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Impact of exogenous rhamnolipids on plant photosynthesis and biochemical parameters under prolonged heat stress

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Abstract

High temperatures severely affect plant growth and development leading to major yield losses. These temperatures are expected to increase further due to global warming, with longer and more frequent heat waves. Rhamnolipids (RLs) are known to protect several plants against various pathogens. To date, how RLs act under abiotic stresses is unexplored. In this study, we aimed to investigate whether RLs could modify *Arabidopsis thaliana* physiology during prolonged heat stress. Measurement of leaf gas exchange and chlorophyll fluorescence showed that heat stress reduces photosynthetic rate through stomatal limitation and reduction of photosystem II yield. Our study reported decreased chlorophyll content and accumulation of soluble sugars and proline in response to heat stress. RLs were shown to have no detrimental effect on photosynthesis and carbohydrate metabolism in all conditions. These results extend the knowledge of plant responses to prolonged heat stress.

Keywords: chlorophyll fluorescence; gas exchange; photosynthesis; soluble sugars; thermotolerance.

Introduction

Global warming poses a significant threat to crop production and food sustainability due to rapid increasing temperatures and precipitation frequency. The IPCC assessment indicates a 0.72°C increase from 1951 to 2012 and predicts a 1.5°C increase in the coming decades (IPCC 2022). A rise in temperature beyond a threshold level sufficient to cause damage to plant growth and development is known as heat stress (HS) (Wahid *et al.* 2007). HS can significantly reduce crop yield, especially in tropical and subtropical regions where it is considered one of the main limiting factors of production (Zhao *et al.* 2017, Wang *et al.* 2020, Guntukula 2020, Khan *et al.* 2023, Stone 2023). High temperatures can reduce yields by 6% in wheat, 3.2% in rice, and 7.4% in maize (Zhao *et al.*

Highlights

- Heat stress reduces net photosynthesis and chlorophyll fluorescence in *Arabidopsis*
- *Arabidopsis* accumulated proline and soluble sugars under prolonged heat stress
- Rhamnolipids did not impact the *Arabidopsis* photosynthesis process during prolonged heat stress

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Abbreviations: ABA – abscisic acid; BR – brassinosteroid; C_i – intercellular CO_2 concentration; CK – cytokinins; E – transpiration rate; ETR – electron transport rate; F_v/F_m – maximal quantum yield of PSII photochemistry; FM – fresh mass; GA – gibberellic acid; g_s – stomatal conductance; HS – heat stress; HSPs – heat shock proteins; IAA – auxin; LEA – late embryogenesis abundant; LHCB – light-harvesting chlorophyll *a*/*b*-binding; MAMPs – microbe-associated molecular pattern; MeJA – methyl jasmonate; MeOH – methanol; P_N – net photosynthetic rate; RLs – rhamnolipids; ROS – reactive oxygen species; SA – salicylic acid.

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2017). Additionally, HS (35°C) causes up to 43 and 30% yield losses in cherry tomatoes and field peas, respectively (Tafesse *et al*. 2019, Park *et al*. 2023). In the literature, crop yield losses due to HS are often associated with a reduction of seed germination potential (Sharma *et al*. 2022), photosynthesis yield, abnormal pollen development (Samtani *et al*. 2022, Smith *et al*. 2023), and alteration of seed-filling rate (Devi *et al*. 2023) to name a few. With global warming, plants are expected to face more frequent, intense, and longer heat waves resulting in more yield losses (Perkins-Kirkpatrick and Gibson 2017, Wedler *et al*. 2023). This calls for a better understanding of the effect of prolonged HS on plants.

HS negatively impacts plant growth and development by affecting various physiological, biochemical, and molecular processes. It increases fluidity and permeability of membranes and denaturation of proteins (Niu and Xiang 2018). It also causes overaccumulation of reactive oxygen species (ROS), thus altering oxidative balance within cells (Suzuki and Mittler 2006, Singh *et al*. 2019, Sharma *et al*. 2020). Moreover, HS can significantly affect photosynthesis components (Sharma *et al*. 2020, Zahra *et al*. 2023) causing damage such as loss of thylakoids membrane integrity (Abdelrahman *et al*. 2020, Rath *et al*. 2022), alteration of chloroplasts (Anderson *et al*. 2021, Zhang *et al*. 2023a), increased chlorophyll degradation (Wang *et al*. 2018), and inactivation of ribulose-1,5-bisphosphate carboxylase (Kim and Portis 2005). HS significantly impacts the photosynthesis system, particularly photosystem II, through ROS-induced damage (Chen *et al*. 2016, Wang *et al*. 2018, Raja *et al*. 2020, Hu *et al*. 2020, Zhao *et al*. 2021, Zahra *et al*. 2023). Together, HS-induced effects lead to decreased chlorophyll (Chl) content, stomatal conductance, and photosystem efficiency, disrupting electron transport, and affecting photosynthesis (Pshybytko *et al*. 2023, Wang *et al*. 2023).

Plants have developed various tolerance mechanisms to protect themselves against the impact of high temperatures. These include the regulation of antioxidant systems to prevent oxidative damage (Wassie *et al*. 2020, Fortunato *et al*. 2023, Suzuki 2023, Ru *et al*. 2023), production of osmoprotectants like proline, glycine betaine, and carbohydrates (Alagoz *et al*. 2023, Anjum *et al*. 2023, Salehi *et al*. 2023, Akram *et al*. 2024) to stabilize cell membranes and prevent PSII dysfunction (Wahid 2007, Gautam *et al*. 2022). Plants also regulate heat shock proteins (HSPs) and the production of secondary metabolites (Rehman *et al*. 2024). These deployed defense mechanisms are insufficient to protect plants against prolonged or severe HS. Various approaches are being explored to improve plant production under HS and ensure sustainable agriculture. Conventional breeding through phenotype selection and identification of heat-tolerant quantitative trait loci (QTL) (Nabavi *et al*. 2020) or genetic engineering (Meng *et al*. 2022) are applied to obtain heat-tolerant crops. Other strategies include the use of plant growth-promoting rhizobacteria (Shaffique *et al*. 2022, Carreiras *et al*. 2023, Mukhtar *et al*. 2023, Zhang *et al*. 2023b), application of exogenous compounds such as proline and glycine betaine (Mo *et al*. 2022, Alagoz *et al*. 2023), minerals (Ali *et al*. 2021), and plant growth regulators including abscisic acid (ABA), cytokinins (CK), methyl jasmonate (MeJA), salicylic acid (SA), gibberellic acid (GA), brassinosteroid (BR), auxin (IAA) (Kumar *et al*. 2012, Yang *et al*. 2016, Zhang *et al*. 2020, Su *et al*. 2021, Guo *et al*. 2022, Wang *et al*. 2022, Lakshmi *et al*. 2023). Once applied, these compounds help improve antioxidant activities, induce the production of osmotic regulators, regulate phytohormone production, and improve photosynthesis efficiency under HS (Feng *et al*. 2023). A recent study showed that the microbe-associated molecular pattern (MAMP) chitosan can alleviate HS impact in bentgrass *via* amelioration of water stress, ROS scavenging, and improvement of photosynthesis efficiency (Li *et al*. 2023).

Among MAMPs are rhamnolipids (RLs), surface-active glycolipids primarily produced by *Pseudomonas* and *Burkholderia* species (Crouzet *et al*. 2020, Cordelier *et al*. 2022). RLs consist of a hydrophobic lipid moiety composed of 2-β-hydroxy fatty acid chains ranging from C_8 to C_{24}, coupled with a hydrophilic moiety made up of mono- or di-(L)-rhamnose (Soberón-Chávez *et al*. 2005). Recently, RLs have gained a lot of attention due to their antimicrobial and insecticidal activities against plant pests and pathogens (Kim *et al*. 2011, Prabakaran *et al*. 2015, Crouzet *et al*. 2020, Monnier *et al*. 2020, Onlamool *et al*. 2022), their low toxicity (Johann *et al*. 2016), and biodegradable properties (Lai *et al*. 2009, Liu *et al*. 2018). Furthermore, RLs have been shown to trigger defense responses in various plants including grapevine (Varnier *et al*. 2009), rapeseed (Monnier *et al*. 2018), and *Arabidopsis* (Sanchez *et al*. 2012, Schellenberger *et al*. 2021). Recent studies hinted that RLs could help mitigate some abiotic stress effects in plants. It was reported that RLs could increase water retention, soil salt rejection, and nutrient availability, change microbial community in favor of plant growth-promoting bacteria, and subsequently alleviate saline stress effect in plants (Hu *et al*. 2023, Liu *et al*. 2023, Chen *et al*. 2024). The direct effects of RLs under extreme conditions such as HS is still poorly explored. Investigating RL effectiveness in the face of abiotic obstacles like high temperatures is crucial for their eventual field application. Several exogenous substances have been shown to alleviate HS's impact on plants through different strategies. This includes the reduction of HS-induced effect on photosynthesis parameters and the production of osmolytes and osmoprotectants that can limit HS impact on cellular processes (Zhang *et al*. 2020, Su *et al*. 2021, Guo *et al*. 2022, Feng *et al*. 2023, Lakshmi *et al*. 2023).

In this study, we assessed the effect of RLs under HS. Mono-RL and di-RL have been shown to display different activities and efficacy (Motta *et al*. 2022, Zhao *et al*. 2022). We thus examined the consequences of both mono- and di-RL treatment on photosynthesis parameters such as gas exchange and chlorophyll fluorescence under prolonged HS. To underline the tolerance mechanism, we also analyzed RL's impact on osmotic adjustment by quantifying carbohydrate and proline contents.

Materials and methods

Plant materials and growth conditions: Wild-type *Arabidopsis thaliana* Colombia-0 ecotypes (*Arabidopsis*) were used for all experiments. Seeds were sown and cultivated in a growth chamber at 20/18°C (day/night), under a 12-h photoperiod with 70% relative humidity, and PAR of 80 µmol(photon) m^{-2} s^{-1}. Conditions in the growth chamber were monitored using a sensor (*KlimaLogg-Pro*, *TFA Dostmann*, Germany).

Rhamnolipids: A mixture of RLs from *Pseudomonas syringae* was purchased from *Jeneil* (*Jeneil Biosurfactant Co*., Saukville, USA). Mono- and di-RLs were purified as described in Schellenberger *et al*. (2021). Stock solutions of di- and mono-RLs (20 mM) were prepared in methanol (100%) and stored at –20°C until use.

Plant treatment: *Arabidopsis*, 5–6-week-old were foliar sprayed with a mono- or di-RLs at 100 µM or methanol (control) solution (3 mL per plant). After RL treatment (72 h), plants were either maintained at a temperature of 20/18°C (day/night) or transferred to another growth chamber set at 38/35°C (day/night) for 7 d.

Leaf gas exchange: Net photosynthesis (P_N), intercellular CO_2 concentration (C_i), stomatal conductance (g_s), and transpiration rate (E) were measured using an IRGA infrared gas analyzer (*Li-6400-XT*, *LI-COR Biosciences*, Lincoln, NE, USA) connected to a leaf chamber fluorometer (LCF) (*6400-40*, *LI-COR Biosciences*, Lincoln, NE, USA) (Su *et al*. 2016). For measurement, chamber conditions were set as follows: CO_2 of 410 ppm, block temperature of 20°C (control) or 38°C (stressed plants), flow rate of 300 ml min^{-1}, and PAR of 300 µmol(photon) m^{-2} s^{-1}, 60% humidity. P_N, C_i, g_s, and E were determined using the equation of von Caemmerer and Farquhar (1981). For each condition, measurements were performed on six mature leaves from seven individual plants in the morning (8:30 and 12:30 h). For each leave, three measurements were taken to ensure chamber stability at 0, 24, 48, 96, and 144 h after stress induction.

Chlorophyll fluorescence: In addition to gas exchange, efficiency of PSII and electron transport rate [$ETR_{(II)}$] were evaluated, every 20 min, for 8 d, post-stress induction using a chlorophyll fluorometer (*MONITORING-PAM*, *Walz*, Effeltrich, Germany) (Porcar-Castell *et al*. 2008). At normal (20/18°C, day/night) and stress conditions (38/35°C, day/night), RL- or methanol-treated leaves were connected to the leaf clamp of the monitoring head, and repetitive saturation pulses [1 saturation light; 3,500 µmol(photon) m^{-2} s^{-1}] were applied to the fixed area.

PAR, quantum efficiency (Y_{II}), and $ETR_{(II)}$ were determined by Moni-head detectors and recorded using *WinCOntrol-3* software (*Heinz Walz GmbH*, *Inc*., Effeltrich, Germany).

Quantification of soluble sugars, chlorophyll, and proline: To evaluate the content of Chl, soluble sugars, and proline, *Arabidopsis* leaves were previously collected, at 0, 24, 48, 96, and 144 h, frozen in liquid nitrogen, and stored at –80°C until use. A single extraction was performed to quantify all metabolites as described by Canal *et al.* (2024). Frozen leaves were ground in liquid nitrogen. Aliquots of each sample (20–100 mg) were mixed with 80% ethanol in 10 mM HEPES/KOH (pH 6.0) (500 µL) and incubated at 80°C for 20 min, then centrifuged at 14,000 rpm, 5 min. The pellets were then extracted again with 300 µL of 80% ethanol in 10 mM HEPES/KOH (pH 6.0) and a third time with 50% ethanol in 10 mM HEPES/KOH (pH 6.0). All resulting supernatants were combined and used for analysis.

Chlorophylls *a* and *b* were immediately quantified after extraction to avoid any degradation by light. An aliquot of the extract (50 µL) was mixed with 120 µL of ethanol (98%) and placed in a 96-well plate. The absorbance at 645 and 665 nm was recorded using a spectrophotometer (*Tecan Infinite F200 Pro*, *Tecan*, Männedorf, Switzerland), and 70% ethanol in 10 mM HEPES/KOH was used as a blank. For each sample, three repetitions were used. Chl *a*, *b*, and the ratio *a*/*b* was estimated using the two equations below: Chl *a* [µg/well] = 5.21 A_{665} – 2.07 A_{645}; Chl *b* [µg/well] = 9.29 A_{645} – 2.74 A_{665} (Canal *et al*. 2024). Chl content was then expressed in mg g^{-1}(fresh mass, FM).

To quantify the soluble sugars, the remaining ethanol extracted was evaporated until completely dry using a concentrator (*Eppendorf Vacufuge Concentrator 5301*, *Eppendorf*, UK). Samples were then resuspended in 100 µL of water, vortex, spin down, and stored at –20°C until use. Sucrose, fructose, and glucose contents were determined using the *Enzytec D-glucose*, *D-fructose*, *D-sucrose* kit (*R-Biopharm AG*, Darmstadt, Germany) and microplate spectrophotometer (*Tecan Infinite F200 Pro*, *Tecan*, Männedorf, Switzerland). The content of sucrose, glucose, and fructose was determined using a standard curve of 0.1 to 0.5 g for glucose and fructose and 0.1 to 0.8 g for sucrose.

Proline content was determined following Rajendran *et al*. (2023) with modifications. Metabolite extract (200 µL) was mixed with 200 µL ninhydrin solution (ninhydrin 1% w/v, glacial acetic acid 60% v/v, ethanol 20% v/v). The mix was incubated at 95°C for 20 min, allowed to cool down to room temperature, and shortly placed on ice to stop the reaction. The absorbance of the 120 µL mixture was measured at 510 nm using a spectrophotometer (*Tecan Infinite F200 Pro*, *Tecan*, Männedorf, Switzerland). The content of proline was determined using an L-proline standard curve (10–100 µM).

Statistical analyses: Statistical analyses were conducted to determine the effect of HS and RLs on gas exchange, chlorophyll fluorescence, soluble sugars, and proline. The normality of the data was verified by a *Shapiro-Wilk* test using *Paleontological Statistics* (*PAST 4*) software. For gas-exchange parameters and chlorophyll and metabolite contents, significant differences were determined by the *Kruskal-Wallis* test followed by a *Mann-Whitney* pairwise and a *Dunn*'s post hoc at $p < 0.05$ using *PAST 4*. Chlorophyll fluorescence data were analyzed with a *Student*'s *t*-test at $p < 0.05$.

Results

HS significantly impacted gas exchange in both RL-treated and control plants: We first measured leaf gas-exchange parameters at different time points to assess the effect of HS with and without RLs (mono- and di-RLs, Fig. 1 and Fig. 2, respectively) on *Arabidopsis*. At 20°C, P_N values varied from 5.93–6.43 µmol(CO_2) m^{-2} s^{-1}. At 38°C, they rapidly decreased below 1.64 µmol(CO_2) m^{-2} s^{-1}, after 96 h of stress (Fig. 1*A*). At 38°C, P_N was significantly reduced in both mono-RL-treated and control plants at all points, compared to plants cultivated at 20°C. There were no significant differences between control and mono-RL-treated plants at 20 and 38°C.

At 20°C, C_i remained stable near 340 µmol(CO_2) mol^{-1} from 0 to 96 h post-stress and slightly decreased to 317 µmol(CO_2) mol^{-1} after 144 h of stress. Like P_N, C_i was reduced at 24 h and 48 h post-stress for plants cultivated at 38°C compared with plants at 20°C (Fig. 1*B*). Regarding the RL effect, the C_i of control plants was significantly higher than that of mono-RL-treated plants at 38°C, after 48 h post-stress.

The g_s values were between 0.15–0.19 mmol(H_2O) m^{-2} s^{-1} from 0 h to 96 h, and 0.10 mmol(H_2O) m^{-2} s^{-1} after 144 h. At 38°C, g_s values were significantly reduced at 24 h, 48 h, and 96 h for all plants and 144 h for mono-RL treated alone compared to plants cultivated at 20°C (Fig. 1*C*). In addition, g_s of plants treated with mono-RLs was lower than that of control plants at 38°C after 48 h of stress.

Concerning *E*, values varied from 1.7–2.26 mmol(H_2O) m^{-2} s^{-1} at 20°C and between 1.7–2.24 mmol(H_2O) m^{-2} s^{-1} at 38°C (Fig. 1*D*). The *E* values of mono-RL-treated plants at 38°C were lower than those of plants at 20°C after 24 h, 48 h, and 96 h post-stress. However, for control plants, *E* values at 38°C were close to those of plants at 20°C from 0 h to 48 h of stress. They only decreased significantly after 96 h of stress. Our results showed that RLs did not impact significantly *E*, at 20°C and 38°C compared to control plants.

Fig. 2 shows that all gas-exchange parameter values (P_N, C_i, g_s, and *E*) were also impacted at 38°C. After 24 h of stress, we found that the P_N of di-RL-treated plants was slightly but significantly higher at 38°C than at 20°C. However, after 96 h and 144 h of stress, P_N values for di-RL-treated plants at 38°C were significantly lower than those at 20°C. Similarly, the P_N of control plants at 38°C was significantly lesser compared to plants at 20°C, after 48 h, 96 h, and 144 h of stress (Fig. 2*A*). There was no significant difference in P_N between di-RL-treated plants and control plants at 20 and 38°C.

At 20°C, C_i values ranged from 352–360 µmol(CO_2) mol^{-1}. When plants were grown at 38°C, C_i values were significantly lower than those observed at 20°C after 24 h and 48 h of stress for all plants. At 38°C, after 48 h and 96 h of stress, we found that C_i values for plants treated with di-RLs were significantly lower than those of control plants (Fig. 2*B*).

At 0 h, g_s values were a bit higher in di-RL-treated plants compared to control plants at 20°C and 38°C

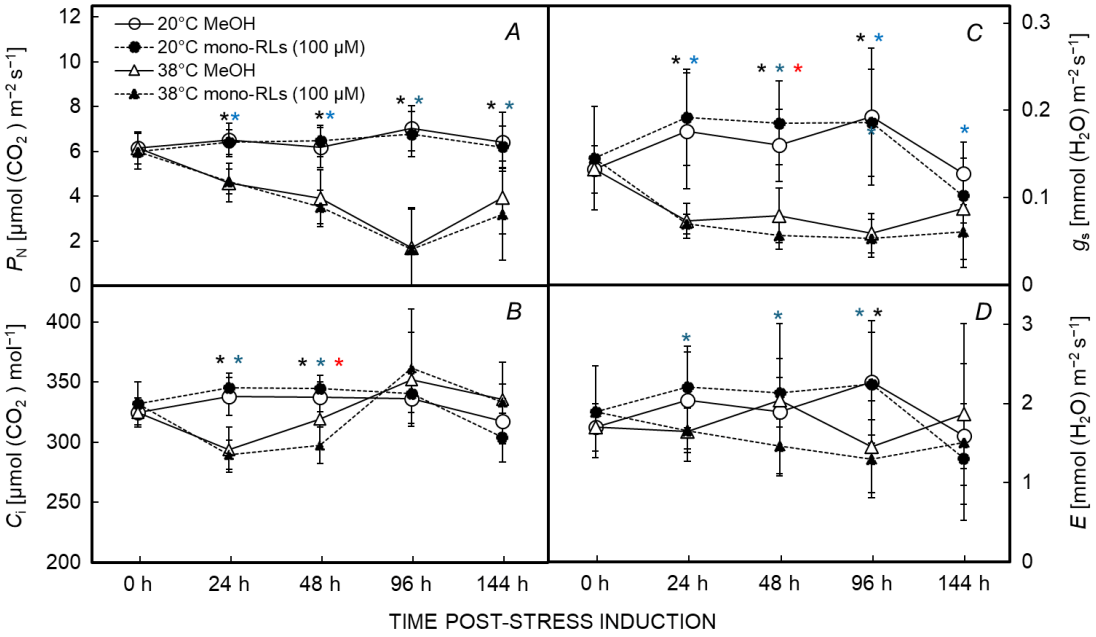

Fig. 1. Leaf gas-exchange parameters assessment in 5-week-old *Arabidopsis* treated with mono-rhamnolipids (mono-RLs) or methanol (MeOH/control) under normal (20/18°C) and heat stress (38/35°C) conditions. (*A*) Net photosynthesis (P_N), (*B*) intercellular CO_2 concentration (C_i), (*C*) stomatal conductance (g_s), and (*D*) transpiration rate (*E*). Mono-RLs (100 µM) and MeOH were sprayed on the leaves 72 h before stress induction. Data represents mean values ± SD (n =18) of three independent experiments with similar results. *Asterisks* represent the significant differences between MeOH at 20°C and MeOH at 38°C (*), mono-RLs at 20°C and mono-RLs at 38°C (*), MeOH at 20°C and mono-RLs at 20°C (*), MeOH at 38°C and mono-RLs 38°C (*) determined by *Kruskal-Wallis* test followed by a *Mann-Whitney* test at $p<0.05$.

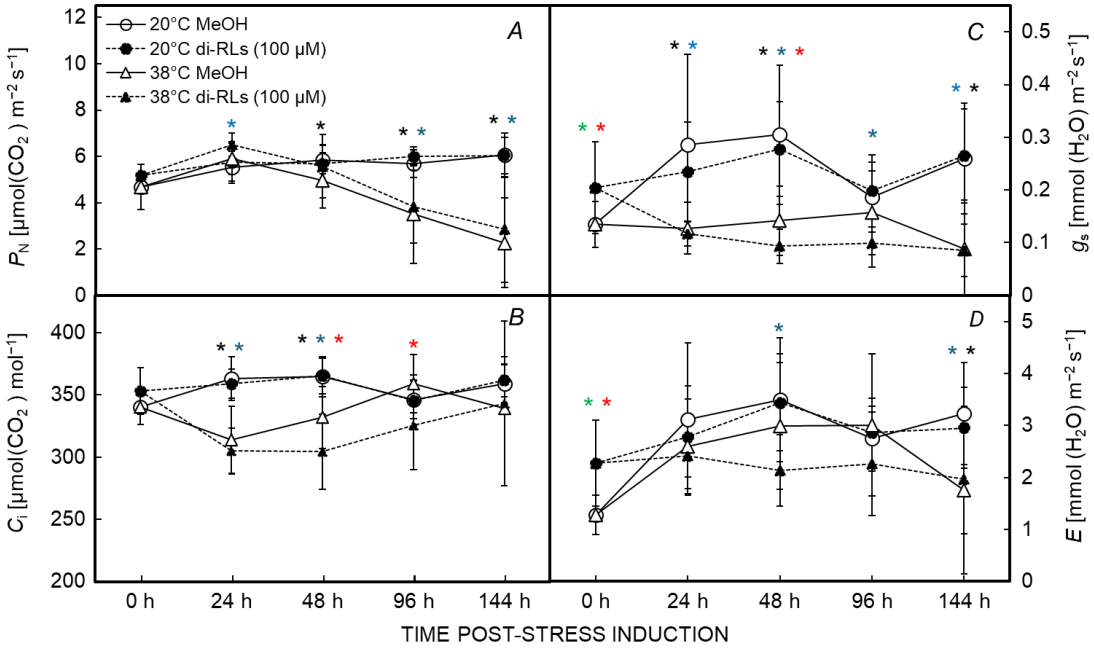

Fig. 2. Leaf gas-exchange parameters assessment in 5-week-old *Arabidopsis* treated with di-rhamnolipids (di-RLs) or methanol (MeOH/control) under normal (20/18°C) and heat stress (38/35°C) conditions. (*A*) Net photosynthesis (P_N), (*B*) intercellular CO_2 concentration (C_i), (*C*) stomatal conductance (g_s), and (*D*) transpiration rate (*E*). Di-RLs (100 µM) and MeOH were sprayed on the leaves 72 h before stress induction. Data represents mean values ± SD (n = 14) of two independent experiments with similar results. *Asterisks* represent the significant differences between MeOH at 20°C and MeOH at 38°C (*), di-RLs at 20°C and di-RLs at 38°C (*), MeOH at 20°C and di-RLs at 20°C (*), MeOH at 38°C and di-RLs 38°C (*) determined by *Kruskal-Wallis* test followed by a *Mann-Whitney* at $p<0.05$.

(Fig. 2*C*,*D*). After 48 h, when plants were grown at 38°C, g_s of di-RL-treated plants was lower than that of control plants. Regarding the HS effect, g_s values of di-RL-treated plants at 38°C were lower than those of plants at 20°C after 24 h, 48 h, 96 h, and 144 h of stress. For the control plants, a reduction of g_s values was also observed at 38°C compared to values at 20°C after 24 h, 48 h, and 144 h of stress.

At 20°C, *E* varied from 1.28 and 3.23 µmmol(H_2O) m^{-2} s^{-1} for control plants and 2.27 and 2.96 µmmol(H_2O) m^{-2} s^{-1} for di-RL-treated plants. Similarly to g_s, the values of *E* at 0 h, were higher in the di-RL-treated plants compared to control plants at 20°C and 38°C. Furthermore, we found that *E* values in control plants at 38°C were similar to those at 20°C after 24 h, 48 h to 96 h of stress. However, *E* significantly decreased after 144 h of stress (Fig. 2*D*). For di-RL-treated plants, *E* values at 38°C were lower than those of plants grown at 20°C after 48 h and 144 h.

HS reduced the efficiency of PSII but not ETR: PAR, the efficiency of PSII, and $ETR_{(II)}$ were continuously monitored for 7 d, during day and nighttime (Fig. 3). PAR was similar for all plants at 20 and 38°C (except day 6). On day 6, at 20°C, the PAR of control plants was significantly lower than that of mono-RL-treated plants (Fig. 3*A*). $ETR_{(II)}$ was not affected either by HS or RL treatment (Fig. 3*B*). Contrary, $Y_{(II)}$, commonly known as the maximum quantum yield of PSII (F_v/F_m) was significantly impacted at 38°C (Fig. 3*C*). $Y_{(II)}$ varied from 0.7 to 0.8 at 20°C, while at 38°C, these levels declined progressively and were below 0.2 at night 7 for all plants. Compared to plants grown at 20°C, $Y_{(II)}$ at 38°C significantly decreased at nights 1, 2, and 3 for all plants and nights 4, 5, 6, and 7 for mono-RL-treated plants only. Results of di-RL treatment are represented in Fig. 4. PAR and $ETR_{(II)}$ were unchanged in all conditions (Fig. 4*A*,*B*). Similar to the experiment with mono-RLs, we observed a decrease of $Y_{(II)}$ at 38°C at night 1, 2, 3, 4, and 5 for all plants and at 6, 7 for di-RL-treated plant only (Fig. 4*C*). Our results reveal no significant differences between RL-treated (mono- and di-) and control plants both at 20 and at 38°C.

Chlorophyll content was reduced following HS in both treated and control plants: The concentration of Chl *a*, Chl *b*, and the ratio Chl *a*/*b* were similar in the control and RL-treated plants at 20°C. Chl *a* was in the range of 0.6–1 mg g^{-1}(FM), 0.1–0.28 mg g^{-1}(FM) for Chl *b*, and between 4–6 for Chl *a*/*b* ratio (Fig. 5*A*–*C*). At 38°C, the contents of Chl *a*, Chl *b*, the ratio Chl *a*/*b* decreased by at least 66, 73, and 82%, respectively, at 144 h post-stress, compared to all plants at 20°C. For di-RLs, the values of Chl *a*, Chl *b*, and the ratio Chl *a*/*b* were similar to those observed with the mono-RLs (Fig. 5). There were no major differences between the di-RL-treated and control plants at 20 and 38°C (Fig. 5*D*–*F*).

HS resulted in the accumulation of proline: To assess the influence of RLs in mitigating HS-induced oxidative stress, we quantified the proline content in all conditions.

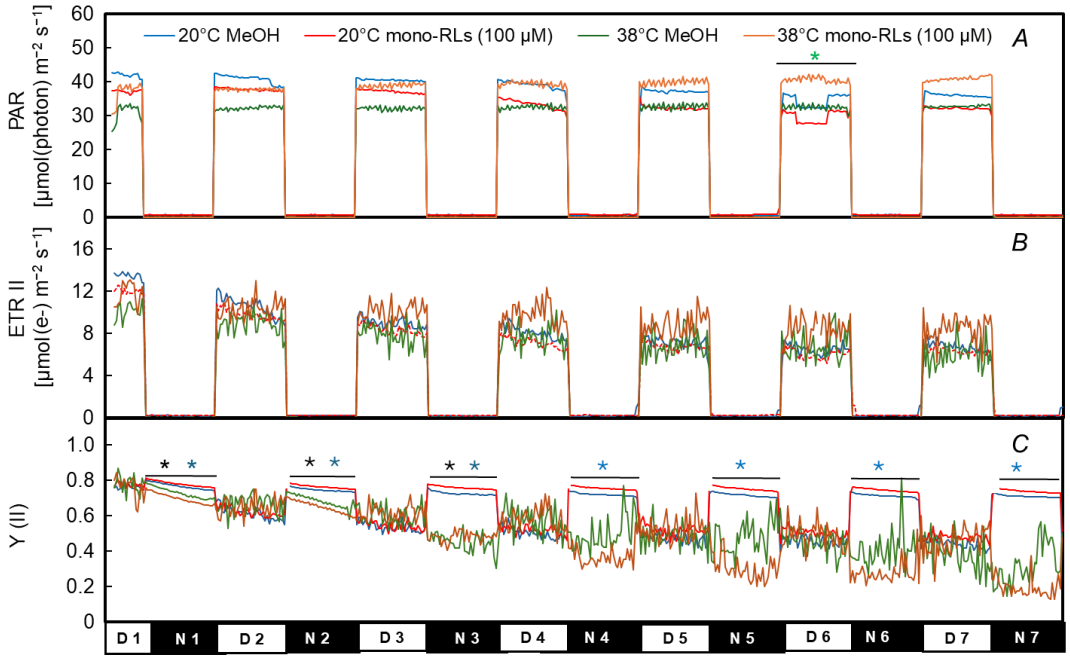

Fig. 3. Chlorophyll fluorescence parameters in leaves of 5-week-old *Arabidopsis* treated with mono-rhamnolipids (mono-RLs) or methanol (MeOH/control) under normal (20/18°C) and heat stress (38/35°C) conditions. (*A*) Photosynthetically active radiation (PAR), (*B*) electron transport rate II [$ETR_{(II)}$], and (*C*) efficiency of PSII [$Y_{(II)}$]. e^- – electron, D – day, N – night. Mono-RLs (100 µM) and MeOH were sprayed on the leaves 72 h before stress induction. Measurements were recorded continuously (day/night) in one leaf, every 20 min. Data represent mean values ± SD (n = 9) of three independent experiments. *Asterisks* represent the significant differences between MeOH at 20°C and MeOH at 38°C (*), mono-RLs at 20°C and mono-RLs at 38°C (*), and MeOH at 20°C and mono-RLs at 20°C (*), *Student*'s *t*-test $p<0.05$.

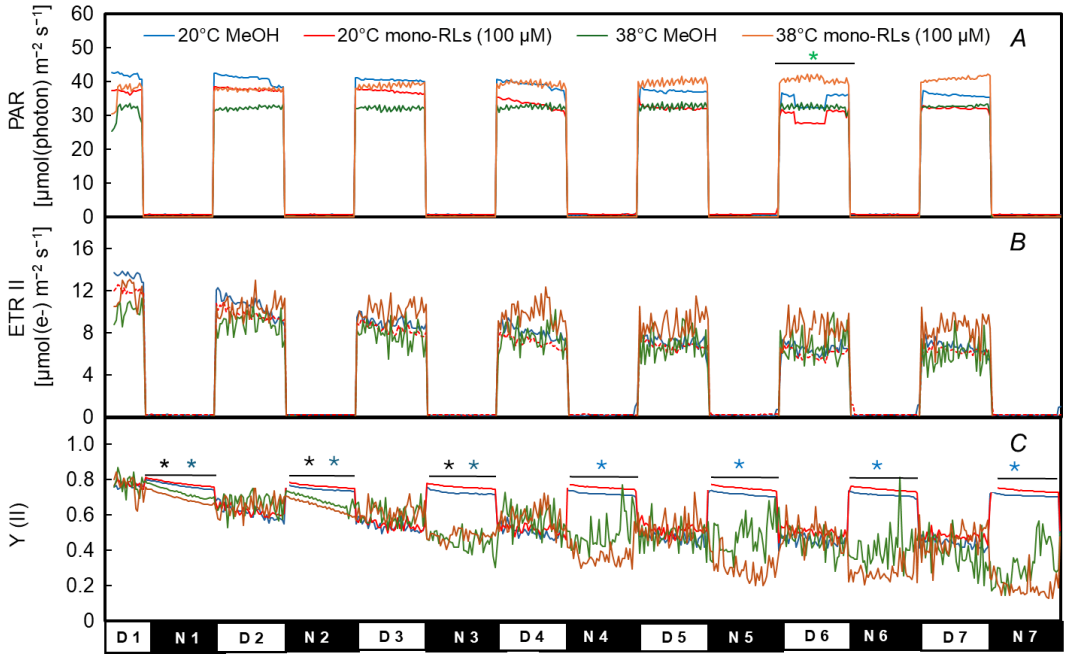

Fig. 4. Chlorophyll fluorescence parameters in leaves of 5-week-old *Arabidopsis* treated with di-rhamnolipids (di-RLs) or methanol (MeOH/control) under normal (20/18°C) and heat stress (38/35°C) conditions. (*A*) Photosynthetically active radiation (PAR), (*B*) electron transport rate II [$ETR_{(II)}$], and (*C*) efficiency of PSII [$Y_{(II)}$]. e^- – electron, D – day, N – night. Di-RLs (100µM) and MeOH were sprayed on the leaves 72 h before stress induction. Measurements were recorded continuously (day/night) in one leaf, every 20 min. Data represents mean values ± SD (n = 9) of three independent experiments. *Asterisks* represent the significant differences between MeOH at 20°C and MeOH at 38°C (*) and di-RLs at 20°C and di-RLs at 38°C (*), *Student*'s *t*-test $p<0.05$.

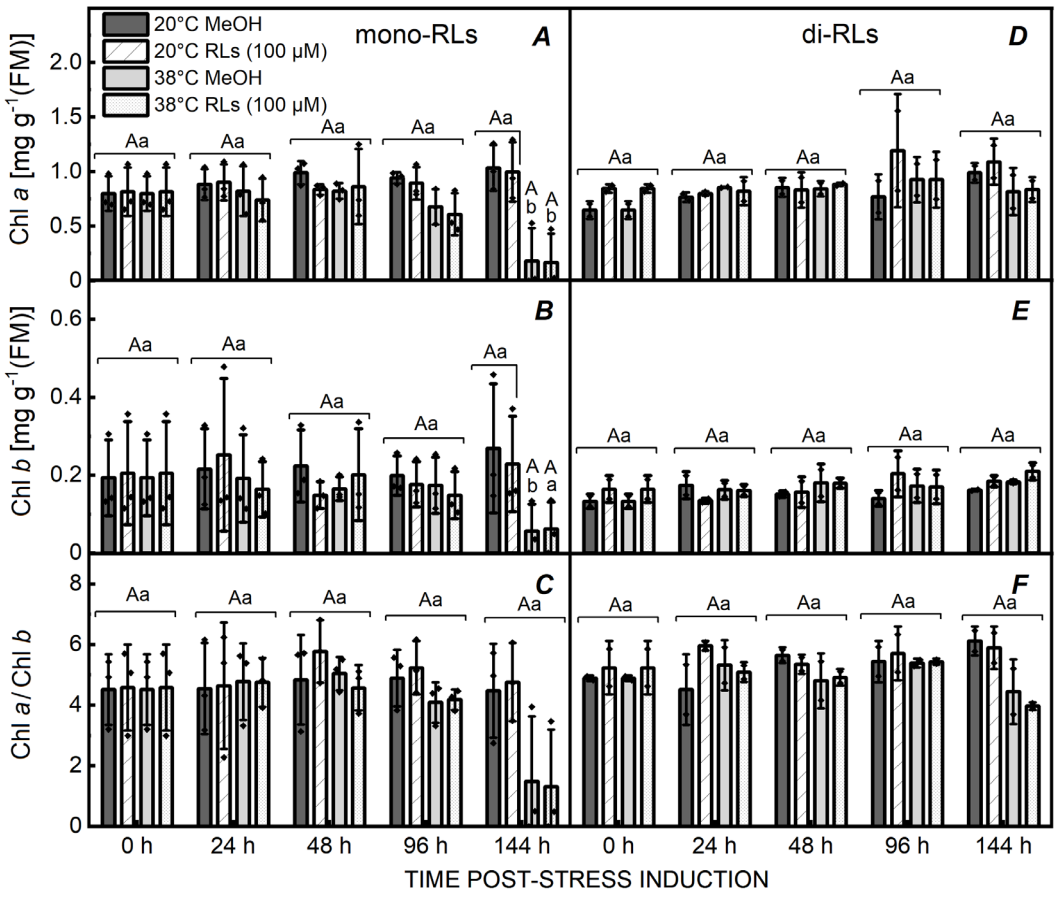

Fig. 5. Content of chlorophyll in leaves of 5-week-old *Arabidopsis* treated with methanol (control) or rhamnolipid (100 µM) and grown under normal (20/18°C) or stress (38/35°C) conditions. Chlorophyll (Chl) *a*, Chl *b*, and Chl *a*/*b* ratio (*A*, *B*, *C* for mono-RLs and *D*, *E*, *F* for di-RLs). FM – fresh mass. Data represents the mean ± SD of three (n = 3) and two (n = 2) independent experiments for mono-RLs and di-RLs, respectively. Each treatment sample was obtained from a pool of three plants per experiment (two leaves/plant). *Diamond-shaped points* represent single data points. *Different lowercase letters* indicate significant differences between plants in normal and stress conditions (effect of heat stress). *Different uppercase letters* represent significant differences between plants treated with MeOH and RLs (RL treatment effect). Statistical analysis determined for each measurement point, using the *Kruskal-Wallis* test followed by a *Dunn*'s post hoc at p<0.05.

The proline content of *Arabidopsis* leaves was around 20 µmol mg^{-1}(FM) for all plants grown at 20°C (Fig. 6). At 96 h post-stress, we observed an increase of proline content in di-RL-treated plants cultivated at 38°C compared to plants at 20°C. For di-RLs, the proline contents were not significantly impacted at 38°C, compared to plants at 20°C. Furthermore, RL-treated plants did not exhibit changes compared to the control plants at 20°C and 38°C.

Soluble sugars strongly increased in stress conditions: When plants were grown at 20°C, glucose, fructose, and sucrose contents were respectively around 100, 40, and 400 µg g^{-1}(FM) in all plants. At 38°C, glucose content was higher (+ 56%) in all plants compared to plants at 20°C from 24 to 144 h after stress (Fig. 7*A*). This increase was significant at 24 h post-stress for mono-RL-treated plants and 48 h post-stress for control plants. Similarly, compared to plants at 20°C, fructose significantly increased by at least 80, 87, 85, and 76% after 24, 48, 96, and 144 h post-stress, respectively, for all plants at 38°C (Fig. 7*B*). The accumulation of fructose at 38°C was significantly different at 24 h (mono-RL-treated plants), and 48 and 96 h post-stress for control plants. The sucrose content rose by at least 60, 76, and 46% in plants at 38°C compared to 20°C after 48, 96, and 144 h (Fig. 7*C*), respectively. The increase of sucrose at 38°C was significant after 24 h (mono-RL) and 48 h (control).

The contents of all three soluble sugars also accumulated at 38°C compared to plants at 20°C (Fig. 7*D–F*). Glucose accumulation was significantly different at 48 h for both control and mono-RL-treated plants and at 96 h for control plants alone (+54%) was observed at 24, 48, 96, and 144 h post-stress (Fig. 7*D*). Fructose contents were higher at 38°C, with an increase of at least 64% at 48, 96, and 144 h post-stress (Fig. 7*E*). Similarly, sucrose increased by minimum 53 and 41% at 48 and 96 h post-stress, respectively (Fig. 7*F*). For all conditions, there were no significant differences in soluble sugars between the plants treated with RLs (mono- or di-) and the control plants.

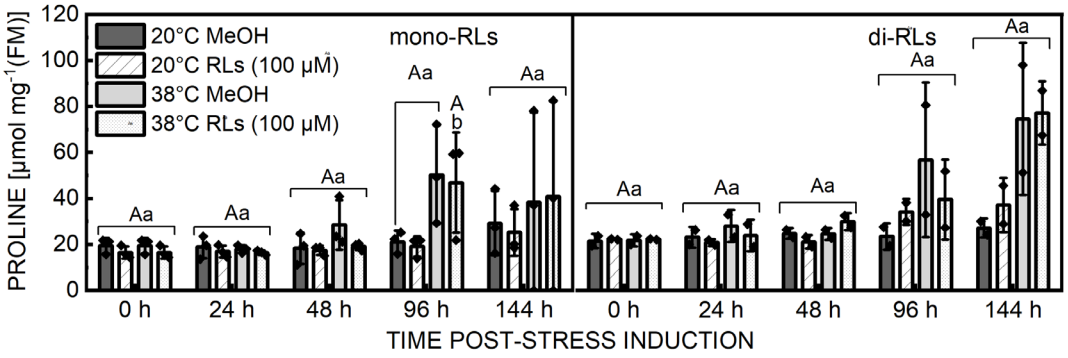

Fig. 6. Content of proline in leaves of 5-weeks-old *Arabidopsis* treated with methanol (control) or rhamnolipids (RLs, 100 µM) and growing under normal (20/18°C) or heat stress (38/35°C) conditions. FM – fresh mass. Data represents the mean values ± SD of three (n = 3) and two (n = 2) independent experiments for mono-RLs and di-RLs respectively. Each sample was obtained from a pool of three plants per experiment (two leaves/plant). *Diamond-shaped points* represent single data points. *Different lowercase letters* represent significant differences between plants in normal and stress conditions (effect of heat stress). *Different uppercase letters* represent significant differences between plants treated with MeOH and RLs (treatment effect). Statistical analysis determined for each measurement point, using the *Kruskal-Wallis* test followed by a *Dunn*'s post hoc at p<0.05.

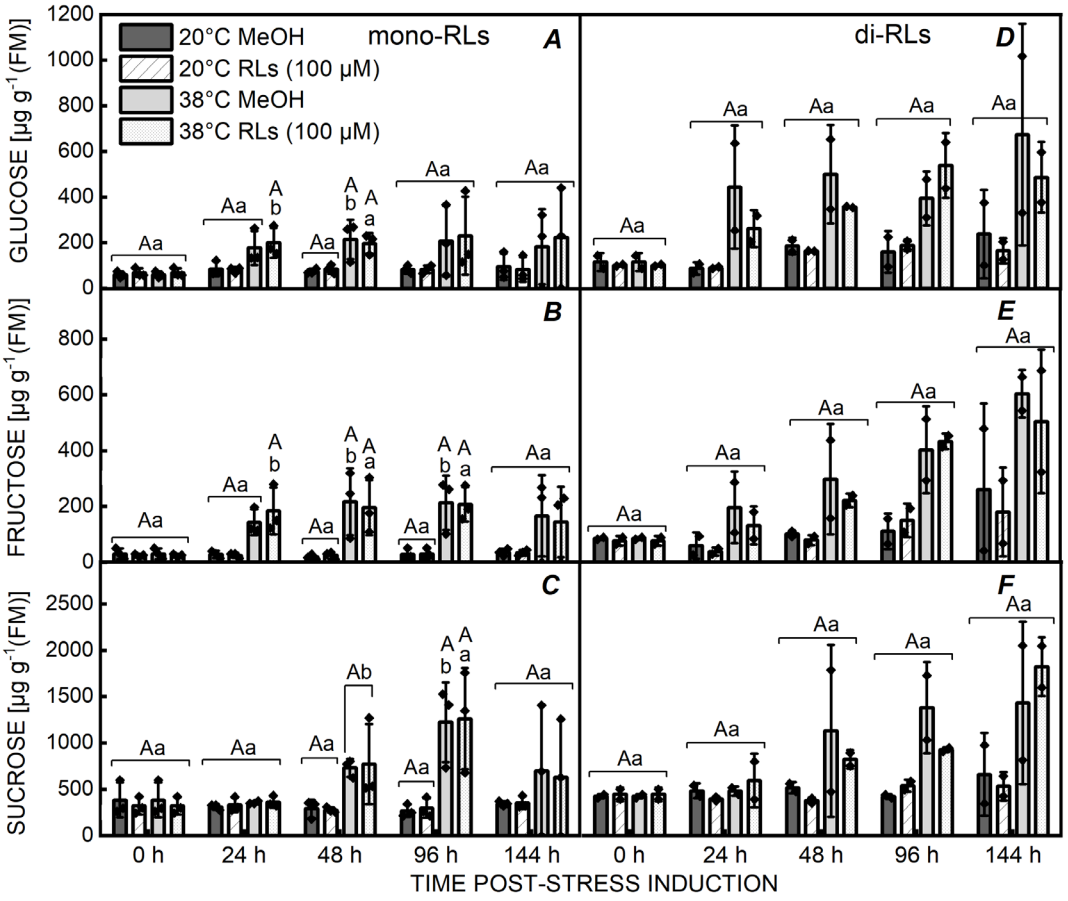

Fig. 7. Content of soluble sugars in leaves of 5-week-old *Arabidopsis* treated with methanol (control) or mono-rhamnolipids (100 µM) grown under normal (20/18°C) or heat stress (38/35°C) conditions. *A*, *B*, *C* (mono-RLs) and *D*, *E*, *F* (di-RLs) for glucose, fructose, and sucrose, respectively. FM – fresh mass. Data represents the mean ± SD of three (n = 3) and two (n = 2) independent experiments for mono-RLs and di-RLs, respectively. Each sample was obtained from a pool of three plants per experiment (two leaves/plant). *Diamond-shaped points* represent single data points. *Different lowercase letters* represent significant differences between plants in normal and stress conditions (heat stress effect). *Different uppercase letters* represent significant differences between plants treated with MeOH and RLs (treatment effect). Statistical analysis determined for each measurement point, using the *Kruskal-Wallis* test followed by a *Dunn*'s post hoc at p<0.05.

Discussion

High temperatures significantly threaten global agricultural production since it hinders plant growth and development. To mitigate these effects, exogenous applications of substances such as nutrients, phytohormones, and elicitors have been identified as effective methods (Feng *et al.* 2023). RLs are glycolipids that are known to induce plant defense mechanisms against a variety of plant pathogens in *Arabidopsis*, wheat, tomato, rapeseed, and grapevine (Varnier *et al.* 2009, Sanchez *et al*. 2012, Monnier *et al.* 2018, 2019; Robineau *et al*. 2020, Platel *et al*. 2022). This study assessed whether RLs (mono- and di-RLs) could contribute to *Arabidopsis* tolerance to HS. In *Arabidopsis*, investigations on HS often focus on the effect of HS stress, which assesses the influence of elevated temperature (5–15°C above normal temperature growth) for a short exposure time (min or few hours) (Paul *et al*. 2020, Cha *et al*. 2020, Kreslavski *et al*. 2023). However, the effect of prolonged HS (days/weeks) is poorly explored. In the context of climate change, the temperature continues to rise and could remain higher for several days in summer. This can influence the plant physiology differently than in a short HS. It is therefore essential to better characterize the physiological responses of plants under a prolonged HS. Here, we exposed plants to prolonged HS (38/35°C, day/night, 7 d) and evaluated various biochemical and physiological responses with and without RLs.

HS reduced photosynthesis efficiency: In this study, measurements of gas-exchange parameters showed that HS significantly reduced P_N. These findings are consistent with other research reporting a decrease in *Arabidopsis* photosynthetic efficiency in response to high temperatures (Paul *et al*. 2020, Kreslavski *et al*. 2023). Furthermore, g_s was lowered, indicating that P_N reduction was most likely caused by stomatal restriction, as previously described (Wang *et al*. 2020). Most likely, g_s reduction led to lower CO_2 uptake and consequently lower P_N. We also found that HS generated a drop in E and C_i. Lower E could indicate that plant leaf temperature cooling was hampered, which may result in the inactivation of Rubisco carboxylase activity (Demirevska-Kepova *et al*. 2005). Although C_i and g_s of all plants at 38°C were impacted, we found that the increase of C_i in RL-treated plants was lesser than that of control plants and that the reduction of g_s was lower in RL-treated plants than in control plants after 48 h of stress (Figs. 1*B*, 2*B*). It could be suggested that RLs helped maintain stomatal closure under HS. Interestingly, RL-induced stomatal closure was also reported by Monnier *et al*. (2018) when applied to *Brassica napus*, under biotic stress.

HS causes serious effects on PSII: Chlorophyll fluorescence is an essential indicator of PSII efficiency under stress (Su *et al*. 2016). Here, monitoring of PSII activity revealed that HS significantly reduces $Y_{(II)}$ in both RL-treated and control plants. These results are consistent with previous reports on *Arabidopsis* (Wang *et al*. 2020, Kreslavski *et al*. 2023) and other plants including tomatoes (Raja *et al*. 2020), peas (Rath *et al*. 2022), and spinach seedlings (Wang *et al.* 2023). Reduction of $Y_{(II)}$ hints towards heat-induced PSII photodamage, which can further explain P_N reduction in this study. Wang *et al*. (2020) have also tested the effect of prolonged heat stress (28°/23°C, day/night) on *Arabidopsis*. Their result hinted that $ETR_{(II)}$ was impacted by prolonged heat stress since $ETR_{(II)}$ related genes, such as *LHCB2.2* and *LHCB2.4* were upregulated. In this study, we found that $ETR_{(II)}$ was not significantly impacted by prolonged HS. Different indicators were utilized in these two studies to evaluate the impact of stress on $ETR_{(II)}$. On the one hand, we measured $ETR_{(II)}$ in real-time, while they assessed the effect at the genomic level. The stress intensity was not the same (28°C *vs.* 38°C). These two reasons might explain the opposite results found by these studies. In addition, it could be suggested that the differential regulation of *LHCB2.2* and *LHCB2.4* was not sufficient to impact $ETR_{(II)}$ or that the plant induced other tolerance changes to balance out this effect.

Reduction of Chl content was observed for both RL-treated and control plants, in response to HS: Photosynthetic pigments are key components of the photosynthesis machinery. Here, HS reduced Chl content and might impact directly photosynthetic efficiency. Other studies also report reducing Chl content under HS in *Arabidopsis* (Todorov *et al*. 2003, Paul *et al*. 2020). Reduction of Chl under HS is often associated with Chl biosynthesis impairment and increases enzymatic Chl degradation as was the case in bentgrass (Jespersen *et al.* 2016). Furthermore, this decrease in Chl may indicate that plants limit the production of ROS by preventing overstimulation of the photosystems. Reduction of Chl content also indicates leaf senescence due to HS (Jahan *et al*. 2021). The exogenous application of RLs had no impact on the Chl content. This is in line with Monnier *et al*. (2018), who also found that RL treatment does not alter the Chl content in rapeseed.

Accumulation of osmolytes and osmoprotectants in response to HS: Accumulation of osmolytes like proline is one of the well-described mechanisms for mitigating the HS effect in plants (Kumar *et al*. 2012, Sihag *et al*. 2024). Increasing evidence demonstrated that accumulation of proline under HS can enhance the antioxidant defense, act as a redox buffer, and stabilize cellular components (Kavi Kishor *et al*. 2022, Alagoz *et al*. 2023, Zulfiqar and Ashraf 2023). In this study, HS led to proline accumulation in mono-RL-treated and control plants. Accumulation of proline under prolonged HS has not been reported yet to the best of our knowledge and contradicts previous findings in *Arabidopsis* (Lv *et al*. 2011). The different nature of the applied HS could explain this discrepancy. In this study, *Arabidopsis* was subjected to prolonged HS (38/35°C, day/night, 7 d), whereas in the cited study the stress was shorter, 24 h at 37°C followed by 50°C for 4 h, and proline was measured at the recovery stage at 22°C for 96 h. It seems that short stress (<48 h) is not sufficient to induce proline accumulation in *Arabidopsis*.

According to the literature, the accumulation of proline in plants can potentially alleviate HS effects but can also inhibit thermotolerance, as previously reported by Lv *et al.* (2011). Exogenous application of RLs did not impact proline content, confirming that RLs do not alleviate HS impact *via* osmolyte regulation.

According to several studies, the buildup of soluble sugars is a crucial strategy for reducing HS (Xalxo *et al.* 2020, Alagoz *et al.* 2023). Soluble sugars help maintain cellular redox homeostasis and participate in ROS scavenging activities (Anjum *et al.* 2023, Akram *et al.* 2024). In this study, HS led to an accumulation of sucrose, fructose, and glucose for both RL-treated and control plants compared to normal conditions. This infers that those plants have activated their thermotolerance. Wang *et al.* (2020) also reported the accumulation of soluble sugars under prolonged stress in *Arabidopsis*. Interestingly our results showed that the accumulation was more significant for fructose and sucrose than glucose. The reason could be that glucose does not have a wide osmoprotectant activity compared to the later sugars (Singh *et al.* 2015). Exogenous application of RLs, however, had no significant impact on this accumulation.

RLs have no significant impact on plant physiology under normal and HS conditions: Our findings showed that RLs did not significantly impact *Arabidopsis* photosynthesis, Chl, and soluble sugar content under normal growth conditions. This aligns with previous results that RLs did not interfere with plant physiological processes such as Chl content, while still being able to protect rapeseed plants against *Botrytis cinerea* (Monnier *et al.* 2018). In support of this, the exogenous application of RLs had no major impact on the regulation of protein associated with primary mechanisms like photosynthesis, and carbohydrates in rapeseed after 7 and 24 h (Pierre *et al.* 2023). Results are interesting for using RLs as an antimicrobial or eliciting agent in the field. Indeed, the effect of elicitors on plant physiological processes, such as photosynthesis, is essential for their future application. It is known that inducing plant resistance can come at a cost to plants, as they struggle to maintain a balance between physiological development and defense strategies due to factors such as nutrient limitation (Godínez-Mendoza *et al.* 2023). RLs at 100 µM induce resistance against biotic stresses in plants. It is therefore encouraging to find that, at the same concentration, they have no negative effect on the physiological parameters evaluated in this study.

The exogenous application of RLs did not improve the *Arabidopsis* thermotolerance mechanism. No difference between mono- and di-RL could be observed. RL eliciting effect against biotic stresses is achieved through the accumulation of ROS, SA, ET, and JA pathways, and regulation of pathogen-related protein and defense-related genes in *Arabidopsis* (Sanchez *et al.* 2012, Schellenberger *et al.* 2021). Some of these signaling and defense responses were involved in inducing HS responses when exogenously applied to plants (Feng *et al.* 2023). Furthermore, in *Brassica napus*, the application of RLs activates HSPs, LEA genes as well as antioxidant-related genes known to be part of plant tolerance to abiotic stresses (Pierre *et al.* 2023). In wheat, synthetic RLs induced transcriptional changes in abiotic stress-related genes (Platel *et al.* 2022) as well. These studies suggest that RLs could moderate the effects of abiotic stress through the activation of transcriptional reprogramming. The lack of RL-induced thermotolerance observed in this study could be due to the intensity and duration of the HS (38/35°C, day/night).

Conclusion: Growing *Arabidopsis* under prolonged heat stress (37/35°C) reduces photosynthetic efficiency by limiting stomata, damaging PSII, and reducing chlorophyll content. Here, we found that prolonged HS has no impact on ETR. Our results revealed a progressive accumulation of proline and soluble sugars in *Arabidopsis* in response to prolonged HS. Regarding RLs, we report that exogenous application of mono- and di-RLs did not affect *Arabidopsis* physiological processes in mature leaves, including photosynthetic activity. In the future, experimentation with a combination of HS and biotic stress could help elucidate the mechanisms induced by RLs under HS. In addition, it will be interesting to test different stress regimes to better understand the impact of RLs.

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