostly stable, even with the lower P_n induced by most treatments, irrespective of location or day, thus without a clear response to source and sink manipulations. By contrast, our data allow concluding that leaf sorbitol and, specially, sucrose are highly sensitive to meteorological conditions and metamitron, since they were severely reduced in most treatments. Sucrose is formed in the cytoplasm and is then exported from source leaves to sink tissues (Zhou and Quebedeaux, 1995). Sorbitol is the most abundant non-structural sugar in the Rosaceae family and is a major phloem-transported sugar (Wang et al., 1995, 1999; Klages et al., 2001; Naschitz et al., 2010). By interrupting the thylakoid electron transfer ATP and $NADPH_2$ and, therefore, CO_2 fixation will be impared (Abbaspoor et al. 2006), as suggested to occur under metamitron application. In this case, a shortage in the soluble sugar production can be expected, as reported by Stander et al. (2018) after applying 75, 150, and 300 mg/L metamitron in citrus. The reductions in P_n rate,

such as the observed in the treatment with low radiation during five days (using shading nets during five days), and in the single 247.5 ppm of metamitron, in all trials, likely contributed to the significant reductions in sucrose (more pronounced) and sorbitol, immediately at 2 days after the meteorological change impositions/spraying, but reaching minimum values generally at 5 days after. Furthermore, the combined exposure to low radiation and metamitron had prolonged aftereffects, with the lowest sucrose and sorbitol content (below control values) being observed 10 days after the metamitron application. Despite the enhancement of the several photosynthetic parameters in trees exposed to higher nighttime temperatures, our data allow concluding that under these conditions there were strong sucrose (more pronounced) and sorbitol decreases comparing to control trees. Moreover, in concordance, several field and growth chamber trials, point that spraying 247.5 ppm of metamitron induces similar soluble sugar reduction as a period of five nights with increased temperature. In the samples collected before sunrise, the greatest impact was found in the trees under higher air temperature. However, in the samples collected at midday, these same trees presented a significant recovery, whereas metamitron treated trees present quite lower values of sucrose and sorbitol due to its inability of photosynthesize at 100%, as indicated by the P_n measurements. In the case of the combined exposure of high nighttime temperatures with metamitron, even lower values were registered, together with a longer recovery period to reach values of these sugars similar to control.

The increased relative humidity, *per se*, did not affect sugar content. However, in its combination with metamitron, the results were controversial between locations, but in agreement within the results obtained in each location. In fact, reductions in sucrose and sorbitol were observed only in Sint-Truiden, where there was higher metamitron absorption, and a consequent reduction in P_n. In addition, the trees used in the trials in this location were younger, suggesting that younger trees (with lower carbohydrate reserves) can be more sensitive to variations.

Regarding starch, our data clearly showed that decreasing radiation and increasing nighttime temperature significantly reduce its content, probably due to remobilization of reserves (Breen et al., 2020), which might have contributed (at least partly) to the most stable values of glucose and fructose.

Since the 15 ºC during the night were actually beneficial for the photosynthetic apparatus, but a strong decrease in sucrose and sorbitol was registered in the field, the growth chamber experiment was set with one of the goals being the assessment of dark respiration and respiratory enzyme activity. An increased respiration rate in high nighttime temperature conditions has been reported in many crops with consequent soluble sugar decreases (Kondo and Takahashi, 1987; Turnbull et al., 2002; Mohammed and Tarpley, 2009; Loka and Oosterhuis, 2010; Peraudeau et al., 2015, Jing et al., 2016). Sugars are the respiratory substrates for the generation of energy molecules and metabolic intermediates synthesis, necessary during the night to maintain the Krebs cycle. Respiration generally consumes

between 30% and 80% of the CO2 taken up by photosynthesis per day (Porter et al., 1990; Loveys et al., 2002), with greater values as temperature rises. This is in line with our findings of a 3-fold increase in Rd under 15 ºC nighttime temperature as compared with control trees exposed to 7.5 ºC, on the first day of the experiment. However, during the following days there was no longer an impact of nighttime temperature on this parameter. Still, PK activity was actually lower under 15 ºC, the reason for the general shortage in sugar content during the night, that remained on sucrose during the day, might be explained by the greater activity of MDH, with consequent consumption of sugar molecules as substrate. In the combined exposure with metamitron, respiration rates were not accompanied by respiratory enzyme activity. When metamitron was sprayed and the trees exposed to 7.5 ºC, PK and MDH activities declined during most of the experiment, whilst under 15 ºC, MDH activity was maintained close to that of at 7.5 ºC. Moreover, PK activity only decreased during a few days, meaning that this normal enzyme activity contributed to sugar consumption as substrate allied to less daily production. Moreover, at 15 ºC the whole tree metabolism would be likely performed at a greater rate than at 7.5 ºC, resulting in higher consumption of carbohydrates.

In the early phase of fruit development, there is a strong sink competition for carbohydrates between the vegetative parts of the tree (shoots) and the small fruitlets. Under limited sugar availability (Table 2.2. and 2.3), as previously described, during the first 40 DAFB, shoot growth has priority over fruit growth for carbohydrate partitioning (Forshey and Elfving, 1989; Bepete and Lakso, 1998; Lakso et al., 2001) therefore this response has a cost, and may contribute to a negative CH balance that can enhance fruit abscission. Chemical fruit thinning strategies are generally applied during cell division phase, in which the fruit grows at an exponential rate, requiring a big demand for carbohydrates (Gillapsy et al., 1993). Our data showed that both metamitron and high nighttime temperature significantly reduced fruit growth rate (Fig. 3.8), both remaining lower than control from five days after the beginning of the experiment on. The metabolism of sorbitol and sucrose fuels fruit growth (Li et al., 2012) and leaves of both treatments experienced a sugar shortage under of both metamitron and high nighttime which resulted in a fruit growth rate limitation. A decrease in growth rate usually leads to fruit drop, since abscising fruits stop growing several days before (Greene et al., 2013; Lakso and Goffinet, 2013, Jakopič et al., 2015). In addition, trees sprayed with metamitron showed significantly higher shoot growth rates than control plants (Fig. 2.4) what denoted a shift of a use of sugars that favours vegetative growth and contributed to enhance fruit abscission. Nevertheless, it should be considered that starch remobilization from the tree structures into soluble sugars might have compensated in some extent the decline of photosynthate production (MacNeill et al., 2017; Dong and Beckles, 2019; Breen et al., 2020). Finally, the stronger shoot growth under metamitron application could also be interpreted as a tree response to metamitron application by producing more fully-functioning leaves capable of restoring the tree photosynthetic capacity and the sugar balance, which would have aggravated the competition for soluble sugars with the developing fruits. The negative carbohydrate balance and the slower fruit growth rate of fruits exposed to high nighttime temperatures and after application of

metamitron, indicate a possible beginning of formation of the abscission zone (Jakopič et al., 2015). All these CH related parameters turn out to indicate an enhancement of fruit abscission.

Shading net placement, simulating a period of low radiation also promoted a significant enhancement of natural fruit drop, even stronger than the effect caused by metamitron application. This effect was irrespective of fruit size (10 or 13 mm) and cultivar ('Gala' and 'Golden') (Table 3.5). Also, high nighttime temperature reduced the number of fruits in all trials, statistically significant in three of them, and with the exception of Girona, the thinning effect induced by five nights of higher nighttime temperature was very similar to applying 247.5 ppm of metamitron (Table 4.3). Low radiation and high nighttime temperature promoted improvements in fruit size and quality. Additionally, both combinations of metamitron with low radiation or with high nighttime temperature resulted in the strongest thinning observed in all trials (Tables 3.5 and 4.3. Some of these combined treatments resulted in serious situations of over thinning with significant losses in yield. The thinning efficacy of these treatments, assessed through the percentage of fruits decrease, was found to be correlated with the percentage of sucrose decrease in leaves (Fig. 6.1), meaning that the content of these sugars could be considered as a proxy for metamitron thinning efficacy.

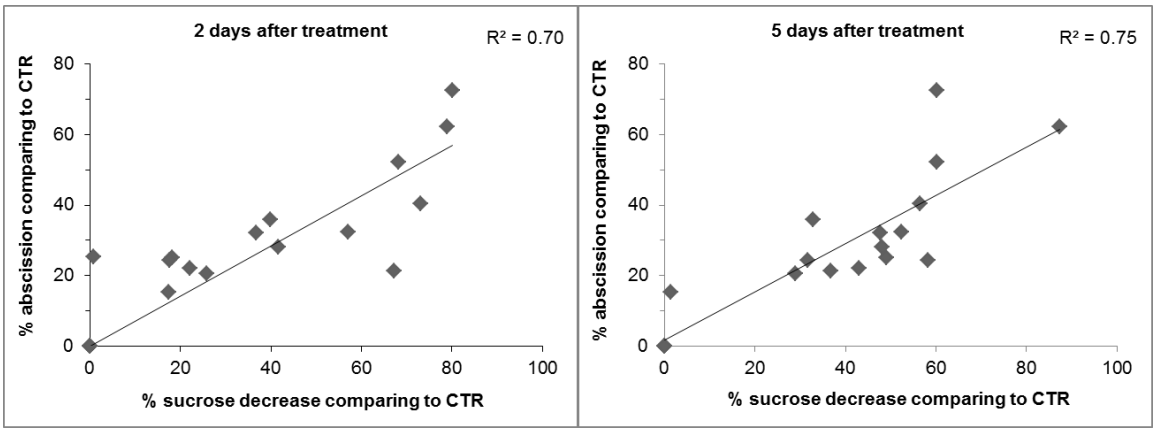

Figure 6.1 – Linear model and correspondent r^2 between the percentage of abscission fruits in all treatments comparing to control trees and the percentage of reduction in sucrose content in leaves in all treatments comparing to control trees. Analysis performed with data from Lleida and Girona trials performed in 2019 (*n* = 21).

The effect of relative humidity on its own was not relevant, but combined with metamitron was significant in both cultivars used in Sint-Truiden ('Braeburn' and 'Elstar'), corroborated by the increase of metamitron absorption, enhanced decrease in P_n as well as in sucrose and sorbitol contents, which resulted in a stronger thinning efficacy and significant fruit size increase. In opposition, the combination of metamitron and increased relative humidity caused no changes in none of the analyzed parameters in the two trials in Spain, in agreement with the thinning efficacy, which was similar in metamitron, *per se*, or in combination with increase relative humidity.

Altogether, the results of these multiple trials are important to understand that certain meteorological conditions, such as radiation, nighttime temperature and relative humidity, may induce significant differences on metamitron thinning efficacy, depending on the characteristics of the trees, namely the age (carbohydrates reserves) and the cultivar.

Due to the interruption in the electron transport chain, metamitron may promote the transfer of electrons to alternative donors such as molecular oxygen, leading to an exacerbation of the oxidative status (Foyer and Noctor, 2000; Noctor et al., 2002). However, shade would reduce the flux of photons reaching the antenna, which can reduce the probability of the transfer of electrons to molecular oxygen. This was in accordance in the absence of leaf MDA and H_2O_2 variation in all treatments in the shading trial treatments, as compared to control (Figure 3.5). There was also an absence of MDA variation in the nighttime temperature trial however, high nighttime temperature, and/or metamitron induced an increase in H_2O_2 levels. Metamitron alone promoted an up-regulation of APX, CAT, GR and SOD activities, whereas its combination with shading nets promoted moderate activity increases of CAT and GPX, and its combination with higher nighttime temperatures promoted CAT, GR, SOD and APX activities. In this way, by reducing the irradiance at leaf surface there will be less unpaired electrons available to form reactive oxygen species, thus contributing to attenuate oxidative effect. Moreover, increased antioxidant levels can detoxify superoxide radicals, thereby preventing oxidative damage, which is in agreement with the high levels of glutathione observed in high nighttime temperature alone, metamitron alone or combined with shading net or high nighttime temperature treatments and justifies the lack of lipid peroxidation (assessed through MDA levels). Overall, our findings pointed that oxidative stress conditions were not present in neither of the applied treatments, although due to a moderate increased antioxidant activity and enhanced antioxidant production, particularly in those treatments where the photochemical use of energy was reduced (under metamitron conditions).

6.2 Conclusions and Future Perspectives

A conceptual model is proposed providing new insights on the effect of radiation, nighttime temperature alone or in combination with metamitron on the physiological processes that trigger fruit abscission (Fig. 6.1).

In summary, the data presented in this study indicates that low radiation and high nighttime temperatures enhance natural fruit abscission, on its own, while high humidity does not affect it. The first, due to a reduced photosynthetic activity and consequent reduction on carbohydrate production. The second, due to a respiratory increase and slight respiratory enzyme activity, on the first day after increasing nighttime temperature, followed by carbohydrate content decrease and a slowdown in fruit growth rate. In the case of higher night temperature, besides the already mentioned factors, there is probably an increased in the whole tree metabolism, that is no longer limited by a reduced temperature that slows down metabolism enzyme Vmax.The relative humidity on its own does not affect the abscission process.

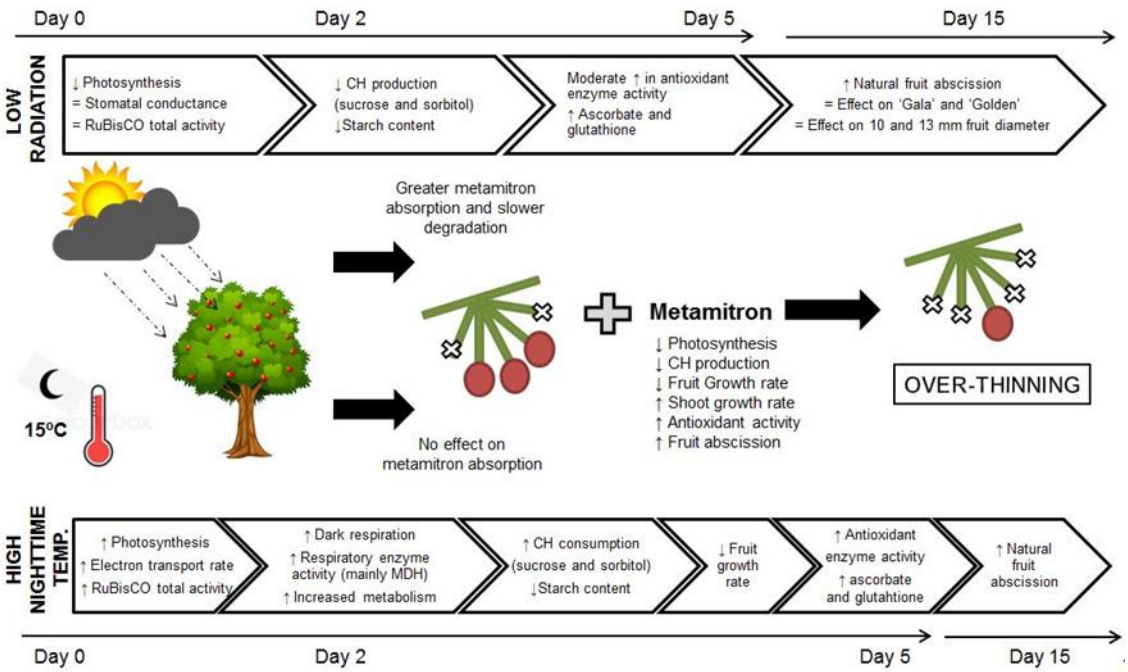

Figure 6.2 – Proposed model of the regulatory events during low radiation (upper side) and high nighttime temperature (lower part) periods per si, during the 5 days after changing the meteorological conditions, and its enhanced effect when combined with 247.5 ppm metamitron application (middle part), and consequent effect on fruit abscission around 15 days after changing meteorological conditions and/or spraying metamitron.

The way of action and pathways until triggering abscission along with its interaction with the meteorological conditions are unlighted in figure 6.1 (middle part). Metamitron binds on D1 protein, inhibiting electron transport rate, finally reducing photosynthesis and ATP and carbon fixation. It reduces sucrose and sorbitol leaf level at a certain extent, finally translating in enhanced fruit abscission. Periods of low radiation after its application enhance metamitron absorption; whilst night temperature has no effect. The higher the absorption, the greater the photosynthetic inhibition. Therefore, the combination of metamitron and low radiation periods induced a very strong photosynthesis inhibition while the combination of metamitron with periods of higher nighttime period, the photosynthetic reduction is similar to metamitron on its own. Nevertheless, both situations triggered an extreme carbohydrate shortage followed by a very strong fruit abscission event causing, in some cases, over-thinning due to the profound yield losses. The explanation for the strong CH reduction under high nighttime temperature is the slight increase in the respiratory activity but mainly in the acceleration of the metabolic processes at 15 ºC which consume sugars as substrate.

This work can also serve as a starting point for studies regarding the effect of relative humidity on metamitron thinning effect. Our data showed that under more humid climates and on younger trees of cultivars 'Braeburn' and 'Elstar', high relative humidity 3 hours before spraying (with an average increase of 7.5 ºC) and one hour after, triggered a chain event of greater

metamitron absorption, with consequent stronger photosynthetic inhibition, which translated in lower leaf sucrose and sorbitol content that finally enhanced fruit drop and increased fruit quality and size, comparing to metamitron alone. In older 'Golden' trees, in two trials performed in the drier climate of Spain, there was no extra thinning effect caused by high relative humidity. This raises many questions, namely how temperature interact with relative humidity, when is relative humidity more likely to affect metamitron efficacy, before or after application, and which cultivars may respond to such environmental change.

In addition, the physiological and biochemical parameters explored in this study clarify the mechanisms that trigger abscission and show that there is a clear correlation between the percentage of fruit drop comparing to control trees (thinning efficacy) and the percentage of sucrose and sorbitol decrease comparing to control leaves, specially the first mentioned one. This could be a very practical future research topic, in which these correlations could be further studied, in order to develop a practical field tool that could assist in the assessment of the efficacy of the first metamitron application, and help the grower to decide if another one needs to be done to achieve its optimal crop load goal.

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