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

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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 °C (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 °C, 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 °C, 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 °C 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 °C, justifying the observed greater thinning potential.

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}); 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}); $_{II,}$ $Y_{(NPQ}$, 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.

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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., 2001; 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., 2020a, b). In fact, it has been reported that a shortage in carbohydrate availability can trigger fruit abscission (Byers et al., 1990, 1991; Lakso and 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., 2020b). 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.

2. Material and methods

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 °C, day/night), RH (75 %), irradiance (600−700 μmol m^{-2} s^{-1}), photoperiod (14 h) 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 °C, no metamitron), MET 7.5 (7.5/25 °C, 247.5 ppm metamitron), CTR 15 (15/25 °C, no metamitron) and MET 15 (7.5/25 °C, 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.

2.2. Metamitron leaf analysis

Metamitron extraction was conducted as described in detail in Rosa et al. (2020a) 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.

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 cm^2 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 °C 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.

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 cm^2 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 °C. 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 °C for 2 h and after cooling. Leakage results were expressed as percentage of total conductivity.

2.5. Leaf gas exchanges analysis

Leaf net photosynthetic rate (P_n) stomatal conductance to water vapor (g_s) 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 (A_{max}) measurements were performed as previously described (Ramalho et al., 2018). Briefly, A_{max} was measured through O_2 evolution in a Clark-type leaf disc O_2 electrode (LD2/2; Hansatech, UK) in leaf discs (1.86 cm2) under saturating conditions of CO_2 (*ca.* 7%), and irradiance (PPFD *ca.* 900 μmol m^{-2} s^{-1}, provided by a Björkman lamp, Hansatech, Norfolk, UK), at 25 °C.

Dark respiration rate (R_d) representing the consumption of O_2 was measured through O_2 evolution in a Clarktype O_2 electrode (LD2/2, Hansatech, Norfolk, UK) using leaf discs (1.86 cm^2), in the dark, at either 7.5 and 15 °C.

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, F_0, and photochemical efficiency of PSII, F_v/F_m, were performed on overnight dark-adapted leaves. F_0 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}). F_v/F_m 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 q_L, q_N, $Y_{(II)}$, $Y_{(NPQ)}$, $Y_{(NO)}$ and F_v'/F_m' (Kramer et al., 2004; Klughammer and Schreiber, 2008) and F_s/F_m' (Stirbet and Govindjee, 2011). The F_0', 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. F_v'/F_m' expresses the PSII efficiency of energy conversion under light exposure. q_L 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 F_s/F_m' 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 (PI_{Chr}), representing the percent reduction in F_v/F_m at each temperature relative to the maximal F_v/F_m obtained during the entire experiment; B) dynamic photoinhibition (PI_{Dyn}), representing the decline in F_v/F_m that is fully reversible overnight, being measured as the percent reduction in midday F_v'/F_m' relative to F_v/F_m at each temperature, relative to the maximal F_v/F_m from the entire experiment; C) total photoinhibition ($PI_{Total} = PI_{Chr} + PI_{Dyn}$), following Werner et al. (2002).

2.7. Thylakoid electron transport rates

Pools of leaves (*ca.* 5 g FW) from four plants were used to obtain subchloroplast 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 O_2 electrode (LW2, Hansatech, Norfolk, UK) at 25 °C, 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 (H_2O→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 ($DCPIPH_2$ →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 cm^2 each) using 80 % (v/v) aqueous acetone and quantified according to procedures described elsewhere (Lichtenthaler, 1987).

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 $MgCl_2$, 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. 04,693,159,001). 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.

2.9. Leaf soluble sugars 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 H_2O, 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 × 6.5 mm, Waters, Milford, USA) at 90 °C, with H_2O as the eluent (containing 50 mg EDTA-Ca L^{-1} H_2O), at a flow rate of 0.5 mL min-1. Standard curves were used for the quantification of each sugar.

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).

3. Results

3.1. Leaf metamitron absorption and degradation

A consistent tendency for greater metamitron (MET) absorption was observed under higher (15 °C) than at lower (7.5 °C) nighttime temperature, although significantly only by D2 when approximately doubled its content (Fig. 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. 1B). Notably, MET degradation metabolite become significantly greater under 7.5 °C than 15 °C from one week onwards after MET application.

3.2. Leaf relative water content and membrane impacts

Among treatments, leaf hydration, here evaluated by means of relative water content (RWC), significantly increased in all treatments when compared with CTR 7.5, only by D4 (Fig. 2A), what might be related, at least partly, with a stomatal conductance to water vapor (g_s) decline of those treatments (see Fig. 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. 2B).

3.3. Leaf gas exchanges analysis

Nighttime temperature did not affect net photosynthetic rate (P_n) 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 °C than 15 °C by D4 and D7, when MET 7.5 when reached minimum values representing 42 and 36 % of their respective CTR 7.5 values (Fig. 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 (g_s), since at 15 °C g_s values with or without MET where lower (D4) or similar (D7) than those at 7.5 °C. Additionally, MET impact on P_n at 7.5 °C from D4 onwards was also independent of g_s, since no significant differences were observed between MET and CTR plants in those days (Fig. 3B). This pointed to lower non-stomatal impacts under 15 °C, as suggested by the slight, but systematic, greater photosynthetic capacity values (A_{max}) in MET 15 along the entire experiment, as compared to MET 7.5 (Fig. 3C). Additionally, greater nighttime temperature reduced g_s until D4 in both CTR and MET conditions, but as for P_n, the g_s values did not differ among treatments by D14, whereas A_{max} still showed an incomplete recovery in MET plants, with a greater impact persisting in MET 7.5.

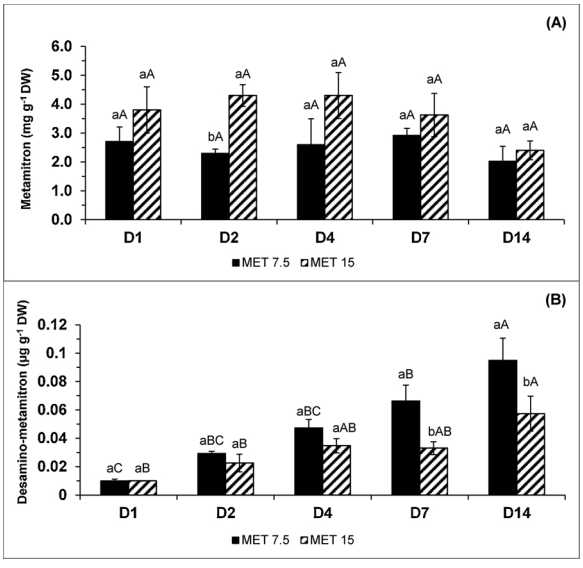

Fig. 1. Variation of metamitron (A) and desamino-metamitron (B) in *Malus* x *domestica* Borkh cv. Golden Reinders plants submitted to nocturnal temperature of 7.5 °C or 15 °C, under in control (CTR) or metamitron sprayed (MET) conditions 1, 2, 4, 7 and 14 days after metamitron application. For each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

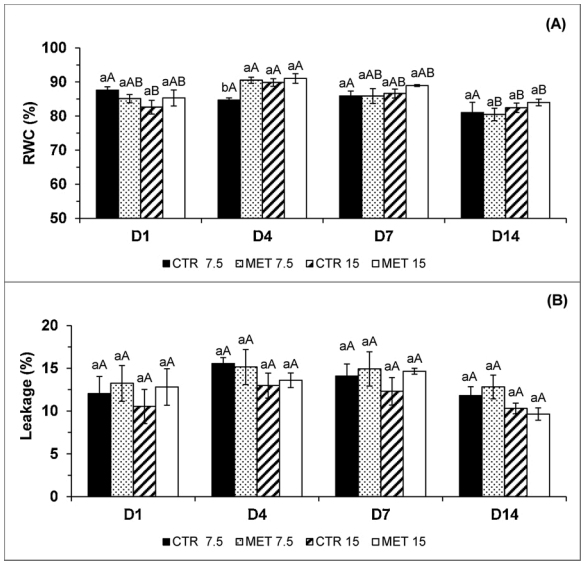

Fig. 2. Changes in relative water content (A) and leakage (B) in *Malus* x *domestica* Borkh cv. Golden Reinders plants submitted to nocturnal temperature of 7.5 °C or 15 °C, under in control (CTR) or metamitron sprayed (MET) conditions 1, 4, 7 and 14 days after metamitron application. For each parameter, 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).

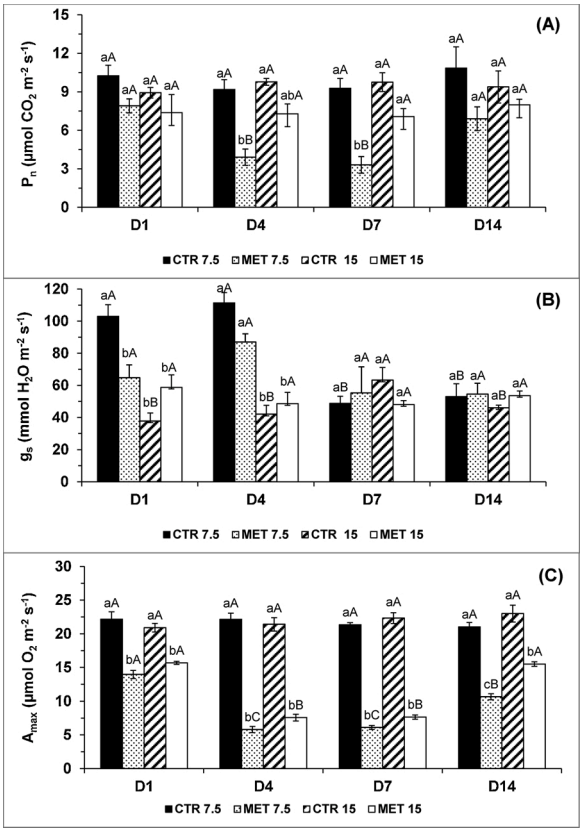

Fig. 3. Changes in leaf net photosynthesis (P_n) (A), stomatal conductance to water vapor (g_s) (B) and photosynthetic capacity (A_{max}) (C) in *Malus* x *domestica* Borkh cv. Golden Reinders plants submitted to nocturnal temperature of 7.5 °C or 15 °C, under in control (CTR) or metamitron sprayed (MET) 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).

3.4. Dark respiration

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

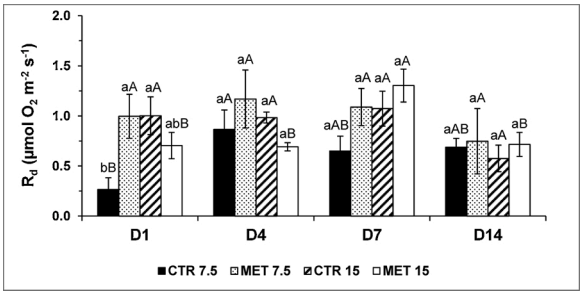

Fig. 4. Changes in dark respiration (R_d) in *Malus* x *domestica* Borkh cv. Golden Reinders plants submitted to nocturnal temperature of 7.5 °C or 15 °C, under in control (CTR) or metamitron sprayed (MET) conditions 1, 4, 7 and 14 days after metamitron application. For each parameter, the mean values ± 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).

3.5. Chlorophyll *a* fluorescence parameters

The increase in nighttime temperature from 7.5–15 °C (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 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).

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. 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 °C, 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. 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 °C, 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.

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 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 7.5–44% than CTR 7.5 only on D4, and in MET 15–49% than CTR 15 only on D14.

Concerning two enzymes from the respiratory pathway, 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

Table 1
Evaluation of leaf chlorophyll *a* fluorescence parameters in *Malus* x *domestica* Borkh cv. Golden Reinders plants submitted to nocturnal temperature of 7.5 °C or 15 °C, under in control (CTR) or metamitron sprayed (MET) conditions, 1, 4, 7 and 14 days after metamitron application. Parameters include the: 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
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
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

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 °C.

3.8. Non-structural carbohydrates during nocturnal 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.

In the leaves obtained at the end of the night period, only a few changes were noted in glucose and sorbitol content (Table 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. Additionally, MET also strongly reduced sucrose values, especially under 15 °C 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 °C. 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 4). Still, greater nighttime temperature (CTR 15) strongly reduced sucrose content, with a maximal

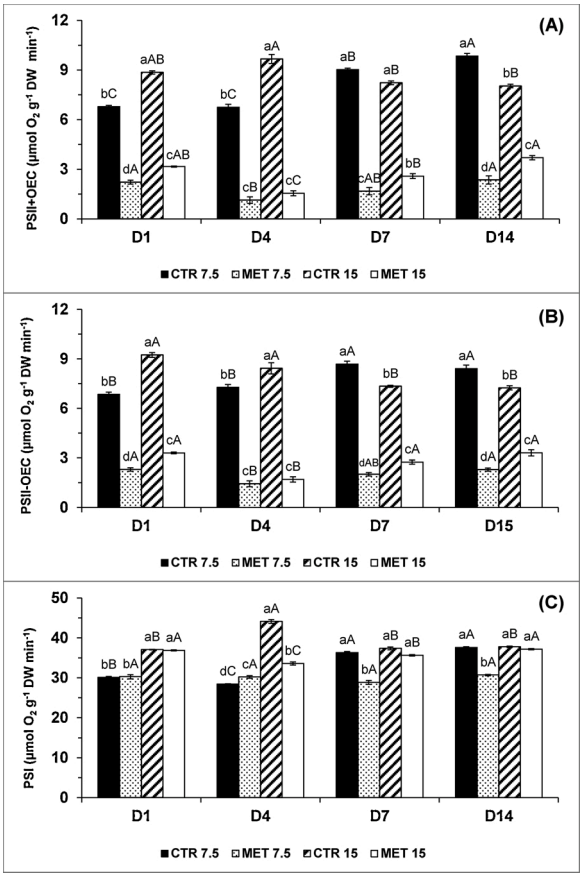

Fig. 5. Changes in the thylakoid electron transport rates associated with PSII (with and without the inclusion of OEC) and PSI in *Malus* x *domestica* Borkh cv. Golden Reinders plants submitted to nocturnal temperature of 7.5 °C or 15 °C, under in control (CTR) or metamitron sprayed (MET) conditions, 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).

decline of 65 % on D7, as compared to CTR 7.5. MET promoted a sucrose reduction only under 7.5 °C, 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 °C. 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.

4. Discussion

Previous studies in apple orchards involving MET application and nighttime temperature have shown strong effects on photosynthesis, soluble sugars and final yield (Rosa et al., 2020b). 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.

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 °C than at 7.5 °C (Fig. 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., 2020a).

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 vapor (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. 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 van Hasselt, 1990), or with the slight changes in F_v/F_m and F_s/F_m' under night temperature of 15 °C (Table 1), reflecting the same stability observed under an increase of the growth temperature by 15 °C 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. 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 °C by D7 and D14) (Fig. 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 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;

Table 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 nocturnal temperature of 7.5 °C or 15 °C, under in control (CTR) or 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 (μmol m^{-2} s^{-1})	**MET 7.5**	47.0	±	10.9	bA	35.2	±	3.8	cA	40.5	±	2.2	cA	42.9	±	7.0	bA
	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 (μmol m^{-2} s^{-1})	**MET 7.5**	157.8	±	9.0	bA	208.2	±	21.3	bA	176.3	±	13.3	bA	204.5	±	9.6	cA
	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

Table 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 nocturnal temperature of 7.5 °C or 15 °C, under in control (CTR) or 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

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 °C.

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 h 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 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 °C. In addition, Turnbull et al. (2002) conducted an experiment in *Populus*

Table 4
Variation in concentrations of soluble sugars in the leaves sampled after 2 h of light in *Malus* x *domestica* Borkh cv. Golden Reinders plants submitted to nocturnal temperature of 7.5 °C or 15 °C, under in control (CTR) or 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

deltoides in which an increase of 10 °C in nighttime temperature resulted in greater R_d (77 %) (Fig. 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 3 and 4). On the other side, pyruvate kinase (PK) activity was lower under 15 °C, and malate dehydrogenase (MDH) activity was similar to CTR 7.5 values (Table 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 °C 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 °C than under 15 °C (Table 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 °C, during the whole experiment. Under 15 °C, PK activity was significantly higher than under 7.5 °C 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 °C promoted the strongest sugar reductions within all treatments, especially on sucrose. The described respiratory activity at moderately higher temperatures (such as 15 °C) 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 °C than at 7.5 °C observed in the trials conducted by Rosa et al. (2020b).

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 °C.

Additionally, MET also inhibit PK and MDH enzymes from the respiratory pathway under 7.5 °C. Although MDH could be in excess and a partial inhibition might not be strongly relevant for glycolysis performance (Schreier et al., 2018; Yokochi 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 °C. 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 °C than at 7.5 °C. 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.

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CRediT authorship contribution statement

Nídia Rosa: Data curation, Investigation, Writing - original draft. **Fernando C. Lidon:** Investigation, Methodology, Validation. **Ana P. Rodrigues:** Investigation, Resources, Validation. **Isabel P. Pais:** Data curation, Investigation, Methodology, Validation. **Paula Scotti-Campos:** Resources, Validation. **Luís Asín:** Funding acquisition, Supervision. **Cristina M. Oliveira:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing - review & editing. **José C. Ramalho:** Conceptualization, Funding acquisition, Investigation, Methodology, Resources, Validation, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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