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Synergistic effects of drought and heat stress on *Medicago truncatula*: understanding growth response and photosynthetic mechanisms

N. RESSAISSI[*,**], W. MNAFGUI[*,***], N. MAIZA[*,**], F. ZRIBI[*], W. ZORRIG[*], N. LUDIDI[***,#],
M.T. SANCHEZ-BALLESTA[##], and M. BADRI[*,+]

Laboratory of Extremophile Plants, Centre of Biotechnology of Borj Cedria, P.O. Box 901, 2050 Hammam-Lif, Tunisia[*]
Faculty of Sciences of Tunis, University of Tunis El Manar, Campus Universitaire El Manar, 2092 Tunis, Tunisia[**]
Plant Stress Tolerance Laboratory, University of Mpumalanga, Private Bag X112831, Mbombela 1200, South Africa[***]
DSI-NRF Centre of Excellence in Food Security, University of the Western Cape, Robert Sobukwe Road, 7530 Bellville, South Africa[#]
Laboratory of Biotechnology and Postharvest Quality, Institute of Food Science, Technology and Nutrition (ICTAN-CSIC), Jose Antonio Novais 6, 28040 Madrid, Spain[##]

Abstract

Drought and heat stress significantly threaten forage crop development and photosynthetic activity in the Mediterranean region. This study investigated the physiological responses and photosynthetic activity of two *Medicago truncatula* lines TN6.18 and F83005.5 (F83), to single and combined heat and drought stress treatments. Biomass traits, leaf gas exchange, and photosystem activities were evaluated. Our findings indicate a reduction in biomass parameters under heat, drought, and combined stress on both lines, particularly in F83. The stomatal conductance and photosynthetic parameters exhibited differential responses, with F83 reducing its stomatal conductance under drought stress, while TN6.18 was adapted by opening its stomata. Moreover, in TN6.18, combined stress enhanced protection mechanisms in PSI, while F83 showed changes in PSII efficiency. These insights deepen our understanding of plant responses to abiotic stresses and offer strategies for improving tolerance and resilience in changing environmental conditions.

Keywords: combined stress; drought; fodder legume; growth; photosynthetic parameters.

Introduction

The increase in global atmospheric temperature leads to frequent droughts, significantly impacting biological systems (Sharma *et al.* 2020). The combination of heat and drought exceeding the plant-specific optimal range may weaken photosynthetic capacity, decreasing photosynthetic area, leaf photochemical efficiency,

Highlights

- Combined drought and heat stress reduced biomass in *Medicago truncatula*
- TN6.18 enhanced photosystem I protection under combined stress
- F83005.5 shows altered photosystem II efficiency under combined stress

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[+]Corresponding author
e-mail: mounawer.badri@cbbc.rnrt.tn

Abbreviations: AFM – aerial fresh mass; *E* – transpiration rate; FC – field capacity; g_s – stomatal conductance; NL – number of leaves; NPQ – nonphotochemical quenching; P_{700m} – maximal fluorescence yield of dark-adapted sample with all PSI centers closed; P_{700m}' – maximal fluorescence yield of illuminated sample with all PSI centers closed; P_{700ox} – oxidized PSI; PAM – pulse amplitude modulation; PCA – principal component analysis; P_N – net photosynthetic rate; ROS – reactive oxygen species; WUE – water-use efficiency (= P_N/E); $Y_{(I)}$ – quantum yield of photosystem I; $Y_{(II)}$ – quantum yield of photochemical energy conversion in PSII; $Y_{(NA)}$ – quantum yield of nonphotochemical energy dissipation in reaction centers limited by acceptor side; $Y_{(ND)}$ – quantum yield of nonphotochemical energy dissipation in reaction centers limited by donor side; $Y_{(NO)}$ – quantum yield of nonregulated nonphotochemical energy dissipation in PSII; $Y_{(NPQ)}$ – quantum yield of regulated nonphotochemical energy dissipation in PSII.

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photosynthetic rate, and ultimately, plant yield (Wassie *et al.* 2020). Photosynthesis, accounting for 90% of plant dry matter, is a crucial physiological process (Lawson and Matthews 2020). It involves the conversion of water and CO_2 into organic compounds like carbohydrates and oxygen through oxygenic photosynthesis which is primarily facilitated by PSII and PSI complexes (Elkhouni *et al.* 2018).

Photosynthesis converts light energy into chemical energy, with the rate of CO_2 assimilation determining the speed of photosynthetic reactions (Ashraf *et al.* 2008). Both PSII and PSI capture light energy and convert it into chemical energy. Plants have evolved precise signaling pathways to adapt to diverse environments. Environmental stressors can rapidly reduce photosynthesis efficiency, serving as an early warning system (Li *et al.* 2018).

Numerous abiotic stresses significantly impact the photosynthetic process by altering chemical reactions mediated by PSII and PSI and affecting chlorophyll production (Dey and Ghosh 2022). PSII is particularly sensitive to heat stress due to increased fluidity of the thylakoid membrane, which dislodges the PSII light-harvesting complex, and its dependence on electron dynamics (Sharma *et al.* 2020). Additionally, these stresses hinder CO_2 assimilation *via* the ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) pathway (Kohli *et al.* 2017). Maintaining photosynthetic equilibrium is crucial for enhancing plant survival and productivity, particularly in nutritional value.

Water is crucial for plant survival, enabling the absorption and transportation of nutrients necessary for photosynthesis (Ashraf *et al.* 2013). CO_2 enters through the stomata, while water exits during transpiration. This process, driven by stomatal opening, is essential for pulling water upward through xylem vessels. Plants regulate water loss by adjusting stomatal openings, and balancing water use with environmental conditions (Arve *et al.* 2011). Water stress can limit photosynthesis *via* stomatal and nonstomatal limitations (Grassi and Magnani 2005). Stomatal closure restricts CO_2 diffusion, while nonstomatal factors affect metabolic processes, such as ATP synthesis and enzyme activities critical for photosynthesis.

Drought stress impacts the entire plant at physiological, morphological, and molecular levels, reducing photosynthetic capacity and yield (Flexas *et al.* 2008). Elevated leaf temperature, accelerated respiration rate, and reduced photosynthetic rate are observed under drought and heat stress (Rizhsky *et al.* 2002). Understanding the combined effects of heat and drought on photosynthesis is vital for developing strategies to enhance plant resilience.

Despite several studies on abiotic stress, there is a lack of data on the influence of combined heat and drought stress on the photosynthetic responses of legume plants. Further research is needed to understand the protective mechanisms under combined stress, which could lead to increased yield.

M. truncatula is an important fodder plant with small genome size and short life cycle, making it an excellent candidate for researching legume biology (Mollinedo *et al.* 2016). Its optimal growth temperature is 23 to 28°C, with annual rainfall ranging from 275 to 400 mm (Irshad *et al.* 2021). It is commonly used as fresh fodder and hay for livestock in Mediterranean countries and is crucial to the ecology and evolutionary dynamics of forage species in grassland farming systems. Understanding the photosynthetic mechanisms involved in *M. truncatula*'s tolerance strategy under combined drought and heat stress is imperative. While the general drought-tolerance strategy of alfalfa contrasting lines was known, their responses to single heat stress and the combination of heat and drought stress effects were not fully characterized by lack of information.

In this context, the photosynthetic mechanisms of two contrasting lines of *M. truncatula* F83 and TN6.18 were studied to understand their tolerance under combined drought and heat stress. This research aims to provide a comprehensive understanding of the physiological impact of these types of stress on plants, ultimately contributing to improved agricultural practices and plant breeding strategies.

Materials and methods

Plant material and growth traits: Two distinct lines of *M. truncatula* seeds, TN6.18, a local Tunisian line, and F83, of a French line (Badri *et al.* 2016a,b; Haddoudi *et al.* 2021) were used. Seeds were germinated in Petri dishes for four days in a growth chamber (14/10 h of light/dark at 25 and 18°C). Subsequently, they were transplanted in 1-L pots of 2:3 peat and sand under a greenhouse (at the Centre of Biotechnology of Borj Cedria, Tunisia, 36°42'32.9"N, 10°25'40.9"E). Plants were subjected to four types of stress, for each treatment, we planted 12 plants: control, drought, heat, and combined heat and drought stress. Stress conditions were initiated after 30 d of the vegetative stage and continued for 20 d (Haddoudi *et al.* 2021, Maiza *et al.* 2021).

The control and heat-stressed plants were irrigated to uphold soil water content at 90% of field capacity (FC). In contrast, the drought and combined stress plants were irrigated to sustain a soil water content equivalent to 40% of FC.

To induce heat stress conditions, a small controlled-growth chamber was utilized, housing a heater (*BioLux*) and a fan. The heater was activated at 07:00 h, resulting in temperatures exceeding 40°C during peak daylight hours, and deactivated at 16:00 h, to mimic the natural day/night cycle (Fig. 1). Plants were sampled after 20 d and divided into shoots and roots. The roots were cleaned twice using chilled distilled water at 4°C and then carefully dried using filter paper. The number of leaves (NL) was counted before collection, and immediately after, the samples were weighed to determine their aerial fresh mass (AFM).

Photosynthetic gas exchange: For the assessment of photosynthetic gas-exchange parameters, such as stomatal conductance (g_s) [mol(H_2O) m^{-2} s^{-1}], net photosynthetic rate (P_N) [µmol(CO_2) m^{-2} s^{-1}], and transpiration rate (E) [mmol(H_2O) m^{-2} s^{-1}], a portable *LC pro+* gas analyzer (*ADC BioScientific Ltd*., Hoddesdon, United Kingdom)

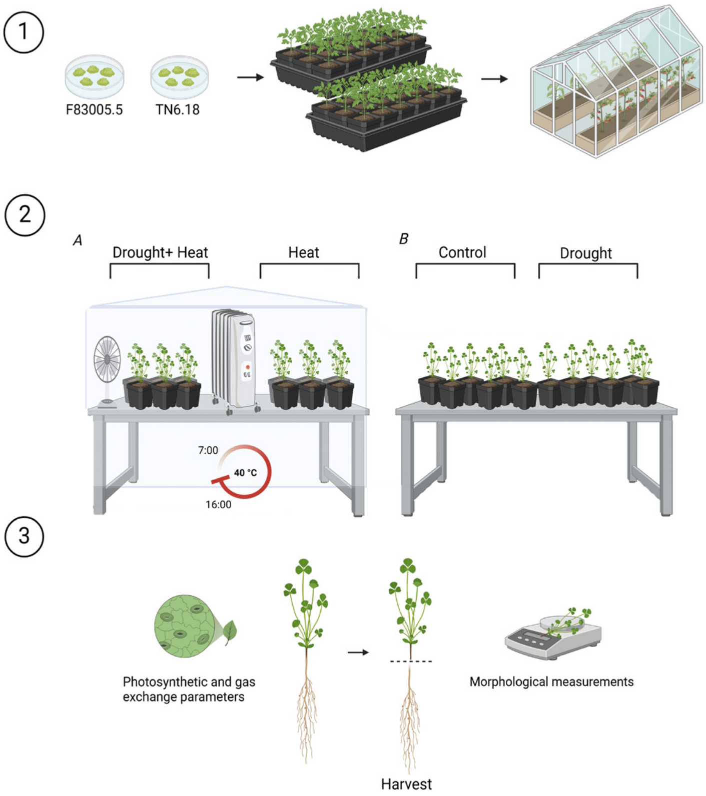

Fig. 1. Seedling and planting of two lines (TN6.18 and F83005.5) of *Medicago truncatula* in the greenhouse for 30 days (1), stress induction of six replicates (n = 6) of control, drought, heat, and combined heat and drought treatments for 20 days (2); (*A*) small greenhouse setup with heater and fan (40°C) for heat stress induction; (*B*) control and drought treatments maintained at 25°C; measurement of photosynthetic and gas-exchange parameters prior to harvesting (3).

was utilized during early day time, from 10:00 until 12:00 h. These measurements were conducted under photosynthetically active radiation (PAR) conditions at an approximate intensity of 1,044 µmol(photon) m^{-2} s^{-1} after 20 d of stress application, the aerial CO$_2$ concentration was 420 ppm, and the flow was 26 µmol s^{-1}. The water-use efficiency (WUE) [mol(CO$_2$) mol(H$_2$O)$^{-1}$] was calculated using the following formula: WUE = rate of photosynthesis/rate of transpiration.

PSI and PSII activities: The evaluation of PSI and PSII activities followed the protocols outlined by Klughammer and Schreiber (2008a,b). A *Dual-PAM-100* device from *Heinz Walz* in Effeltrich, Germany, was used to measure the leaves of *M. truncatula* lines TN6.18 and F83. Before measurements, the leaves underwent a 30-min dark-adaptation period to acclimatize to low-light conditions.

Leaves were exposed to different levels of actinic light using the method set up by Klughammer and Schreiber (2008a). Measurements were taken using *Dual-PAM-100* (*Heinz Walz*, Effeltrich, Germany) preconditioning to darkness (before storage) on *M. truncatula* leaves measured in the dark for 30 min (Falouti *et al.* 2022). To record the yields of photochemical energy conversion in PSII [$Y_{(II)}$], regulated nonphotochemical energy dissipation in PSII (NPQ), and nonregulated nonphotochemical energy dissipation in PSII [$Y_{(NO)}$], leaves were exposed to actinic light (0, 6, 12, 21, 56, 107, 146, 257, 412, 652, and 1,017 µmol(photon) m^{-2} s^{-1}) that initiated electron transport between photosystems. Additionally, the absorbance of PSI was measured using P700 dual-wavelength emitter-detector systems (830 and 875 nm, *Dual-PAM-100*, *Heinz Walz*, Effeltrich, Germany). In this photosystem, the loss of nonphotochemical energy in reaction centers is regulated by the acceptor side [$Y_{(NA)}$] or the donor side [$Y_{(ND)}$], while the converted photochemical energy in PSI [$Y_{(I)}$] was measured (Klughammer and Schreiber 2008b).

Statistical analysis: Means comparisons were performed using the *Duncan*'s test in *IBM SPSS Statistics 20* software. In this analysis, the *Snedecor-Fisher* coefficient (F) was utilized to measure significance, with $p \leq 0.05$ considered statistically significant. This approach was used to analyze and compare the means of measured gas exchange and growth trait parameters, identifying significant differences between treatment groups and pinpointing which groups differed. Principal Component Analysis (PCA) was conducted using *XLSTAT* version *2014.5.03*, considering variables centered on their means. Graphs of PSI and PSII were demonstrated using *SigmaPlot 14.0* software (*Systat Software Inc.*, USA).

Results

Morphological responses of *M. truncatula* contrasting lines: NL decreased in all of the treatments in F83 with a pronounced negative effect under combined stress for F83, while it remained lower under heat in TN6.18 (Fig. 2*A*). The F83 line reduced the NL by 57% in drought stress, 51% in heat stress, and 71% in combined heat and drought stress, compared to the control. Meanwhile, TN6.18's tolerance to drought stress resulted in maintaining a similar NL to the control. On the other hand, this parameter decreased by 50% under heat stress and 27% under combined stress.

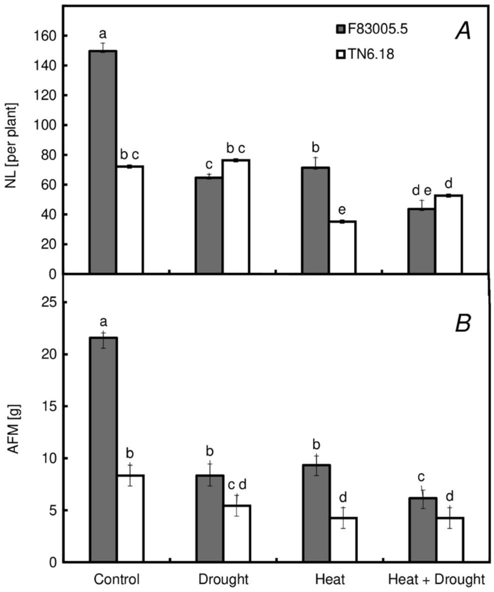

Fig. 2. Effect of drought (40% FC), heat, and combined heat and drought stress on (*A*) number of leaves (NL) and (*B*) aerial fresh mass (AFM) of two lines of *Medicago truncatula* TN6.18 and F83005.5 compared to control treatment (25°C). Means followed by *the same letters* are not significantly different between both lines according to *Duncan*'s test at 5%. The error bars correspond to standard errors, $n = 6$.

As shown in Fig. 2*B*, drought stress significantly reduced AFM by 62% in F83 and 29% in TN6.18 compared to the control condition. However, heat stress caused a slight decrease in AFM for TN6.18. At the same time, it remained at similar levels for F83 as in drought conditions (Fig. 2*B*). Combined heat and drought stress reduced AFM in both lines, particularly by 71% in F83 compared to the control treatment.

Leaf gas exchange: The variation in g_s, E, WUE, and P_N in the leaves of both *M. truncatula* lines F83 and TN6.18 under drought and/or heat stress is represented in Table 1. Under drought stress, TN6.18 showed no significant difference in all the measured parameters. When subjected to heat stress, TN6.18 exhibited a reduction in g_s, WUE, and P_N, with reduction percentages of 25 (g_s), 73 (WUE), and 63% (P_N), respectively, and an increase of 16% in E compared to the control. Under combined treatment, TN6.18 reduced in g_s, P_N, WUE, and E by 58, 72, 60, and 30%, respectively.

F83 exhibited a significant decrease in the drought treatment compared to the control, with reduction percentages of 37 (g_s), 83 (E), and 78% (P_N), and a slight reduction of 14% in WUE. Under heat stress, F83 showed an increase of 3% in g_s and a reduction of 8% in E, P_N was 19% less than the control treatment, and there was a reduction of 23% in WUE. Meanwhile, under combined

Table 1. Comparison of means of measured traits in *Medicago truncatula* lines TN6.18 and F83005.5 under control treatment, drought, heat stress, and combined heat and drought stress. *F* is the *Fisher–Snedecor* coefficient indicating significance at $P \leq 0.05$. The measured parameters include transpiration rate (E) [mmol(H_2O) m^{-2} s^{-1}], stomatal conductance (g_s) [mol(H_2O) m^{-2} s^{-1}], net photosynthetic rate (P_N) [μmol m^{-2} s^{-1}], and water-use efficiency (WUE) [mol(CO_2) mol(H_2O)$^{-1}$]. Means followed by *the same letters* are not significantly different between the two lines according to *Duncan*'s test at 5%. Values are averages of six replicates, $n = 6$.

Treatment	g_s [mol(H_2O) m^{-2} s^{-1}]	P_N [μmol m^{-2} s^{-1}]	WUE [mol(CO_2) mol(H_2O)$^{-1}$]	E [mmol(H_2O) m^{-2} s^{-1}]
Control				
F83005.5	0.038 ± 0.001^{b}	4.629 ± 0.170^{b}	4.301 ± 0.052^{b}	1.070 ± 0.033^{b}
TN6.18	0.036 ± 0.002^{b}	5.206 ± 0.204^{a}	7.822 ± 0.476^{b}	0.686 ± 0.048^{d}
F	1.010	4.204	108.401	45.897
P	0.325	0.051	0.000	0.000
Drought				
F83005.5	0.024 ± 0.004^{d}	1.024 ± 0.690^{ab}	3.698 ± 0.391^{cd}	0.177 ± 0.050^{a}
TN6.18	0.034 ± 0.000^{bc}	5.117 ± 0.108^{e}	7.587 ± 0.421^{bc}	0.685 ± 0.009^{e}
F	208.327	30.481	8.831	464.315
P	0.000	0.000	0.009	0.000
Heat				
F83005.5	0.039 ± 0.002^{a}	3.768 ± 0.213^{c}	3.294 ± 0.107^{cd}	1.156 ± 0.070^{c}
TN6.18	0.027 ± 0.006^{c}	1.917 ± 0.381^{d}	2.068 ± 0.197^{e}	0.797 ± 0.143^{b}
F	0.392	5.237	19.674	0.447
P	0.540	0.035	0.000	0.513
Heat and drought				
F83005.5	0.010 ± 0.002^{f}	1.024 ± 0.142^{e}	3.807 ± 0.790^{d}	0.269 ± 0.025^{f}
TN6.18	0.015 ± 0.002^{f}	1.477 ± 0.190^{de}	3.006 ± 0.254^{a}	0.482 ± 0.047^{e}
F	13.800	5.191	29.295	2.062
P	0.001	0.032	0.000	0.164

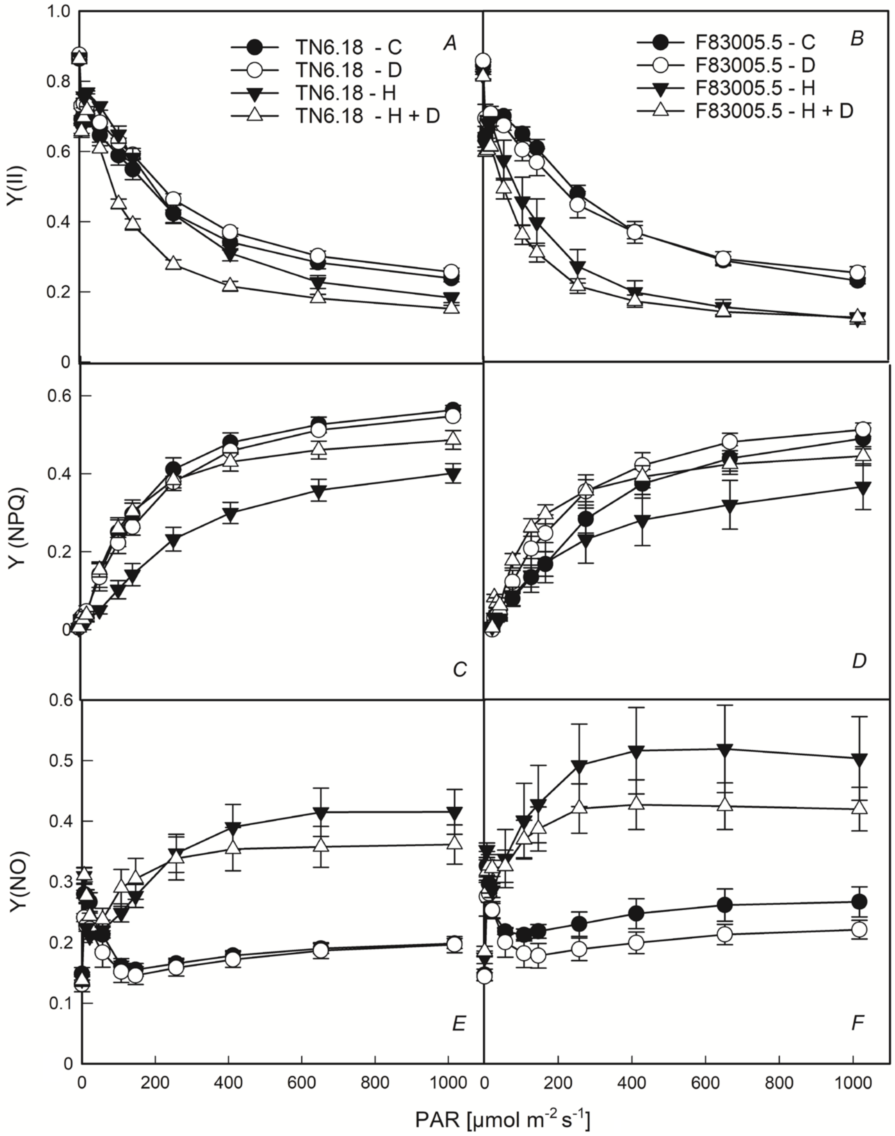

Fig. 3. Effect of drought and heat stress on fluorescence parameters of PSII for dark-adapted leaves (dark test) and light test in two lines of *Medicago truncatula* TN6.18 and F83005.5. (*A*,*B*) Quantum yield of photochemical energy conversion in PSII, $Y_{(II)}$; (*C*,*D*) quantum yield of regulated nonphotochemical energy dissipation in PSII, $Y_{(NPQ)}$; (*E*,*F*) quantum yield of nonregulated nonphotochemical energy dissipation in PSII, $Y_{(NO)}$. Error bars correspond to standard errors, $n = 5$. C – control; D – drought; H – heat; H + D – heat and drought.

heat and drought stress, stomatal conductance (g_s), water-use efficiency (WUE), transpiration (E), and photosynthetic rate (P_N) showed a significant decrease, with reduction percentages of 74 for g_s, 75 for WUE, 11 for E, and 78% for P_N, respectively, as shown in Table 1.

PSI and PSII activities: Individual treatments elicited significant variation in the TN6.18 and F83 lines regarding PSI and PSII activity. For the PSII variation, $Y_{(II)}$ in TN6.18 showed a decrease in combined treatments, and $Y_{(NPQ)}$ increased under heat in TN6.18 (Fig. 3), whereas no effect was noted under drought. In addition, $Y_{(NO)}$ reached its maximum under combined stress compared to other treatments.

In contrast, when exposed to combined stress, the sensitive F83 showed a drop in $Y_{(II)}$ (Fig. 3*B*). $Y_{(NPQ)}$ rose under drought compared to the control and heat and drought combined treatment which were equivalent but decreased under heat (Fig. 3*C*,*D*). $Y_{(NO)}$ increased significantly under heat in both lines, surpassing combined heat and drought treatment, and decreased during drought (Fig. 3*E*,*F*).

Furthermore, the $Y_{(I)}$ (Fig. 4) decreased in both lines grown under combined stress compared to other treatments. However, the $Y_{(ND)}$ increased more especially in TN6.18 under combined stress compared to the sensitive F83. Moreover, the $Y_{(NA)}$ in TN6.18 declined regardless of treatments, with the lowest value noted under combined stress (Fig. 4*E*), whereas it showed a slight increase in the sensitive line (Fig. 4*F*). For instance, the different treatments resulted in increased P_{700m} and P_{700ox} in the tolerant TN6.18 (Fig. 5*A*–*C*), whereas drought treatment notably increased P_{700m} and P_{700ox} in F83 (Fig. 5*B*–*D*).

Principal Component Analysis (PCA) was performed to assess the physiological responses of the *Medicago truncatula* lines TN6.18 and F83 under control, heat, drought, and combined heat and drought stress conditions. The first two principal components (F1 and F2) accounted for 81% of the total variance, with F1 explaining 55% and F2 explaining 25%. Fig. 6 shows the PCA biplot, with

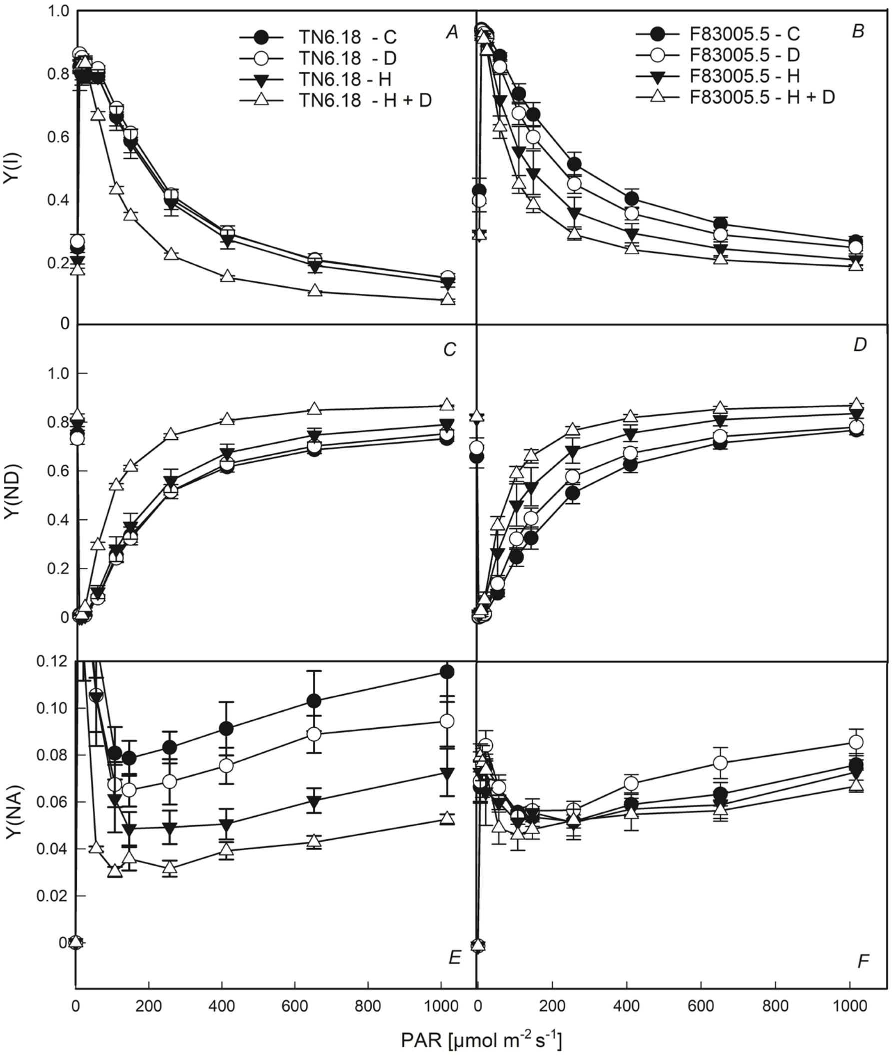

Fig. 4. PSI quantum yields parameters in leaves of *Medicago truncatula*. (*A*,*B*) Quantum yield of photochemical energy conversion in PSI, $Y_{(I)}$; (*C*,*D*) quantum yield of nonphotochemical energy dissipation in reaction centres limited by donor side, $Y_{(ND)}$; (*E*,*F*) quantum yield of nonphotochemical energy dissipation in reaction centres limited by acceptor side, $Y_{(NA)}$. Error bars correspond to standard errors, $n = 5$. C – control; D – drought; H – heat; H + D – heat and drought.

the positions of the TN6.18 and F83 samples under different stress conditions plotted along the F1 and F2 axes. The vectors represent the contributions of various physiological traits, including g_s, *E*, WUE, P_N, number of leaves (NL), and aerial fresh mass (AFM).

TN6.18 maintained higher WUE, NL, AFM, and P_N under individual stress conditions compared to F83, as evidenced by the clustering of TN6.18's control and stress conditions. TN6.18 exhibited less variability in response to stress, indicating greater resilience. F83 showed significant declines in physiological parameters under stress conditions, particularly drought and combined stress. The dispersed positions of F83 under stress conditions indicate greater variability and sensitivity to stress. Both lines exhibited the most severe reductions under combined heat and drought stress. TN6.18 demonstrated superior stress tolerance, maintaining relatively better physiological performance than F83 under combined stress.

Discussion

The observed alterations in biomass demonstrate the susceptibility of *M. truncatula* lines TN6.18 and F83 to various stress treatments. Our findings highlighted that the number of leaves in F83 decreased significantly across all stress treatments, with the most pronounced reduction under combined drought and heat stress. This indicates a severe inhibition of leaf production and maintenance under compounded stresses, likely due to impaired cell division or accelerated senescence. Biomass loss is consistent with earlier research on plants' physiological responses to stress such as drought and heat, as revealed by Kumar and Verma (2018) and Li *et al.* (2018). In particular, Semerci *et al.* (2017) found that drought stress reduced plant growth including shoot height, biomass, and number of leaves, leading to stunted growth.

In this study, the significant decrease in aerial fresh mass and the number of leaves under single and combined stress highlighted the sensitivity of both lines to simultaneous heat and drought, which is consistent with studies showing lower growth and biomass accumulation under combined stress, as noted by Zandalinas *et al.* (2018). These findings highlight the importance of investigating line-specific responses to understand the mechanisms controlling stress adaptation in legumes.

F83 exhibited considerably lower AFM and NL under heat and drought stress than TN6.18. In addition,

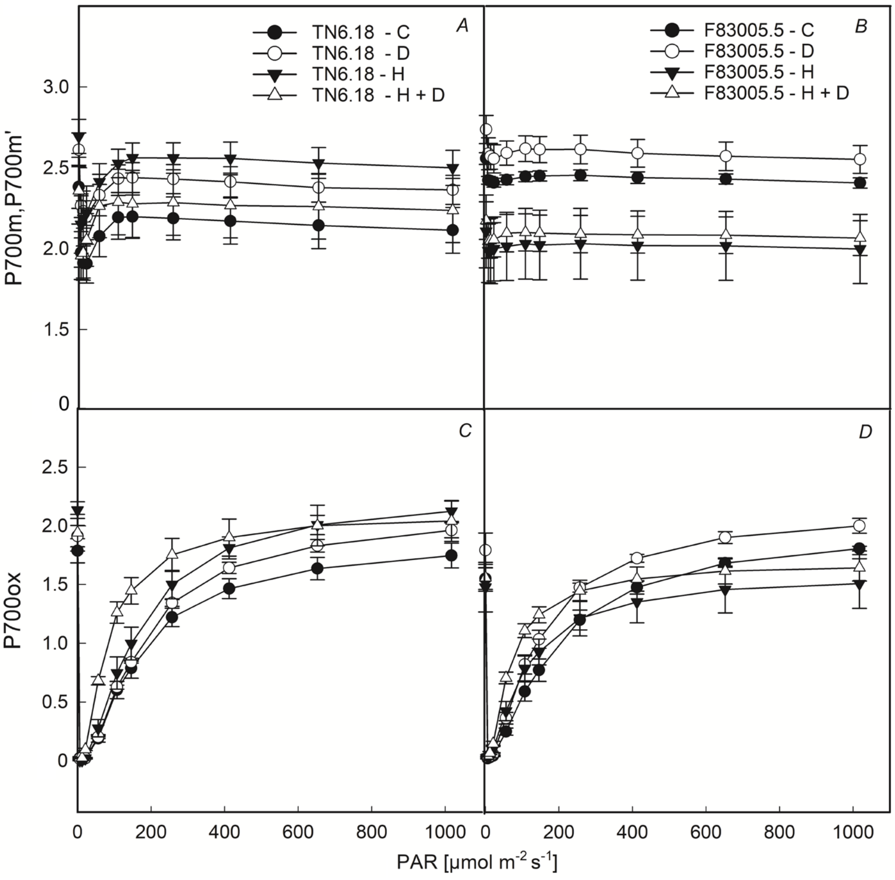

Fig. 5. Means of fluorescence parameters of PSI quantum yields parameters in leaves of *Medicago truncatula*. (*A*,*B*) Maximal fluorescence yield of dark-adapted sample with all PSI centres closed, $P700_m$, and maximal fluorescence yield of illuminated sample with all PSI centres closed, $P700_m'$; (*C*,*D*) oxidized PSI, $P700_{ox}$. Error bars correspond to standard errors, *n* = 5. C – control; D – drought; H – heat; H + D – heat and drought.

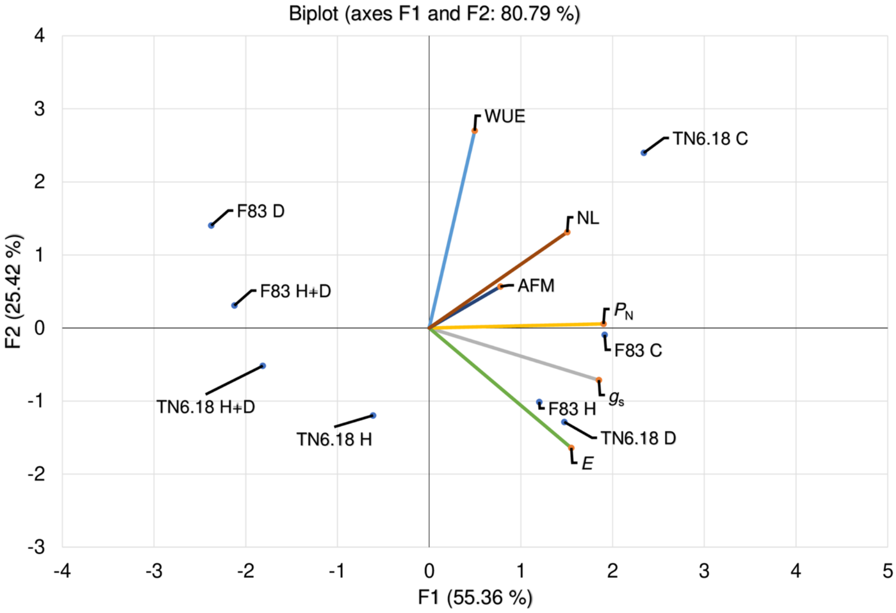

Fig. 6. Two-dimensional plots generated by Principal Component Analysis (PCA) using morphological and gas-exchange parameters for TN6.18 and F83005.5 lines of *Medicago truncatula*. C – control; D – drought; H – heat; H + D – heat and drought; P_N – net photosynthetic rate; AFM – aerial fresh mass; *E* – transpiration rate; WUE – water-use efficiency; g_s – stomatal conductance; NL – number of leaves.

heat stress alone caused only a slight reduction in AFM compared to drought, suggesting that F83 might be more sensitive to water limitation than to elevated temperatures. Furthermore, the severe decline in both NL and AFM under combined stress and a single one in F83 compared to TN6.18 suggests a lack of effective adaptive mechanisms, such as maintaining cellular turgor or managing oxidative stress, which are critical for sustaining growth under adverse conditions. The stability of NL and AFM in TN6.18 under drought and its limited reductions under heat demonstrate better physiological strategies to cope with stress suggesting its thermal-tolerance behavior. This unique behavior of *M. truncatula* lines under abiotic stress has been highlighted in a recent study by Haddoudi *et al.*

(2021) on TN6.18 under drought stress. Our findings add to this by showing a comparison framework of sensitivity. TN6.18's better performance under stress conditions shows the presence of stronger mechanisms for adaptation, especially when combined stress is present. The effect on aerial fresh mass, a key parameter influencing photosynthetic parameters in plants, revealed a differential response in gas exchange under different treatments (Bhandari *et al.* 2017).

Under drought stress, TN6.18 exhibited no significant changes in all measured parameters (g_s, E, WUE, and P_N). This stability indicates a robust physiological mechanism to cope with water deficits, through osmotic adjustment and efficient use of available water to maintain photosynthetic capacity (Chaves *et al*. 2003, Zhu *et al.* 2003, Haddoudi *et al.* 2021). F83 exhibited a markedly different response to individual and combined stress treatments. Under drought stress, there were significant reductions in g_s, E, and P_N, with a slight reduction in WUE. The sharp decline in g_s and E suggests a severe restriction in the stomatal opening, which greatly limits CO_2 uptake and reduces photosynthetic rates (Lawlor and Cornic 2002, Grassi and Magnani 2005, Zhang *et al.* 2008).

When subjected to heat stress, TN6.18 showed a significant reduction in g_s, WUE, and E but an increase in P_N. The reduction in WUE and P_N suggests that elevated temperatures impair carbon-assimilation efficiency, while the rise in E may reflect enhanced evaporative cooling, possibly as a protective mechanism to reduce leaf temperature (Medrano *et al.* 2002, Xu *et al.* 2010). The significant increase in P_N despite reduced stomatal conductance might be attributed to enhanced photosynthetic efficiency under higher temperatures or a possible acclimation mechanism that improves CO_2 assimilation efficiency under heat stress (Hu *et al*. 2020). The modest increase in g_s and a lower reduction in P_N compared to drought stress indicate that heat alone might not impose as severe limitations on photosynthetic activity in F83 as drought did. These observations are consistent with increased leaf stomatal conductance under heat stress (Tzortzakis *et al.* 2020). Consistently, high temperatures led to a decrease in relative leaf water content and stomatal conductance in lentils (Kaushal *et al.* 2013) and chickpea (Bhandari *et al.* 2017).

The comparative behavior between both lines under heat stress proved that heat stress had a more pronounced negative effect on TN6.18 than F83, as seen in the greater reductions in WUE and P_N for TN6.18. This suggests that while TN6.18 excels in drought tolerance, its thermal tolerance mechanisms might be less effective compared to F83's modest resilience under heat.

Under combined stress, both lines showed a synergistically low response in g_s, especially in the sensitive line F83. These results are consistent with those of previous studies on the effects of drought and heat stress on the photosynthetic characteristics of alfalfa (Mu and Chen 2021). Thus, stomatal closure in TN6.18 and F83 is an adaptive strategy to minimize water loss under combined stress. This strategy affects its photosynthetic rate by reducing the CO_2 uptake and transport of nonstructural carbon, an important component of photosynthesis, potentially leading to carbon starvation, as described by Steppe *et al.* (2015). This finding was consistent with the common observations on single stress, such as drought or heat stress, and the combination of drought and heat stress (Jiang and Huang 2001, Rizhsky *et al.* 2002). Overall, combined stress significantly reduced g_s, WUE, P_N, and E in both lines. However, the reductions were more severe in F83, reflecting its heightened vulnerability, and for TN6.18 its superior adaptability to multiple abiotic challenges.

The increase in $Y_{(NPQ)}$ in TN6.18 under heat stress indicates an enhanced capacity for nonphotochemical quenching, which protects PSII from excess light by dissipating it as heat. This protective mechanism was less pronounced under combined stress, suggesting additional stress-induced constraints (Mu and Chen 2021). However, the rise in $Y_{(NPQ)}$ in F83 under drought compared to its reduction under heat indicates that F83's energy dissipation strategy varies by stress type. Reduced $Y_{(NPQ)}$ under heat and combined stress likely reflects the limited capacity for thermal dissipation (Golding *et al.* 2004). The significant rise of $Y_{(NO)}$ under combined stress highlights increased energy dissipation through nonregulated pathways, indicating photodamage or less effective stress mitigation (Oh *et al.* 2022).

The different responses in quantum yields for PSII and nonphotochemical energy dissipation indicated that F83 had several adaptation mechanisms to deal with limited sources. The decrease in $Y_{(II)}$ in both lines under combined and heat stress indicates a compromised capacity for photochemical conversion, accompanied by reduced electron flow from PSII to PSI (Takagi *et al.* 2019).

Under the heat, drought, and combined treatments, TN6.18 showed an increase in P_{700m} and P_{700ox} in PSI compared to the control. P_{700} oxidation is anticipated to shield PSI against photoinhibition through ROS mitigation (Takagi *et al.* 2019) and is deemed a crucial regulatory mechanism for PSI photoprotection (Farhat *et al.* 2023). In contrast, under drought conditions, F83 considerably influenced P_{700m} in PSI, indicating a major effect on the redox state and electron-transfer efficiency within PSI when P_{700} oxidation was stimulated. This difference in behavior reveals that under combined stresses, TN6.18 maintained larger P_{700} oxidation which may be a critical feature for photoprotection under combined stress, a property that could guide future stress physiology studies. In the present study, F83 displayed a more nuanced response, with lower $Y_{(I)}$ and $Y_{(II)}$ values under combined stress, suggesting a compromise in PSII functionality. Similarly, Tattini *et al.* (2015) showed a downregulation of photosynthesis due to the negative effects of drought and heat on PSII by reducing the quantum yield of PSII. This might be explained by the improved ability to dissipate excess light energy, leading to photoprotection.

These thorough and line-specific variations in dark-adapted photosynthetic measurements under combined and drought stress offered valuable insights into the adaptive strategies of TN6.18 and F83. Investigation of PSI and PSII activities yielded mixed results, revealing

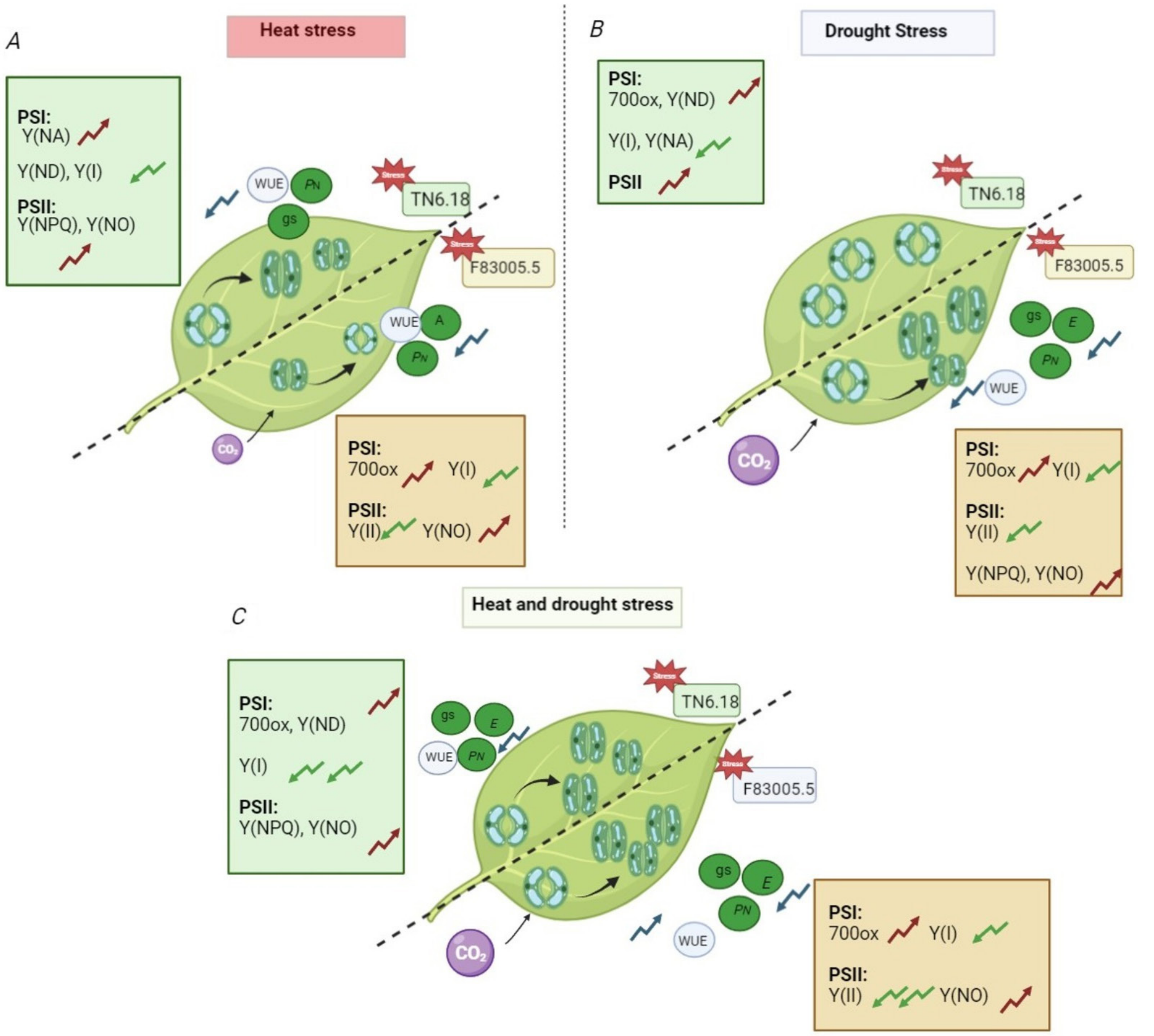

Fig. 7. Effect of different stress treatments on gas exchange and PSI and PSII activity in *Medicago truncatula* lines TN6.18 and F83005.5. (*A*) Heat stress, (*B*) drought stress, (*C*) combined drought and heat. P_N – net photosynthetic rate; E – transpiration rate; g_s – stomatal conductance; WUE – water-use efficiency; $Y_{(I)}$ – quantum yield of photosystem I; $Y_{(NA)}$ – quantum yield of nonphotochemical energy dissipation in reaction centres limited by acceptor side; $Y_{(ND)}$ – quantum yield of nonphotochemical energy dissipation in reaction centres limited by donor side; $Y_{(NO)}$ – quantum yield of nonregulated nonphotochemical energy dissipation in PSII; $Y_{(NPQ)}$ – quantum yield of regulated nonphotochemical energy dissipation in PSII; $P700_{ox}$ – oxidized PSI.

that TN6.18 employs effective protective strategies under combined stress, such as enhanced $Y_{(NPQ)}$ for thermal dissipation, increased $Y_{(ND)}$ to prevent PSI over-reduction and efficient P_{700} regulation. F83 presented a high tolerance to heat stress and sensitivity to combined heat and drought stresses. These mechanisms contribute to its resilience under combined stress. Overall, these results help us understand the complex and line-specific regulatory systems involved in the response of *M. truncatula* to heat and drought stress. This study provides useful information that could be used to develop strategies to improve plant tolerance to abiotic stress, potentially leading to more sustainable agriculture in the face of changing environmental conditions. By focusing on TN6.18's unique physiological and photoprotective mechanisms, this study provides new insights that can be used to improve breeding methods for climate-resistant crops.

Conclusion: The study examined the morphological and physiological responses of *M. truncatula* lines F83 and TN6.18 to drought, heat, and combined stress (Fig. 7). F83 exhibited pronounced negative effects under combined stress, particularly in the number of leaves (NL) and aerial fresh mass (AFM). In contrast, TN6.18 maintained better performance under drought and showed moderate declines under heat and combined stress. Under drought stress, TN6.18 exhibited no significant changes in gas-exchange parameters, whereas heat stress led to reductions in g_s, WUE, and E but an increase in P_N. Combined stress caused significant decreases in all parameters for TN6.18. F83 showed substantial reductions in E and P_N under drought and combined stress, with a moderate decrease under heat stress. PSI and PSII activities varied significantly, with TN6.18 demonstrating better adaptation, particularly under combined stress, while F83 showed marked declines in photosynthetic efficiency. Overall, TN6.18 displayed greater tolerance to individual and combined stress, suggesting its potential for enhancing crop resilience in stressful environments.

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