Title

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

Authors

Nídia Rosa[1*](https://orcid.org/0000-0001-5516-7818), Glória Àvila[2] (http://orcid.org/0000-0002-4643-4758), J. Carbó[2] (http://orcid.org/0000-0001-7492-2663), Wim Verjans[3], J. Bonany[2] (http://orcid.org/0000-0003-3738-4725), J.C. Ramalho[4,5*](https://orcid.org/0000-0002-7639-7214), L. Asín[6*] (http://orcid.org/0000-0003-3431-2988), C. M. Oliveira[1*] (https://orcid.org/0000-0002-3480-9380)

Affiliations

[1] Linking Landscape, Environment, Agriculture and Food (LEAF), Instituto Superior de Agronomia (ISA), Universidade de Lisboa , 1349-017, Lisbon, Portugal

[2] IRTA-Mas Badia Field Station, La Tallada de l'Empordà, 17134, Girona, Spain

[3] PCFruit Research Station, BE-3800, Sint-Truiden, Belgium

[4] PlantStress & Biodiversity Lab, Centro de Estudos Florestais (CEF), Instituto Superior Agronomia (ISA), Universidade de Lisboa, 1349-017, Lisboa, Portugal.

[5] Unidade de Geobiociências, Geoengenharias e Geotecnologias (GeoBioTec), Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, 2829-516, Caparica, Portugal

[6] IRTA Fruitcentre, PCiTAL, Park of Gardeny, Fruitcentre Building, 25003, Lleida, Spain

*Corresponding authors:

N. Rosa - nidia.heleno.rosa@gmail.com; J.C. Ramalho - cochichor@mail.telepac.pt; cochichor@isa.ulisboa.pt; L. Asín - luis.asin@irta.cat; C.M. Oliveira - crismoniz@isa.ulisboa.pt;

HIGHLIGHTS

- Increased nighttime temperature and metamitron application, per si, retarded fruitlet growth rate by 30%, reduced sucrose and sorbitol leaf content and enhanced abscission, in a similar way.
- The greatest reductions in sucrose and sorbitol were caused by metamitron combined with higher nighttime temperature, followed by the strongest thinning efficacy and fruit size improvement.
- Metamitron inhibit photosynthesis and reduced carbohydrate (CH) production and high nighttime temperature increased CH consumption likely due to greater respiration rates.
- Enzyme related with oxidative stress control were moderately enhanced under the combined use of metamitron and nighttime temperature.

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

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.

2. MATERIAL AND METHODS

2.1 Plant material and experimental design

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.

160 **2.1.2 Treatment implementation**

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

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

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

177 To monitor the environmental conditions in each trial, sensors for temperature and relative
178 humidity record were installed inside and outside of the structures on both sides and in the middle
179 (with and without HNT); in each case in the upper (2 m) and lower (1 m) level of the trees. In Girona,
180 six EasyLog USB Data Loggers (Lascar Electronics, Wiltshire, UK) were used; in Lleida, six Testo
181 177-h1 sensors were used (Testo, Titisee-Neustadt, Germany); and six Testo 174H sensors (Testo,
182 Titisee-Neustadt, Germany USA) were used in Sint-Truiden.

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

187 **2.2 Metamitron leaf analysis**

188 In 2017, leaf samples for metamitron concentration were collected only in Lleida, 1 and 3 days
189 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).

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.

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

219 constituted by 2 shoot leaves and 2 cluster leaves, 4 and 8 repetitions per treatment, in 2017 and
220 2018, respectively.

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

227 **2.5 Leaf oxidative status evaluation**

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

231 **2.5.1 Lipoperoxidation and H_2O_2 content**

232 Sample extraction was performed using 200 mg FW frozen material, homogenized with 2.0
233 mL of 0.1% trichloroacetic acid (TCA), and centrifuged (12000 *g*, 15 min, 2 ºC). Lipid peroxidation
234 was estimated by measuring malondialdehyde (MDA) content, using the thiobarbituric acid (TBA)
235 method, as described by Demiral and Turkan (2005). After extraction, 4 mL of 20% TCA containing
236 0.5% TBA was added to a 1 mL aliquot of the supernatant. This mixture was heated (95 ºC, 30 min)
237 followed by quick cooling in an ice bath and centrifugation (10000 *g*, 15 min, 2 ºC). The amount of
238 MDA was calculated from the coefficient of absorbance at 532 nm after subtracting the non-specific
239 absorption at 600 nm. The extinction coefficient 155 mM^{-1} cm^{-1} for MDA was used. Hydrogen
240 peroxide (H_2O_2) content was measured using the method described by Singh et al. (2006). To a 50
241 µL aliquot of the supernatant obtained in the extraction, 959 µL 100 mM phosphate buffer, pH 7.6,
242 and 1 mL 1 M potassium iodide were added. The absorbance of the supernatant was measured at
243 390 nm and for quantification was used a standard curve of hydrogen peroxide (0, 1.1, 2.2, 3.3, 4.4
244 and 5.5 µg mL^{-1}).

245 **2.5.2 Antioxidative enzyme assays**

246 For catalase (CAT), guaiacol peroxidase (GPOD), superoxide dismutase (SOD) and
247 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.

2.5.2.1 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}.

2.5.2.2 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}.

2.5.2.3 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}.

2.5.2.4 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}.

2.5.2.5 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}.

2.5.2.6 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}.

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.

2.5.3.1 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.

2.5.3.2 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.

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.

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).

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).

3. RESULTS

3.1 Environmental conditions

A brief characterization of the environmental conditions in the five performed trials is shown in Table 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 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

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. 1).

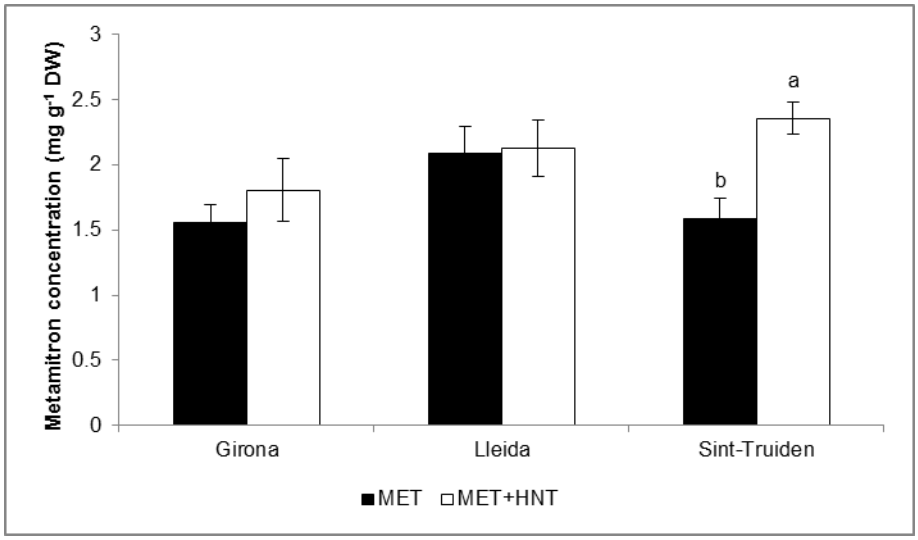


Figure 1 - Metamitron content (mg g^{-1} DW) evaluated 2 DAS, in the trials of 2018 in Girona, Lleida and Sint-Truiden. Trees were exposed to artificially increased nighttime temperature for 5 nights after spraying, from 20:00-8:00 h. For each parameter, the mean values ± SE (*n*=8) followed by different 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

3.3 Leaf gas exchanges

High nighttime temperature, did not promote changes in P_n, in comparison with CTR along the entire trial (Fig. 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. 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. 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. 2B). During the experiment, g_s was not a limiting factor of P_n, since its value remained stable and similar within treatments.

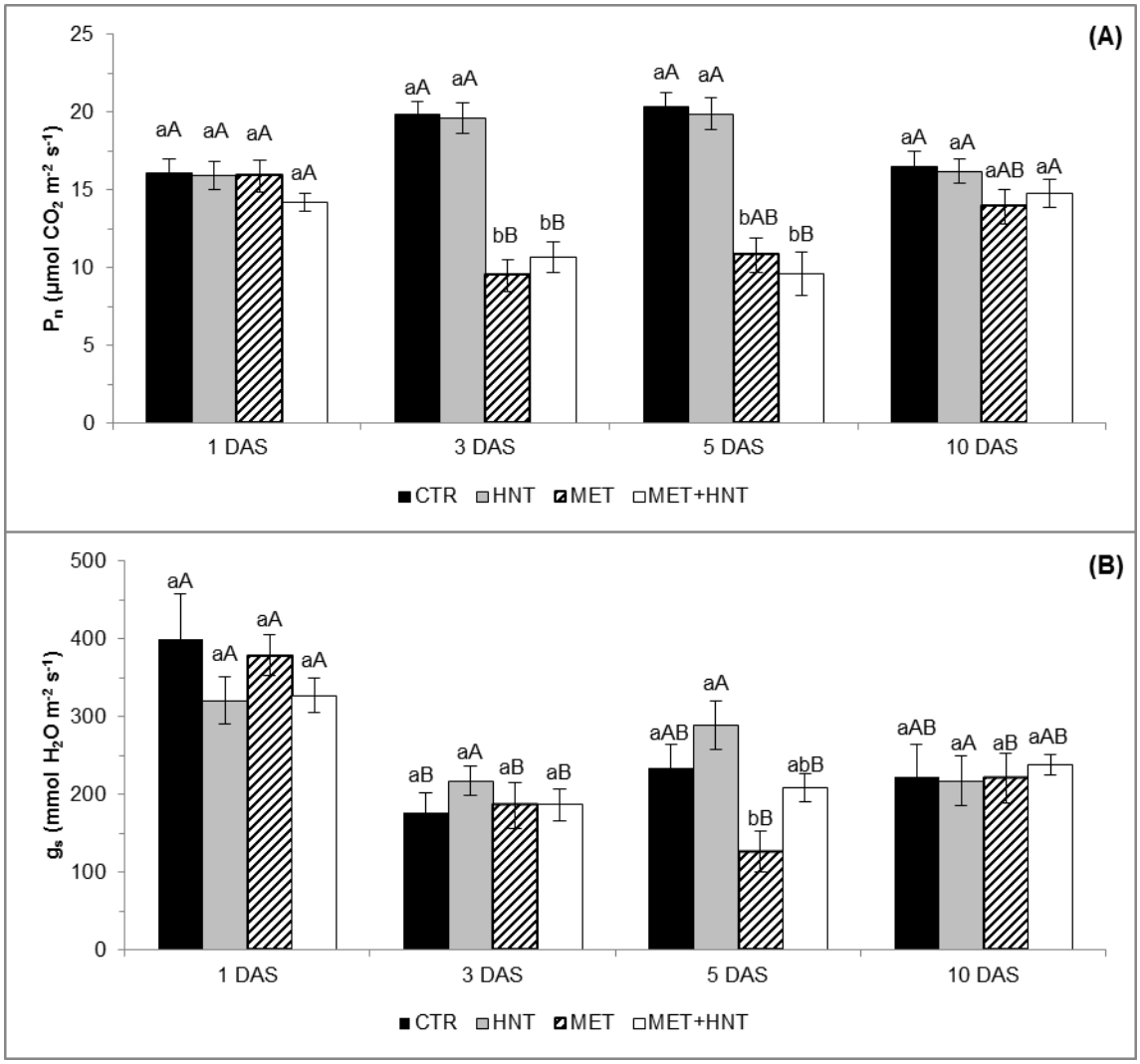


Figure 2 – Leaf net CO_2 gas exchange (P_n) (µmol CO_2 m^{-2} s^{-1}) (A) and stomatal conductance to water vapor rate (g_s) (mmol H_2O m^{-2} s^{-1}) (B) evaluated 1, 3, 5 and 10 days after spraying (DAS), in the trial of 2017, in Lleida. Trees were exposed to artificially increased nighttime temperature 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

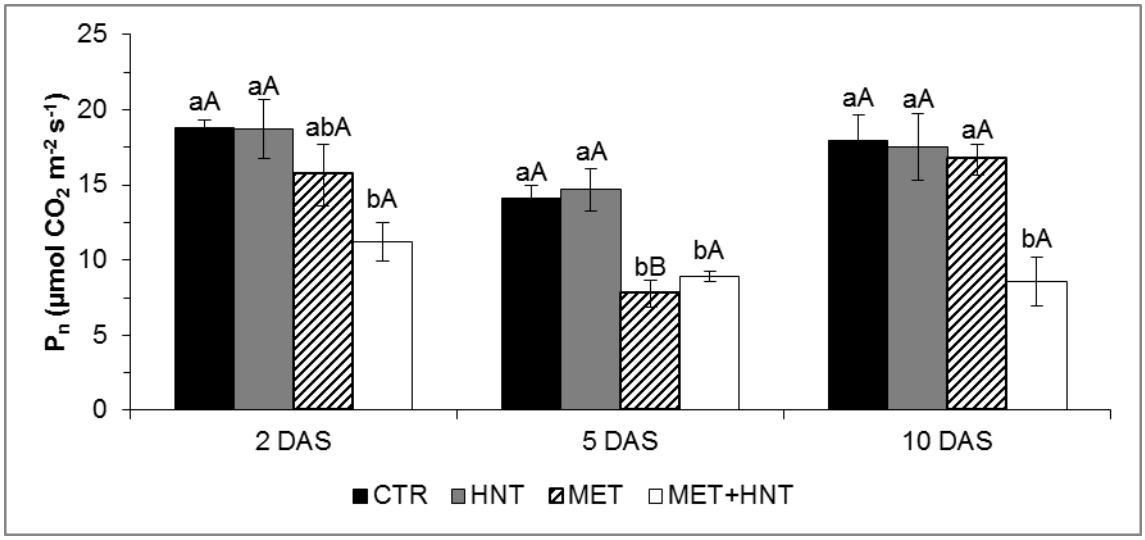


Figure 3 – Leaf net CO_2 gas exchange (P_n) (µmol CO_2 m^{-2} s^{-1}) evaluated 2, 5 and 10 DAS, in the trial of 2018, in Sint-Truiden. Trees were exposed to artificially increased nighttime temperature for 5 nights after spraying, from 20:00-8:00 h. For each parameter, the mean values ± SE (*n*=8) followed 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). 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.

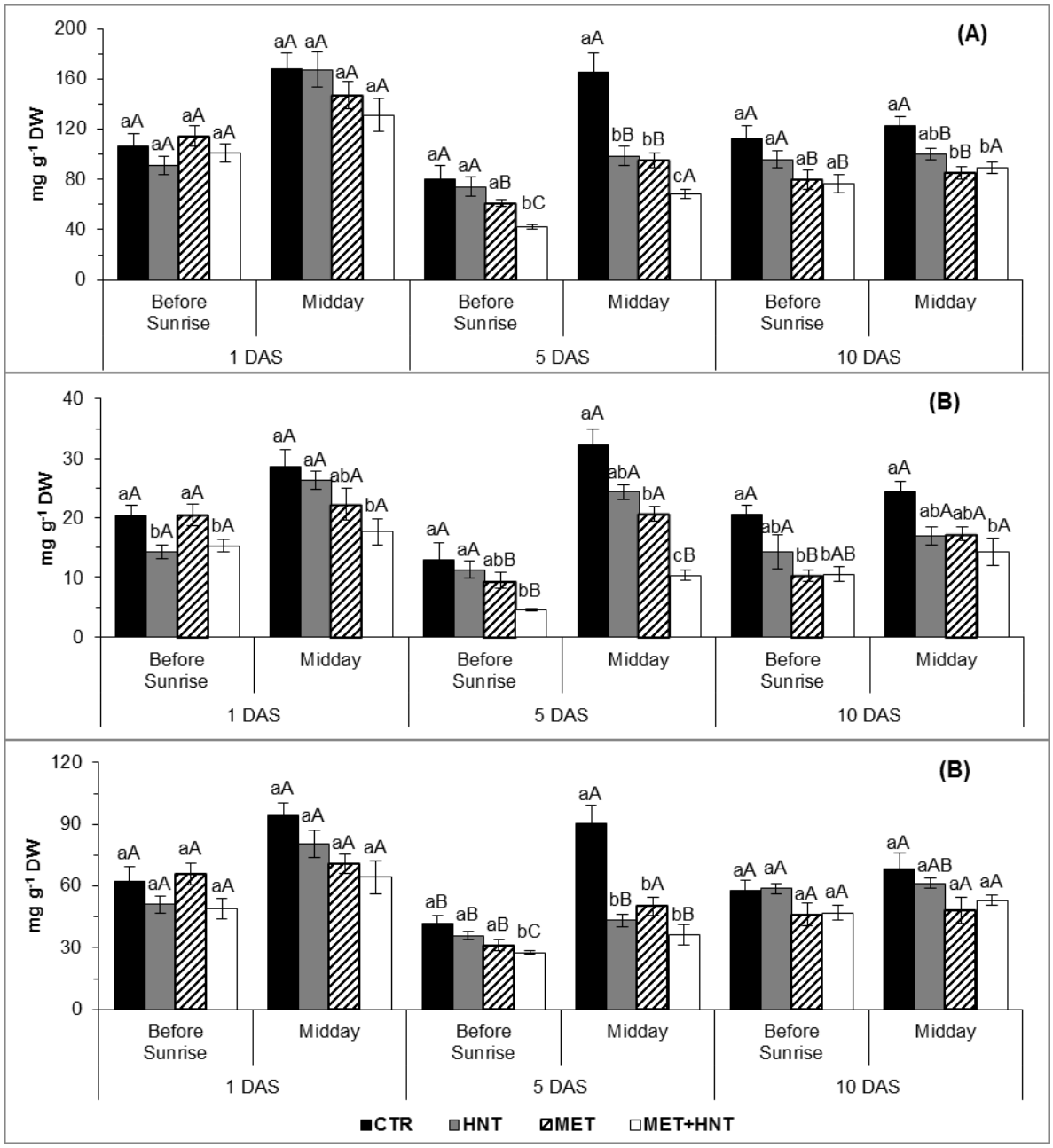


Figure 4 – Total sugars (A), sucrose (B) and sorbitol (C) concentration in the leaves (mg g^{-1} DW) evaluated 1, 5 and 10 days after spraying (DAS), before sunrise and at midday, in the trial of 2017, in Lleida. Trees were exposed to artificially increased nighttime temperature for 5 nights after spraying, 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

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.

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 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

3.5 Leaf oxidative status

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. 5).

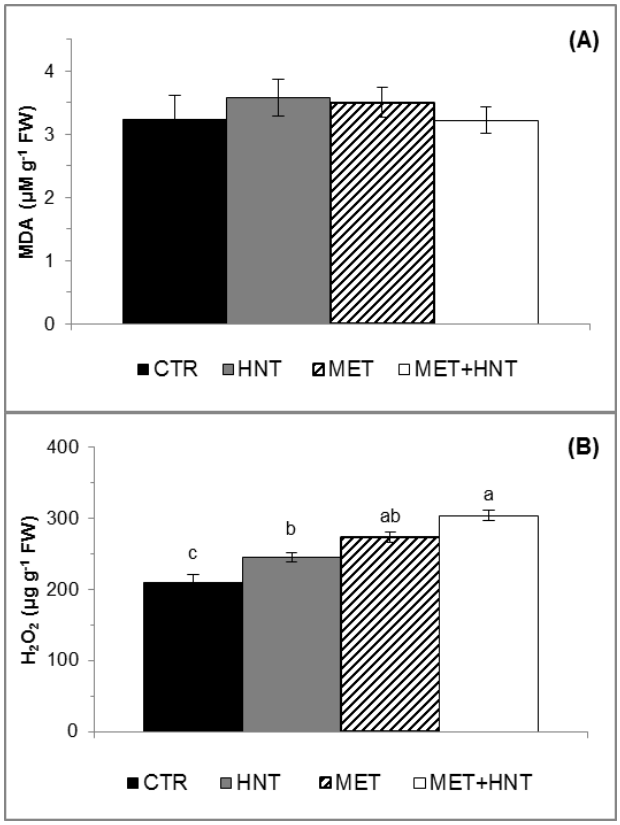
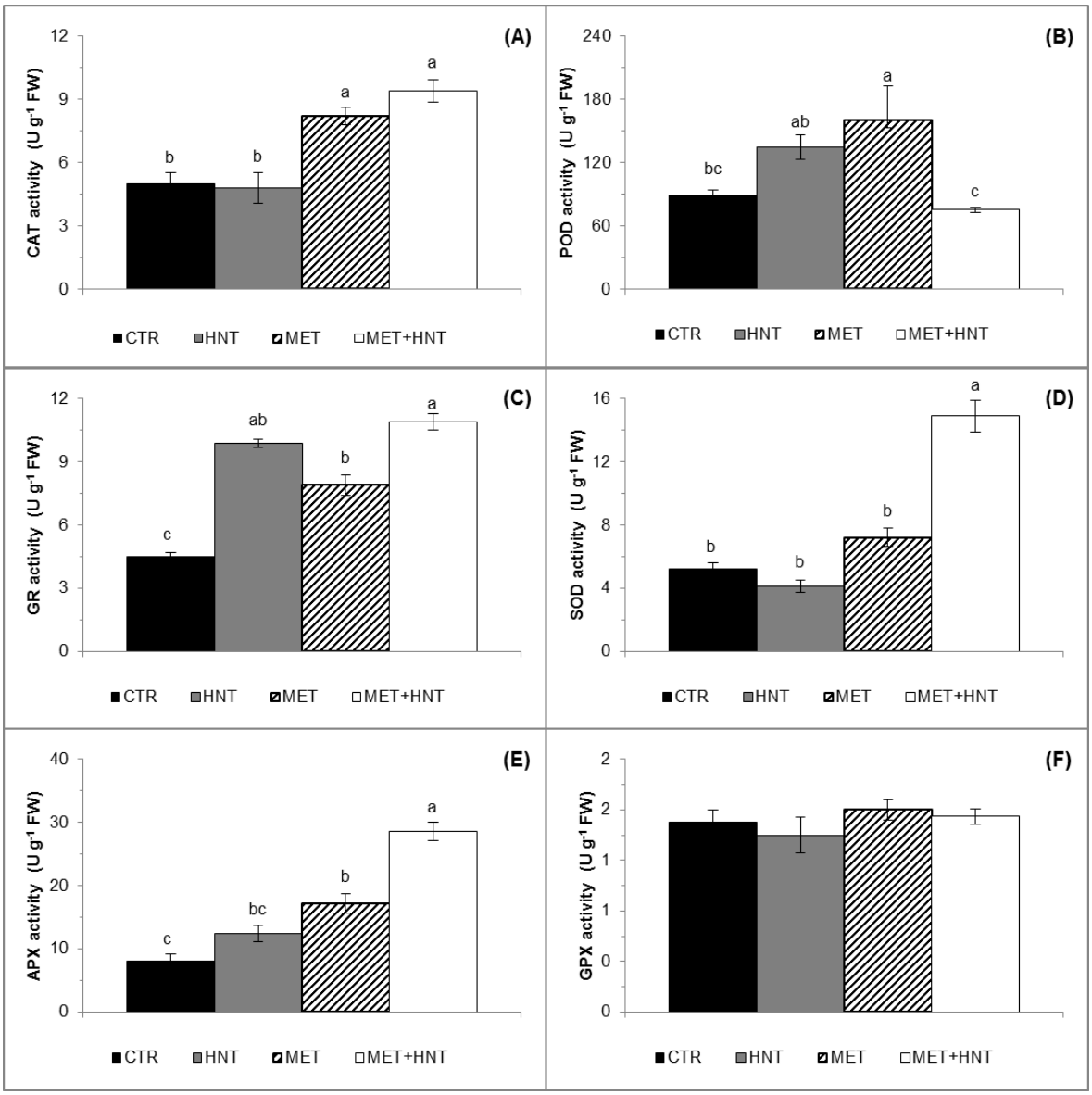


Figure 5 – Leaf average contents of malondialdehyde (MDA) (µM g^{-1} FW) (A) and hydrogen peroxide (H_2O_2) (µg g^{-1} FW) (B) evaluated 5 DAS, in the trial of 2018, in Sint-Truiden. Trees were exposed to artificially increased nighttime temperature for 5 nights after spraying, from 20:00-8:00 h. The 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

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. 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.



476 Figure 6 – Catalase (CAT) (A), guaiacol peroxidase (POD) (B), glutathione reductase (GR) (C),
477 superoxide dismutase (SOD) (D), ascorbate peroxidase (APX) (E) and glutathione peroxidase
478 (GPX) (F) activities (U g^{-1} FW) evaluated 5 DAS, in the trials of 2018, in Sint-Truiden. Trees were
479 exposed to artificially increased nighttime temperature for 5 nights after spraying, from 20:00-8:00 h.
480 For each parameter, the mean values ± SE (*n*=4) followed by different letters express significant
481 differences between treatments using Tukey's HSD test (*α*-value ≤ 0.05). No letters indicate no
482 significant difference between means. HNT – High nighttime temperature; MET – Metamitron, DAS
483 – Days after spraying

484

485

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. 7). Ascorbate showed an inverse pattern of that displayed by glutathione. All treatments promoted the reduction in total (AsA+DHA) ascorbate (Fig. 7). Concerning total ascorbate, the sharpest decrease was promoted by MET+HNT, reducing to values 34% lower than CTR.

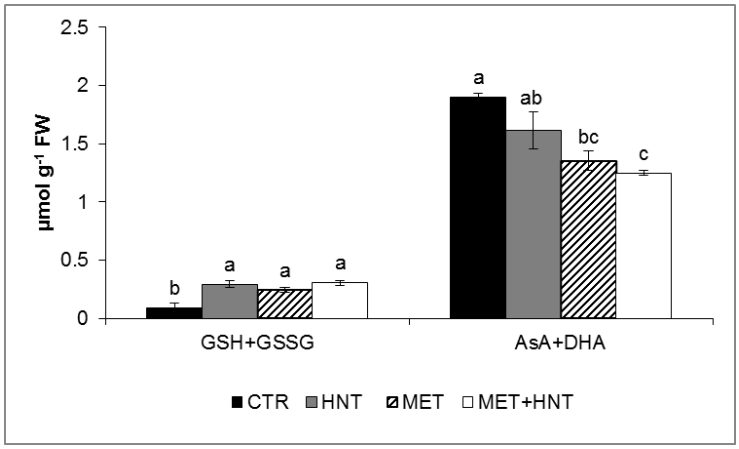
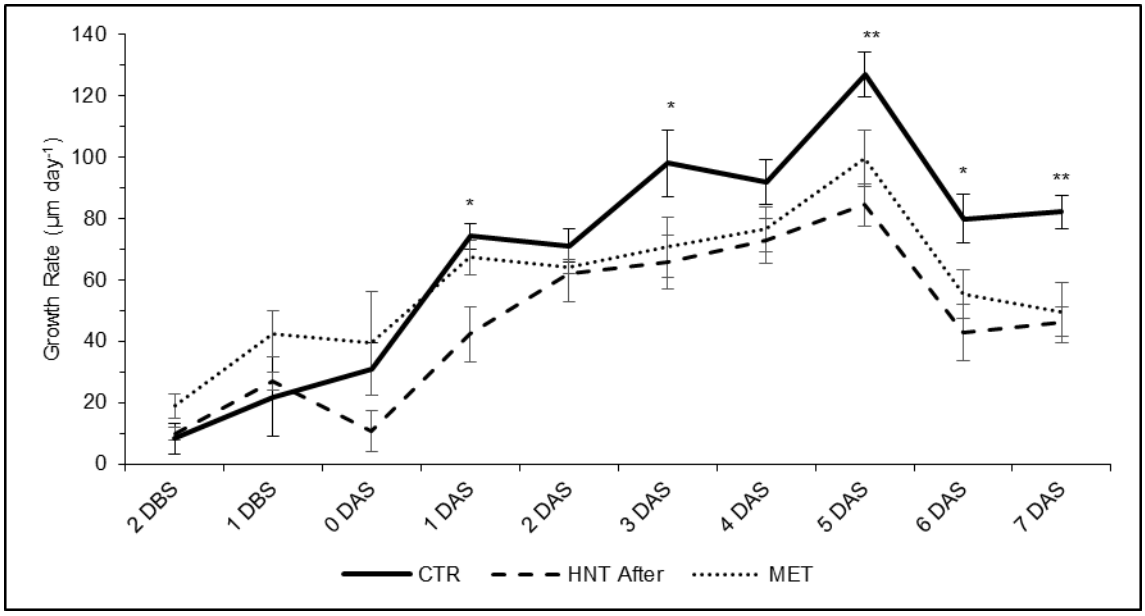


Figure 7 - Total glutathione (GSH+GSSG) (A) and total ascorbate (AsA+DHA) (B) (µmol g^{-1} FW) evaluated 5 DAS, in the trials of 2018, in Sint-Truiden. Trees were exposed to artificially increased nighttime temperature for 5 nights after spraying, from 20:00-8:00 h. For each parameter, the mean values ± 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

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. 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.



508

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

514

515 **3.7 Yield parameters**

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

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

523 Metamitron significantly reduced the number of fruits per 100 flower clusters, in both years
524 in Lleida (2017 and 2018), 40 and 26%, and in Girona (2018) and Sint-Truiden, both 40% less fruits
525 comparing to CTR. Consequently, there was a significant improvement on average fruit weight in
526 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 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, DAS – Days after spraying

			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
			Fruits/100 flower clusters	**Average fruit weight (g)**	**Yield/tree (kg)**	**% fruits > 70 mm**
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. DISCUSSION

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.

4.2 Effect on non-structural carbohydrates

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 agreement 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. Regarding sucrose, in Lleida (2018), 2 and 5 DAS, the mentioned treatment showed significantly lower content with no signs of recovery 10 DAS (still significant differences comparing to the other 5 treatments) and in Sint-Truiden, 5 DAS sucrose content was significantly lower than all the other treatments. Sorbitol followed the same trend, showing significantly lower values than the other treatments at 10 DAS. 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.

4.3 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 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. 8). High nighttime temperature showed signs of slowing down fruit growth around 1 DAS whereas the effect of metamitron arise 3 DAS, both remaining lower than CTR from 5 DAS on. The metabolism of sorbitol and sucrose fuels fruit growth (Li et al., 2012) and as discussed in 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 18ºC 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

706 metamitron is not always linear between years and locations. When metamitron was combined with
707 high nighttime temperatures, in some cases there was not an extra thinning effect as compared with
708 MET, whereas in both years in Lleida and in Sint-Truiden, the combination of MET+HNT
709 consistently promoted tendencies for stronger fruit abscission as compared with MET alone. In
710 some trials, MET+HNT was the only treatment that promoted a significantly increased fruit weight
711 and fruit size. Moreover, the only yield reduction (over-thinning) observed within the 5 performed
712 trials was caused by MET+HNT, in Sint-Truiden. The effect of environmental conditions, namely
713 nighttime temperature, on the efficacy of chemical thinners has been described by several authors
714 (Lakso et al., 1999; Byers et al., 2002) and included in models, either to estimate carbohydrate
715 balance (Lordan et al., 2019) or to accurately predict the thinning effect of metamitron based on
716 irradiance and nighttime temperature (Clever, 2018). Stern (2015) sprayed 190 ppm of metamitron
717 in 'Golden Delicious' trees in the warm climate of Israel, in which nighttime temperature during the 3
718 weeks after spraying varied between 12.8 and 14.6 ºC in the three trials, and observed a strong fruit
719 abscission (with a 10 fold increase in kg in fruit size class > 70 mm). Metamitron efficacy in 'Golden
720 Delicious' trials set up by Stern (2015) were higher than the ones obtained by Brunner (2014), using
721 247.5 ppm, by Gabardo et al. (2017) using higher metamitron dosages in 'Fuji', or than the results
722 here obtained. All the previously mentioned studies were performed in regions in which average
723 nighttime temperature is about 10 ºC lower than in Israel by the time application was made. Similar
724 strong abscission results were obtained in 'Gala' by Stern (2014). This author attributes the efficacy
725 of the relatively low dose compared to those used in Europe and the USA, to the higher night
726 temperatures for 3 weeks after application, which increased dark respiration at a critical point of
727 fruitlet growth and caused assimilation deficiencies that triggered the abscission process. The same
728 explanation applies to our results.
729
730 **5. CONCLUSIONS**
731 It is more and more accepted that thinning is highly depend on carbon hydrate balance being
732 the nighttime temperature an environmental factor that has a great impact on carbohydrate content.
733 Consequently, nighttime temperature after metamitron application has an influence on fruit
734 abscission and on the chemical thinning response. High nighttime temperature did not affect
735 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, 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.

Conflict of Interest Statement

The authors declare that there are not any potential conflicts of interest.

Funding

This study was supported by ADAMA-Israel, as well as by Fundação para a Ciência e a Tecnologia (FCT) through the research units UID/AGR/04129/2020 (LEAF), UIDB/00239/2020 (CEF), and UIDP/04035/2020 (GeoBioTec).

765 **REFERENCES**

766 Abbaspoor, M., Teicher, H.B. and Streibig, J.C., 2006. The Effect of Root-Absorbed PSII Inhibitors
767 on Kautsky Curve Parameters in Sugar Beet. Weed Res., 46, 226-235. DOI: 10.1111/J.1365-
768 3180.2006.00498.X

769 Aebi, H.E., 1983. Catalase. In: Bergmeyer, H.U., Ed., Methods of Enzymatic Analysis, 3, 273- 286.

770 Anderson, J., Chevone, B. and Hess, J., 1992. Seasonal Variation in the Antioxidant System of
771 Eastern White Pine Needles: Evidence for Thermal Dependence. Plant Physiol., 98, 501-
772 508. DOI: 10.1104/pp.98.2.501

773 Aravind, P. and Prasad, M.N.V., 2005. Modulation of cadmium-induced oxidative stress in
774 Ceratophyllum demersum by zinc involves ascorbate-glutathione cycle and glutathione
775 metabolism. Plant Physiol. Biochem., 43, 107-116. DOI: 10.1016/j.plaphy.2005.01.002

776 Arevalo, L. M., Oosterhuis, D.M. and Brown, R.S., 2008. The Physiological Response of Cotton to
777 High Night Temperatures. AAES Research Series, 533, 44-50.

778 Basak, A., 2011. Efficiency of Fruitlet Thinning in Apple 'Gala Must' by Use of Metamitron and
779 Artificial Shading. J. Fruit Ornam. Plant Res., 19, 51-62.

780 Bieleski, R. L., 1969. Accumulation and Translocation of Sorbitol in Apple Phloem. Aust. J. Biol.
781 Sci., 22, 611–620. DOI: 10.1071/bi9690611

782 Brunner, P., 2014. Impact of Metamitron as a Thinning Compound on Apple Plants. Proc. XIIth IS on
783 Plant Bioregulators in Fruit Production. Acta Hort., 1042, ISHS 2014. DOI:
784 10.17660/ActaHortic.2014.1042.21

785 Byers, R.E., 2002. Influence of temperature and darkness on apple fruit abscission and chemical
786 thinning. J. Tree Fruit Prod. 3, 41-53. DOI: 10.1300/J072v03n01_04

787 Carvalho, L.C. and Amâncio, S., 2002. Antioxidant Defense System in Plantlets Transferred from in
788 Vitro to ex Vitro: Effects of Increasing Light Intensity and CO_2 Concentration. Plant Sci., 162,
789 33-40. DOI: 10.1016/S0168-9452(01)00524-6

790 Cheng, L., Zhou, R., Reidel, E.J., Sharkey, T.D. and Dandekar, A.M., 2005. Antisense Inhibition of
791 Sorbitol Synthesis Leads to Up-regulation of Starch Synthesis Without Altering CO_2
792 Assimilation in Apple Leaves. Planta, 220, 767–776. DOI: 10.1007/s00425-004-1384-5

793 Chong, C. and Taper, C.D., 1970. Daily Variation of Sorbitol and Related Carbohydrates in Malus
794 Leaves. Can. J. Bot., 49 (1), 173-177. DOI: 10.1139/b71-029

Chong, C., 1971. Study of the Seasonal and Daily Distribution of Sorbitol and Related Carbohydrates Within Apple Seedlings by Analysis of Selected Tissues and Organs. Can. J. Bot., 51, 519-525. DOI: 10.4141/cjps71-100

Clever, M., 2018. Effects of Solar Irradiation and Nighttime Temperature on the Thinning Efficacy of Metamitron (Brevis®) in Apple. Proc. EUFRIN Thinning working Group Symposia, Acta Hortic., 1221, 23-30. DOI:10.17660/ActaHortic.2018.1221.4

Corelli-Grappadelli, L. and Lakso, A.N., 2004. Fruit Development in Deciduous Tree Crops as Affected by Physiological Factors and Environmental Conditions. Proc. XXVI IHC – Deciduous Fruit and Nut Trees, Acta Hort., 636, 425-441. DOI: 10.17660/ActaHortic.2004.636.52

Demiral, T. and Turkan, I., 2005. Comparative Lipid Peroxidation, Antioxidant Defense System and Proline Content in Roots of Two Rice Cultivars Differing in Salt Tolerance. Environ. Exp. Bot., 53, 247-257. DOI: 10.1016/j.envexpbot.2004.03.017

Denne, M.P., 1963. Fruit Development and Some Tree Factors Affecting It. N. Z. J. Bot., 1, 265-294. DOI: 10.1080/0028825X.1963.10428999

Doerflinger, F., Lakso, A. and Braun, P., 2015. Adapting MaluSim Apple Tree Model for the 'Gala' Cultivar. Proc. IXth IS on Modeling in Fruit Research and Orchard Management. Acta Hort., 1068, 267-271. DOI: 10.17660/ActaHortic.2015.1068.33

Foyer, C.H. and Noctor, G.. 2000. Oxygen processing in photosynthesis: regulation and signaling. New Phytol., 146, 359-388. DOI: 10.1046/j.1469-8137.2000.00667.x

Gabardo, G., Kretzschmar, A., Petri, J., Couto, M., Hawerooth, F. and Silva, C., 2017. Taxa Fotossintética em Macieiras Tratadas com Metamitron. Rev. Elet. Cient. UERGS, 3, 617-633. DOI: 10.21674/2448-0479.33.617-633

Gajewska, E., Sklodowska, M., Slaba, M. and Mazur, J., 2006. Effect of nickel on antioxidative enzyme activities, proline and chlorophyll contents in wheat shoots. Biol. Plant., 50, 653-659. DOI: 10.1007/s10535-006-0102-5

Gillapsy, G., Ben-david, H. and Gruissem, W., 1993. Fruits: a Developmental Perspective. Plant Cell, 5, 1439–1451. DOI: 10.1105/tpc.5.10.1439

Gonzalez, L., 2019. Metamitrona, Una Nueva Herramienta Para Optimizar el Aclareo Químico en Manzano. Tesis Doctoral. Universitat de Lleida, Spain

825 Greene, D.W, Lakso, A.N., Robinson, T.L. and Schwallier, P., 2013. Development of a Fruitlet
826 Growth Model to Predict Thinner Response on Apples. Hortscience, 48 (5), 584-587. DOI:
827 10.21273/HORTSCI.48.5.584
828 Guidi, L. and Degl'innocenti, E., 2011. Imaging of Chlorophyll a Fluorescence: a Tool to Study
829 Abiotic Stress in Plants. Abiotic Stress in Plants - Mechanisms and Adaptations. Intech. DOI:
830 10.5772/22281
831 Jing, P., Wang, D., Zhu, C. and Chen, J., 2016. Plant Physiological, Morphological and Yield-
832 Related Responses to Night Temperature Changes across Different Species and Plant
833 Functional Types. Frontiers in Plant Science, 7, 1774. DOI: 10.3389/fpls.2016.01774
834 Klages, K., Donnison, H., Wünschem J. and Boldingh, H., 2001. Diurnal Changes in Non-Structural
835 Carbohydrates in Leaves, Phloem Exudate and Fruit in 'Braeburn' Apple. Aust. J. Plant
836 Physiol., 28, 131-139. DOI: 10.1071/PP00077
837 Kondo, S. and Takahashi, Y., 1987. Effects of High Temperature in the Nighttime and Shading in
838 the Daytime on the Early Drop of Apple Fruit 'Starking Delicious'. J. Japan. Soc. Hort. Sci.,
839 56(2), 142-150. DOI: 10.2503/jjshs.56.142
840 Kumar, S., Gupta, D. and Nayyar, H., 2012. Comparative Response of Maize and Rice Genotypes
841 to Heat Stress: Status of Oxidative Stress and Antioxidants. Acta Physiol Plant , 34, 75–86.
842 DOI 10.1007/s11738-011-0806-9
843 Jones, K. M., Bound, S. A., Oakford, M. J. and Gillard, P., 2000. Modeling Thinning of Pome Fruits.
844 Plant Growth Regul., 31, 75–84. DOI: 10.1023/a:1006315000499
845 Lakso, A.N., Wünsche, J.N., Palmer, J.W. and Corelli-Grapadelli, L., 1999. Measurement and
846 Modeling of Carbon Balance of Apple Tree. HortScience, 34 (6), 1040-1047. DOI:
847 10.21273/HORTSCI.34.6.1040
848 Lakso, A.N. and Goffinet, M. C., 2013. Apple Fruit Growth. New York Fruit Quarterly, 21 (1), 11-14.
849 Lakso, A. and Robinson, T.L., 2013. Decision Support for Apple Thinning Based on Carbon
850 Balance Modeling. Proc. IXth IS on Modeling in Fruit Research and Orchard Management,
851 Acta Hort. 1068, 235-242. DOI: 10.17660/ActaHortic.2015.1068.29
852 Lesueur, C., Knittl, P., Gartner, M., Mentler, A. and Furhacker, M., 2008. Analysis of 140 pesticides
853 from conventional farming foodstuff samples after extractions with the modified QuECHERS
854 method. Food Control, 19, 906-914. DOI: 10.1016/j.foodcont.2007.09.002

Li, M., Feng, F. and Cheng, L., 2012. Expression Patterns of Genes Involved in Sugar Metabolism and Accumulation during Apple Fruit Development. PLoS ONE 7(3): e33055. doi:10.1371/journal.pone.0033055

Li, M., Li, P., Ma, F., Dandekar, A.M. and Cheng, L., 2018. Sugar Metabolism and Accumulation in the Fruit of Transgenic Apple Trees With Decreased Sorbitol Synthesis. Hortic. Res., 5(60). DOI 10.1038/s41438-018-0064-8

Loescher, W., Marlow, G. and Kennedy, R., 1982. Sorbitol Metabolism and Sink-Source Interconversions in Developing Apple Leaves. Plant Physiol., 70, 335-339. DOI: 0032-0889/82/70/0335/05/$00.50/0

Loka, D.A. and Oosterhuis, D.M., 2010. Effect of High Night Temperatures in Cotton Respiration, ATP Levels and Carbohydrate Content. Environ. Exp. Bot., 68, 258-263. DOI: 10.1016/j.envexpbot.2010.01.006

Lordan, J., Reginato, G.H., Lakso, A.N., Francescatto, P. and Robinson, T.L., 2019. Natural Fruitlet Abscission as Related To Apple Tree Carbon Balance Estimated With the MaluSim Mode. Sci. Hortic., 247, 296-309. DOI: 10.1016/J.Scienta.2018.11.049

Ma, Y., Ma, F., Zhang, J., Li, M., Wang, Y. and Liang, D., 2008. Effects of High Temperature on Activities and Gene Expression of Enzymes Involved in Ascorbate-Glutathione Cycle in Apple Leaves. Plant Sci., 175, 761-766. DOI: 10.1016/j.plantsci.2008.07.010

Mohammed, A. and Tarpley, L., 2009. Impact of High Nighttime Temperature on Respiration, Membrane Stability, Antioxidant Capacity and Yield of Rice Plants. Crop Sci., 49, 313-322. DOI: 10.2135/cropsci2008.03.0161

Moing, A., 2000. Sugar Alcohols as Carbohydrate Reserves in Some Higher Plants. Develop. Crop. Sci, 26, 337-358. DOI: 10.1016/S0378-519X(00)80017-3

Moura, D.S., Brito, G.G., Campos, A.D., Moraes, I.L., Porto, F.G.S., Teixeira, S.B., Fagundes, P.R.R., Andres, A., Schreiber, F. and Deuner, S., 2017. Non-Structural Carbohydrates Accumulation in Contrasting Rice Genotypes Subjected to High Night Temperatures. J. Agric. Sci., 9 (12), 302-315. DOI: 10.5539/jas.v9n12p302

Noctor, G., Gomez, L., Vanacker, H. and Foyer, C.H., 2002. Interactions Between Biosynthesis, Compartmentation and Transport in the Control of Glutathione Homeostasis and Signaling. J. Exp. Bot., 53, 1283-1304. DOI: 10.1093/jexbot/53.372.1283

885 Okamura, M., 1980. An Improved Method for Determination of L-ascorbic Acid and L-
886 dehydroascorbic Acid in Blood Plasma. Clin. Chim. Acta, 103, 259-268. DOI: 10.1016/0009-
887 8981(80)90144-8
888 Orbovic, V., Achor, D., Petacek, P., Syvertsen, J., 2001. Air Temperature, RH, and Leaf Age Affect
889 Penetration of Urea Through Grapefruit Leaf Cuticles. J. Amer. Hort. Sci., 126 (1), 44-50.
890 DOI: 10.21273/JASHS.126.1.44
891 Pandey, S., Fartyal, D., Agarwal, A., Shakla, T., James, D., Kaul, T., Negi, Y.K., Arora, F. and
892 Redyy, M., 2017. Abiotic stress tolerance in plants: myriad roles of ascorbate peroxidase.
893 Front. Plant Sci., 8, 851. DOI: 10.3389/fpls.2017.00581
894 Pereaudeau, S., Lafarge, T., Roques, S., Quiñones, CO., Clement-Vidal, A., Ouwerkerk, P.B.F.,
895 Rie, J.V., Fabre, D., Jagadish, K.S.V. and Dingkuhn, M., 2015. Effect of Carbohydrates and
896 Night Temperature on Night Respiration in Rice. J. Exp. Bot., 66 (13), 3931-3944. DOI:
897 10.1093/jxb/erv193
898 Prasad, P.V.V., Pisipati, S.R., Ristic, Z., Bukovnic, U. and Fritz, A., 2008. Impact of Nighttime
899 Temperature on Physiology and Growth of Spring Wheat. Crop Sci., 48, 2372-2380. DOI:
900 10.2135/cropsci2007.12.0717
901 Ramalho, J.C., Rodrigues. A.P., Semedo. J.N., Pais. I.P., Martins. L.D., Simões-Costa, M.C.,
902 Leitão, A.E., Fortunato, A.S., Batista-Santos, P., Palos, I.M., Tomaz, M.A., Scotti-Campos,
903 P., Lidon, F.C. and Damatta, F.M., 2013. Sustained Photosynthetic Performance of Coffea
904 Spp. Under Long-Term Enhanced [CO2]. PLoS ONE, 8 (12).
905 DOI:10.1371/journal.pone.0082712
906 Rees, T., 1984. Sucrose Metabolism. In: Storage Carbohydrates in Vascular Plants, Distribution,
907 Physiology and Metabolism. Society of Experimental Biology, 53-76. Seminar Series 19,
908 Cambridge University Press, Cambridge, United Kingdom. ISBN 0521236983
909 Robinson, T.L., and Lakso, A.N., 2011. Advances in Predicting Chemical Thinner Response of
910 Apple Using a Carbon Balance Model. New York Fruit Quarterly, 19 (1), 15-20.
911 Robinson, T.L., Hoying, S., Sazom M.M. and Rufato, A., 2013. Precision Crop Load Management
912 Part 2. New York Fruit Quarterly, 22 (1), 9-13.
913 Rosa, N., Verjans, W., Oliveira, C., Bylemans, D., and Remy, S., 2018. Comparison Between 6-
914 Benzyladenine and Metamitron as Thinning Agents in “Royal Gala”, “Cripps Pink” and “Red

915 Delicious" Apple Cultivars. Acta Hortic., 1221, 51–58. DOI:10.17660/actahortic.2018.1221.8
916 Rosa, N., Verjans, W., Àvila, G., Carbò, J., Bonany, J., Ramalho, J.C., Asín, L. and Oliveira, C.M.,
917 2020. Effects of Metamitron under Different Relative Humidity Conditions on the Fruit
918 Abscission of Malus domestica Borkh. Cultivars. Horticulturae, 6 (4), 89-103. DOI:
919 10.3390/horticulturae6040089
920 Rubio, M., González, E., Minchin, F., Webb. J., Arrese-Igor, C., Ramos, J. and Becana, M., 2002.
921 Effects of water stress on antioxidant enzymes of leaves and nodules of transgenic alfafa
922 overexpressing superoxide dismutases. Physiol. Plant., 115, 531-540. DOI: 10.1034/j.1399-
923 3054.2002.1150407.x
924 Singh, R.P, Banerjee, S., Kumar, PV.V.S., Raveesha, K.A and Rao, A.R., 2006. Tinospora
925 Cordifolia Induces Enzymes of Carcigen/drug Metabolism and Antioxidant System and
926 Inhibits Lipid Peroxidation in Mice. Phytomedicine, 13, 74-84. DOI:
927 10.1016/j.phymed.2004.02.013
928 Shanker, A.K., Djanaguiranam, M., Sudhagar, R., Chandrashekar, C.N., Pathmanabhan, G., 2004.
929 Differential antioxidative response of ascorbate glutathione pathway enzymes and
930 metabolites to chromium speciation stress in green gram (Vigna radiata (L.) R. Wilezek. cv.
931 CO4) roots. Plant Sci., 166, 1035-1043. DOI: 10.1016/j.plantsci.2003.12.015
932 Sharma, P. and Dubey, R.S., 2004. Ascorbate peroxidase from rice seedlings: properties of enzyme
933 isoforms. effect of stresses and protective roles of osmolytes. Plant Sci., 167, 541-550. DOI:
934 10.1155/2012/217037
935 Sharma, P., Jha, A.B., Dubey, R.S. and Pessarakli, M., 2012. Reactive oxygen species, oxidative
936 damage, and antioxidative defense mechanism in plants under stressful conditions. J. Bot.,
937 .2012, Article ID 217037. DOI: 10.1155/2012/217037
938 Stander, O.P.J, Botes, J. and Krogscheepers, C., 2018. The Potential Use of Metamitron as a
939 Chemical Fruit-Thinning Agent in Mandarin. HortTechnology, 28(1), 28-34. DOI:
940 10.21273/HORTTECH03913-17
941 Stern, R.A., 2014. The Photosynthesis Inhibitor Metamitron is an Effective Fruitlet Thinner for 'Gala'
942 Apple in the Warm Climate of Israel. Sci. Hortic., 178, 163-167. DOI:
943 10.1016/j.scienta.2014.08.005
944 Stern, R.A., 2015. The Photosynthesis Inhibitor Metamitron is a Highly Effective Thinner for 'Golden

945 Delicious' Apple in a Warm Climate. Fruits, 70 (3), 127-134. DOI: 10.1051/fruits/2015007
946 Tausz, M., Sircelj, H. and Grill, D., 2004. The Glutathione System as a Stress Marker in Plant
947 Ecophysiology: is a Stress-response concept valid? J. Exp. Bot., 55, 404. DOI:
948 10.1093/jxb/erh194
949 Turnbull, M.H., Murthy, R. and Griffin, K.L., 2002. The Relative Impacts of Daytime and Nighttime
950 Warming on Photosynthetic Capacity in Populus deltoides. Plant Cell Environ., 25, 1729-
951 1737. DOI: 10.1046/j.1365-3040.2002.00947.x
952 Wang, Z., Pan, Q. and Quebedeaux, B., 1999. Carbon Partitioning into Sorbitol, Sucrose, and
953 Starch in Source and Sink Apple Leaves as Affected by elevated CO_2. Environ. Exp. Bot., 41,
954 39-46. DOI: 10.1016/S0098-8472(98)00054-9
955 Wünsche, J.N., Greer, D.H., Laing, W.A. and Palmer, J.W., 2005. Physiological and Biochemical
956 Leaf and Tree Responses to Crop Load in Apple. Tree Physiol., 25, 1253-1263. DOI:
957 10.1093/treephys/25.10.1253
958 Yoon, T.M., Robinson, T.L., Reginato, G.H., 2011. Effects of Temperature and Light Levels on
959 Efficiency of Chemical Thinner on 'Empire' Apple Trees. Proc. IXth IS on Orchard Systems,
960 Acta Hort., 903, 1085-1094. DOI: 10.17660/ActaHortic.2011.903.151
961 Zhou, R. and Quebedeaux, B., 2003. Changes in Photosynthesis and Carbohydrate Metabolism in
962 Mature Apple Leaves in Response to Whole Plant Source-Sink Manipulation. J. Amer. Soc.
963 Horti. Sci., 128 (1), 113-119. DOI: 10.21273/JASHS.128.1.01
964
965