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Unveiling novel contrasting photosynthetic responses in *Medicago truncatula* under combined drought stress and *Phoma medicaginis* infection

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ABSTRACT

Forage legumes face simultaneous abiotic and biotic stresses, causing substantial yield losses. This study explores the combined impacts of drought and *Phoma medicaginis* infection on the growth and photosynthetic activity of *Medicago truncatula* contrasting lines TN6.18 and F83005.5. The tolerant TN6.18 line exhibits superior tolerance to combined drought and *P. medicaginis* infection, manifesting in minimal leaf area reduction, the highest number of healthy leaves, increased carotenoids content, a consistently high and stable photosynthesis rate, and enhanced performance of photosystems PSI and PSII. On the contrary, the sensitive F83005.5 line shows pronounced leaf chlorosis, particularly under drought stress, and decreased pigment levels under the combination of drought and *P. medicaginis* infection stresses (combined stress). Moreover, the drought-stressed F83005.5, experiences reduced hydration and photosynthetic performance, linked to diminished gas exchange and chlorophyll fluorescence parameters. Chlorophyll fluorescence revealed more severe PSI impairment than PSII under combined stress. In conclusion, understanding the Pm8 infection-drought interaction enhances insights into *M. truncatula* resistance mechanisms to the combination of these stresses.

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Introduction

Climate change induces various adverse environmental conditions that detrimentally affect plant life (Farooq et al. 2022). Among these challenges, drought stands out as a prominent abiotic stressor, up to 45% of the world's agricultural land faces recurring water shortages, disrupting physiological and biochemical processes and ultimately reducing crop yields (Farooq et al. 2022). Raza et al. (2023) affirmed also that drought stress stands out as a particularly damaging natural threat affecting food security. It significantly impacts a large segment of the global population, especially those residing in arid and semi-arid regions.

Under drought stress, plants may become more susceptible to pathogen attacks. For instance, *Parthenocissus quinquefolia* (L.) Planch. plants infected with *Xylella fastidiosa*, causing leaf scorch, in low moisture soil exhibited severe symptoms, including reduced leaf area, shoot length, leaf water potential, and stomatal conductance, compared to pathogen-infected plants in normal soil moisture conditions (McElrone et al. 2001; 2003; McElrone and Forseth 2004). Similarly, common bean (*Phaseolus vulgaris* L.) plants exposed to a combination of drought stress and the fungal pathogen *Macrophomina phaseolina* exhibited higher transpiration rates and leaf temperatures than plants subjected to drought stress alone (Mayek-PÉrez et al. 2002). Hybrid poplar (*Populus nigra* L. × *Populus maximowiczii* Henry) exposed to both drought stress and infection by *Septoria musiva* developed larger cankers (Maxwell et al. 1997), and red pines (*Pinus resinosa* Aiton) under moderate drought stress were vulnerable to *Sphaeropsis sapinea* (Fr.) Dyko & Sutton causing blight (Blodgett et al. 1997).

Conversely, drought-stressed plants resist pathogens requiring consistently wet conditions. For example, in tomato, *Medicago sativa* L., and *Arabidopsis thaliana* (L.) Heynh. plants, drought stress reduced infection by the fungal pathogen *Botrytis cinerea* by 50% and suppressed the spread of *Oidium neolycopersici*, responsible for powdery mildew, attributed to increased endogenous abscisic acid (ABA) levels. Drought-acclimated *Nicotiana benthamiana* Domin plants exhibited fewer disease symptoms when infected with *Sclerotinia sclerotiorum* and *Pseudomonas syringae* pv. tomato, compared to well-watered plants (Achuo et al. 2006; Gupta et al. 2016). Moreover, endophytic fungi improved gas exchange, chlorophyll content, photosynthesis, and chloroplast fluorescence in red oak and *Sorghum bicolor* (L.) Moench seedlings under to drought stress (Pandey et al. 2017; Zhou et al. 2021).

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The combined occurrence of drought stress and pathogen infection can have either positive or negative effects on plants, as demonstrated in various combinations of biotic and abiotic stresses (Mayek-PÉrez et al. 2002; McElrone et al. 2003; Ramegowda et al. 2013; Rejeb et al. 2014). Efforts have been directed toward unraveling how plants respond to combined stresses of drought and pathogen attack, which are crucial for enhancing crop production. Legume plants, vital as food sources, are substantially impacted by simultaneous biotic and abiotic stresses, necessitating the elucidation of mechanisms for developing climate-resilient varieties (Pandey et al. 2017). However, economically important legumes pose challenges for in-depth genetic studies due to traits such as large genomes and complex polyploidies. Therefore, a simple model for legumes is essential, and *Medicago truncatula* Gaertn. an annual forage legume, serves this purpose with its small genome, short life cycle, self-pollination ability, and high genetic transformation efficiency (Castañeda and González 2021; Zhang et al. 2022).

Uncovering the mechanisms of adaptation to dual constraints of drought and fungal stress in *M. truncatula* using physiological and photosynthetic responses is essential to enhance forage crop yield. Moreover, to our knowledge, there is a lack of available information regarding the responses underlying certain photosynthetic traits affected by combined stresses, particularly concerning the concurrent tolerances of *P. medicaginis* infection and drought stress in *M. truncatula*.

This study aims to understand the effects of infection with *P. medicaginis* on morphological, photosynthetic responses of two contrasting lines of *M. truncatula* (the tolerant TN6.18 and the sensitive F83005.5) grown initially under drought stress compared to normal conditions.

Materials and methods

Fungal material

Phoma medicaginis was isolated from *M. truncatula* plants and identified as the reference strain Pm8 of *P. medicaginis* (Djebali 2013). Conidial suspensions were prepared from 2-week-old cultures by adding 10 mL of distilled water to each plate and rubbing the surface of each culture with a spreader bar. Conidial suspensions were adjusted to 10^6 conidia mL^{-1} following hemocytometer counts.

Plant material and experimental conditions

Two contrasted *M. truncatula* lines were utilized in our study including the tolerant Tunisian line TN6.18 and the sensitive French line F83005.5 to drought stress and to Pm8 strain infection (Limami et al. 2014; Haddoudi et al. 2021; Maiza et al. 2021). The seeds from both lines were subjected to scarification using q60 sandpaper and then kept in darkness at 4 °C for 72 h. Afterward, they were transferred to a temperature of 21 °C for 24 h to promote germination. The germinated seeds were sown into black pots with a 1-liter capacity, and filled with a mixture of sand and peat (3:1, v/v). For controlled growth, the plants were cultivated in a growth chamber under specific conditions, including a temperature of 25/18 °C (day/night), a relative humidity of 60–80%, and a photoperiod of 16/8 h (light/dark). Regular irrigation was carried out every two days using the Fåhraeus nutritive solution (Fåhraeus 1957).

The plants were cultivated under three treatments: (i) control conditions (100% field capacity (FC)), (ii) drought stress (40% FC), and (iii) a combination of drought stress (40% FC) and Pm8 strain of *P. medicaginis* infection. The water deficit (40% FC) was applied after six weeks of cultivation of the plants under control conditions (100% FC) and watered with a Fåhraeus nutritive solution. Fourteen days after the start of the water deficit treatment, plants were inoculated with the Pm8 strain and were harvested at 14 days post-inoculation (dpi). Five replicates were used per line and treatment.

Inoculum production and inoculation tests

After 2 weeks of drought stress (40% FC), drought-stressed plants were separated into two groups: the first group was sprayed on the leaves with 20 mL of H_2O (non-inoculated plants) in leaves, while the second group was spray-inoculated on the leaves with 20 ml of the conidial suspension at a concentration of 10^6 conidia per mL of the Pm8 strain until run-off (inoculated plants) as described by Maiza et al. (2021). The inoculated plants were covered with transparent plastic bags to maintain an atmosphere saturated with humidity to promote infection. They were then placed in the dark for 48 h at 25 °C. The experimental design consisted of two treatments, four blocks, and five plants per block (one plant per pot).

Morphological traits measurement

Measurement of the parameters on the plants was carried out at harvest after 14 days post-inoculation. The measured parameters were the number of healthy leaves (NHL), the number of chlorotic leaves (NCL) and leaf area.

The measurements of leaf area were conducted for each line. We selected five regular-shaped leaves for the three treatments. Each leaf lamina was sandwiched in a clear file folder (310 mm × 220 mm) and placed on a white clipboard (Koyama 2023). Immediately after the photography procedure, we sampled the same five leaves per species. The adaxial surface of the leaf laminas was then scanned using a digital scanner. Individual leaf area is defined as the area of one side of each leaf lamina. For images obtained using scanner, the area of the leaves was measured using the EPSON scanner.

Photosynthetic gas exchange parameters and water status

The determination of photosynthetic gas exchange parameters was conducted using an infrared gas analyzer (LCi portable photosynthesis system, ADC BioScientific Ltd., Hoddesdon, Herts, UK). The following parameters were recorded at saturating light: Photosynthesis rate (*A*), stomatal conductance (*gs*), and transpiration rate (*E*). The water use efficiency (*WUE*)

was estimated using the following formula: *WUE* = *A*/*E* (Hatfield and Dold 2019). All measurements were performed in fully expanded leaves (the same leaf stage from the bottom). Values of the above-mentioned parameters were taken after the stabilization of the photosynthetic levels.

Photosynthetic pigment concentrations

The extraction of photosynthetic pigments was done using the method of Lichtenthaler (1987). Fresh leaf discs (50 mg) were introduced into vials containing 3 ml of 80% acetone. The vials were then placed in the dark at 4 °C for 7 d. The absorbance of the extracts was determined at 470, 646, and 663 nm for Chl. *a*, Chl. *b*, and total carotenoid assays, using a UV-visible spectrophotometer (Specord 210 Plus, Analytik Jena, Germany). Chl (*a* and b) and carotenoid contents were calculated according to the following formulas:

$$\text{Chl. } a\left[\mu g ml^{-1}\right] = \left(12.21 \times A_{663}\right) - \left(2.81 \times A_{646}\right)$$

$$\text{Chl. } b\left[\mu g ml^{-1}\right] - \left(20.13 \times A_{646}\right) - \left(5.03 \times A_{663}\right),$$

Carotenoids [μg ml^{-1}] = (1,000 × A_{470} − 3.27 [Chl. *a*] − 104 [Chl. *b*])/227, where A_{470}, A_{646}, and A_{663} represent extract absorbance at 470, 646, and 663 nm, respectively.

PSI and PSII activities

PSI and PSII activities were assessed following the procedures outlined by Klughammer and Schreiber (2008a,2008b) using a Dual-PAM-100 fluorometer (Heinz Walz, Effeltrich, Germany) on *M. truncatula* leaves that were preadapted to darkness for 30 min before measurements. Operational photochemical efficiency and energy dissipation of PSII were also investigated.

To determine the minimal fluorescence yield of the dark-adapted state (F0), low-intensity light was applied, allowing all reaction centres of PSII to be open and minimizing fluorescence. Subsequently, a flash of saturating light induced the closure of all PSII reaction centres, resulting in a maximal fluorescence yield of the dark-adapted state (Fm) and a reduction in overall photochemical efficiency. Following this, leaves were exposed to actinic light with varying intensities (0, 6, 12, 21, 56, 107, 146, 257, 412, 652, and 1,017 μmol (photon) m^{-2} s^{-1}) to initiate electron transport between photosystems, enabling the recording of the quantum yield of photochemical energy conversion in PSII [Y(II)], the non-photochemical quenching (NPQ), and quantum yield of non-regulated non-photochemical energy dissipation in PSII [Y(NO)].

For PSI absorbance, P700 dual-wavelength emitter-detector units (830 and 875 nm) were employed, following the methodology of Klughammer and Schreiber (2008b). Measurements for this photosystem included the quantum yield of photochemical energy conversion in PSI [Y(I)], the quantum yield of non-photochemical energy dissipation in reaction centres limited by acceptor side [Y(NA)], and the quantum yield of non-photochemical energy dissipation in reaction centres limited by donor side [Y(ND)].

Statistical analysis

The statistical analyses were conducted using the SPSS software (version 17.0, SPSS Inc., Chicago, IL, USA). To compare the means of the measured parameters, Duncan's multiple range test at a 5% significance level was employed. PCA was carried out using XLSTAT software, version 2014 (https://www.xlstat.com), utilizing the means of the lines.

Results

Assessment of morphological parameters under drought or combined stresses

The morphological parameters were assessed, including the number of healthy leaves (NHL) (Figure 1(A)), chlorotic leaves (NCL) (Figure 1(B)) and leaf area (Figure 1(C)). The results revealed a significant decrease in NHL and an increase in NCL under both stresses in both lines, with this effect being particularly evident in the sensitive line F83005.5 compared to the control. Moreover, the most substantial reduction in NHL occurred under combined stresses compared to only-drought stress with a 35% of reduction in TN6.18 compared to an 80% of reduction in F83005.5, while the highest NCL was observed when plants experienced drought stress (Figure 1(A, B)).

In contrast, the sensitive line F83005.5 exhibited a greater leaf area than the tolerant line under all treatment conditions, with the most notable decrease in this parameter observed under combined stress (22%) in comparison to drought conditions. Otherwise, TN6.18 showed a higher decrease in leaf area under combined drought and *P. medicaginis* infection compared to only-drought stress (48.5%) (Figure 1(C)).

Photosynthetic gas exchange determinations

The imposition of drought stress had a notable impact on the photosynthetic gas exchange parameters, as depicted in Table 1. In the sensitive line F83005.5, both photosynthesis rate (*A*) and water use efficiency (*WUE*) decreased by 78% and 84% respectively, under drought stress, accompanied by an increase in transpiration rate (13%). However, when subjected to combined drought and *P. medicaginis* infection, the tolerant line TN6.18 showed a reduction in *WUE* (45%) and increase in transpiration rate (*E*) (21%) and stomatal conductance (g_s) (62%), although it did not show any significant difference in all parameters under drought stress alone. In addition, g_s peaked under the combined stresses in the F83005.5 line, compared to only-drought stress (85%). (Table 1).

Pigments content

Chlorophyll *a* (Chl. *a*), chlorophyll b (Chl. *b*), total chlorophylls (Chl. *a* + *b*), and carotenoids content exhibited notable differences between the lines under various treatments. However, under drought stress, no significant effects were observed on Chl. *b* content in both lines and Chl. *a* + *b* in TN6.18 when compared to the control (Figure 2(B, C)). In contrast, the

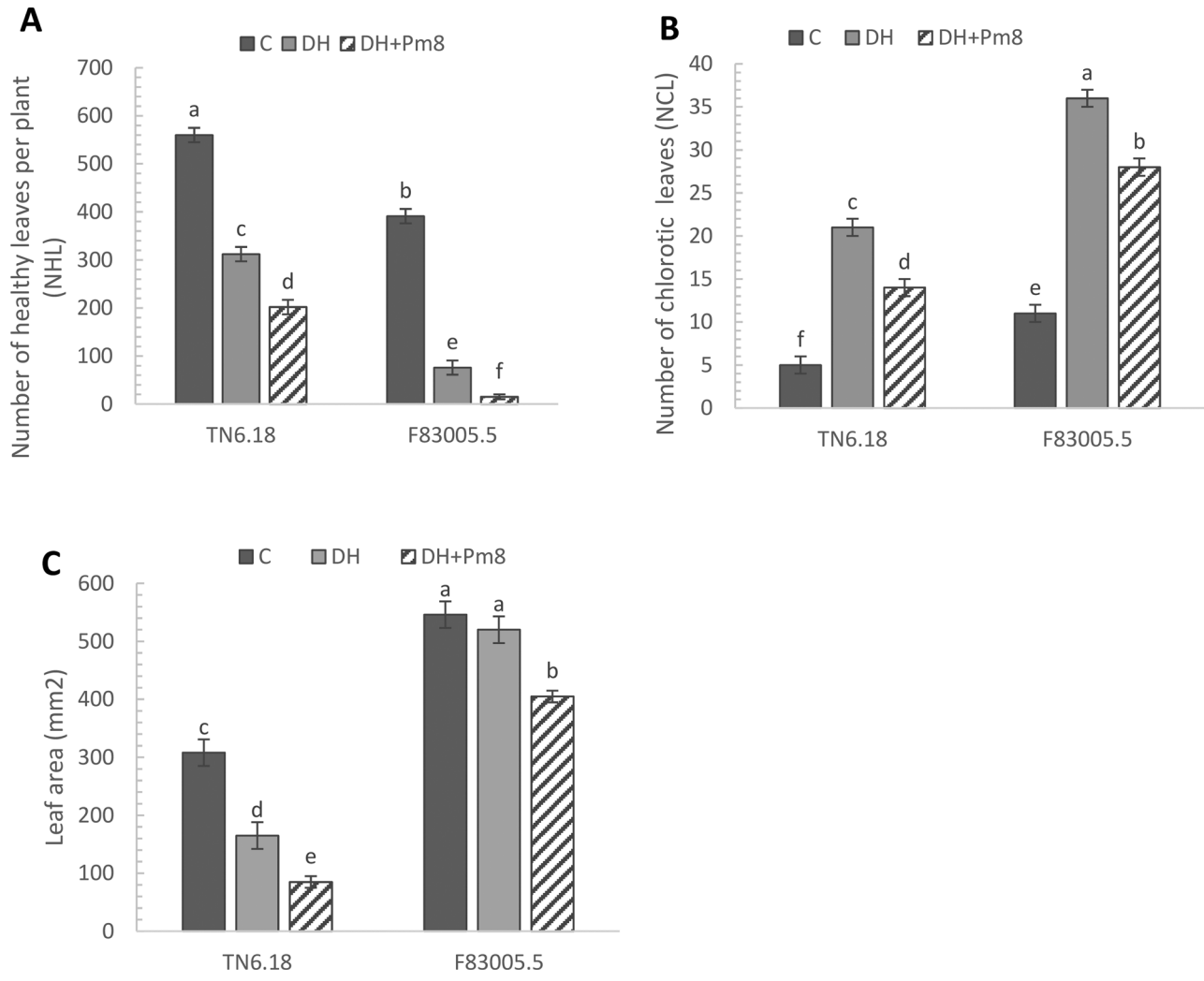

Figure 1. Effects of drought on the number of healthy leaves per plant (NHL) (A), number of chlorotic leaves per plant (NCL) (B) and leaf area (C) in two *Medicago truncatula* lines TN6.18 and F83005.5. These lines were cultivated under control conditions (C), drought stress (DH), and a combination of drought stress and infection with the *Phoma medicaginis* Pm8 strain (DH + Pm8). Means denoted by the same letter(s) are not significantly different among the studied lines for each trait, based on Duncan's test at the 5% significance level. Values are averages of five replicates. The error bars correspond to standard errors.

Table 1. Comparison of the mean values of measured traits across both *Medicago truncatula* lines under various conditions: control, drought stress, and a combination of drought stress and infection with the *Phoma medicaginis* Pm8 strain.

	E	*gs*	*A*	*WUE*
		Control		
TN6.18	4.76 ± 0.17 abc	0.29 ± 0.02 c	6.78 ± 0.56 b	1.42 ± 0.07 b
F83005.5	3.82 ± 0.63 c	0.32 ± 0.01 c	13.06 ± 1.47 a	3.48 ± 0.18 a
F	2.04	2.00	15.00	103.00
P	0.000	0.000	0.016	0.001
		Drought		
TN6.18	4.40 ± 0.11bc	0.33 ± 0.01c	6.96 ± 0.2 b	1.60 ± 0.11 b
F83005.5	5.34 ± 0.11ab	0.20 ± 0.02 c	2.82 ± 0.04c	0.53 ± 0.01d
F	14	47	409	83
P	0.019	0.002	0.000	0.001
		Drought + Pm8 infection		
TN6.18	5.55 ± 0.29 a	0.86 ± 0.16b	4.92 ± 0.54 bc	0.88 ± 0.087 c
F83005.5	5.35 ± 0.28 ab	1.28 ± 0.25 a	2.89 ± 0.03 c	0.54 ± 0.035 d
F	0.000	2.01	13.00	13.00
P	0.000	0.000	0.021	0.022

Mean ± Standard Deviation (SD), F is the Snedecor-Fisher coefficient, indicating significance at $p \leq 0.05$. The measured parameters include transpiration rate (*E*), stomatal conductance (*gs*), photosynthesis rate (*A*), and water use efficiency (*WUE*). Different letters indicate significant differences between both lines for the same parameter treatments under each treatment according to the Duncan test at 5%. Values are averages of five replicates.

sensitive-F83005.5 showed a significant 10% decrease in total Chl. *a* + *b* compared to the control under drought stress.

Infection with the Pm8 strain significantly affected pigment content in both drought-stressed lines. Specifically, the impact of strain Pm8 was more pronounced on the drought-stressed F83005.5, resulting in a 34%, 47%, and 42% reduction in *chl. a*, *chl. b* and *chl. a* + *b*, respectively, compared to a 24%, 22%, and 18% reduction, respectively, in the drought-stressed TN6.18 (Figure 2(A–C)). However, a significant 16% increase in carotenoids was observed in drought-stressed TN6.18 due to *P. medicaginis* infection, restoring the levels to those of the control. In contrast, the drought-stressed F83005.5 exhibited a higher increase of 32% in carotenoids due to *P. medicaginis* infection. (Figure 2(D)).

Principal component analysis

To consolidate our findings, Principal Component Analysis (PCA) was conducted on all assessed parameters characterizing the response of contrasting lines of *M. truncatula* to drought stress and combined drought and *P. medicaginis* infection. PC1 and PC2 accounted for 77.93% and 26.41% of

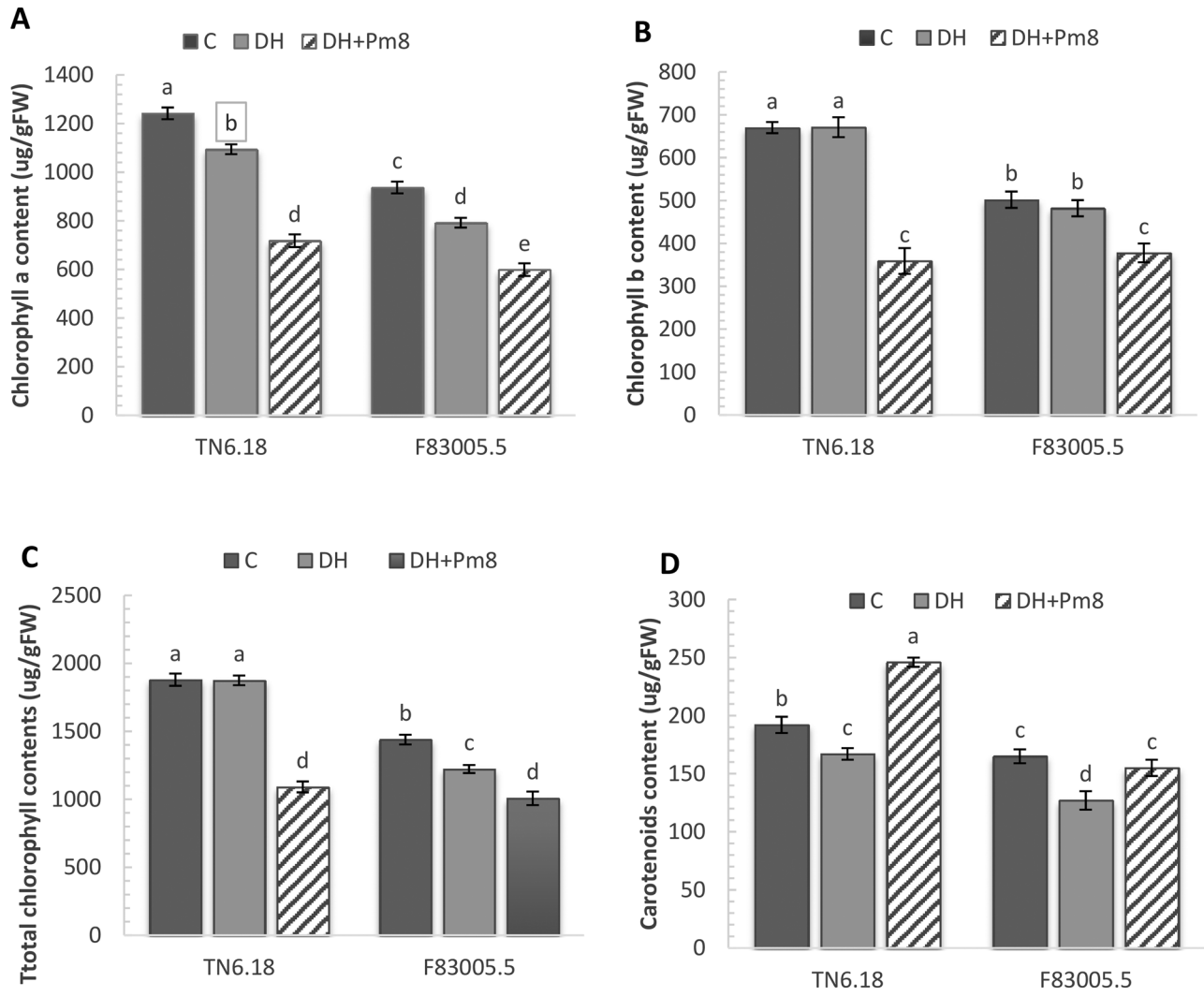

Figure 2. Mean values of chlorophyll a content (a), chlorophyll b content (B), total chlorophylls content (C), and carotenoids content (D) in two *Medicago truncatula* lines TN6.18 and F83005.5 cultivated under control conditions (C), drought stress (DH), and the combination of drought and infection with the *Phoma medicaginis* Pm8 strain (DH + Pm8). Means denoted by the same letter(s) is not significantly different among the studied lines for each trait, based on Duncan's test at the 5% significance level. The Duncan test was performed for each pigment for both lines separately. Values are averages of five replicates. The error bars correspond to standard errors.

biological variability, respectively (Figure 3). According to PC1, DH + Pm8–TN6.18, DH + Pm8- F83005.5, and DH-F83005.5 were separated from the rest of the treatments C-F83005.5, C-TN6.18, and DH-TN6.18. Most analyzed parameters positively correlated with PC1, except for transpiration rate (E), stomatal conductance (*gs*), and the number of chlorotic leaves (NCL), all of which showed a positive correlation with the F83005.5 line under both drought and combined stress conditions (Figure 3).

Under drought stress, TN6.18 exhibited the highest levels of photosynthesis rate (A), water use efficiency (*WUE*), and chlorophyll *a* and *b* content. The separation of inoculated plants from all other treatments confirms our previous findings that fungal attack was more deleterious than drought stress.

Chlorophyll fluorescence determinations

PSI and PSII activities in dark test

The dark test revealed a significant increase in the maximal quantum yield of PSII photochemistry (Fv/Fm) in TN6.18 under drought and combined drought and *P. medicaginis* infection, with a more pronounced increase in the latter case (0.86 compared to the control's 0.79) (Figure 4). Conversely, in F83005.5, Fv/Fm decreased, particularly under drought conditions (0.708 compared to the control's 0.768). In the sensitive F83005.5, similar patterns were observed for oxidized PSI (P700ox) and maximal fluorescence yield of dark-adapted sample with all PSI centres closed (P700m). However, the levels of P700ox and P700m decreased by 33.5% and 34.6%, respectively, under drought stress in F83005.5 compared to the control, but they returned to control levels under combined stress conditions. While, in the tolerant TN6.18, there was no significant difference in P700ox and P700m under the treatments.

PSI and PSII activities in light test

Concerning the light test, our overall analysis indicates that drought stress led to a more pronounced reduction in the operational photochemical yield (Y) and the electron transfer rate (ETR) in PSI and PSII in the sensitive line F83005.5 compared to TN6.18 which showed an increase in Y and ETR in

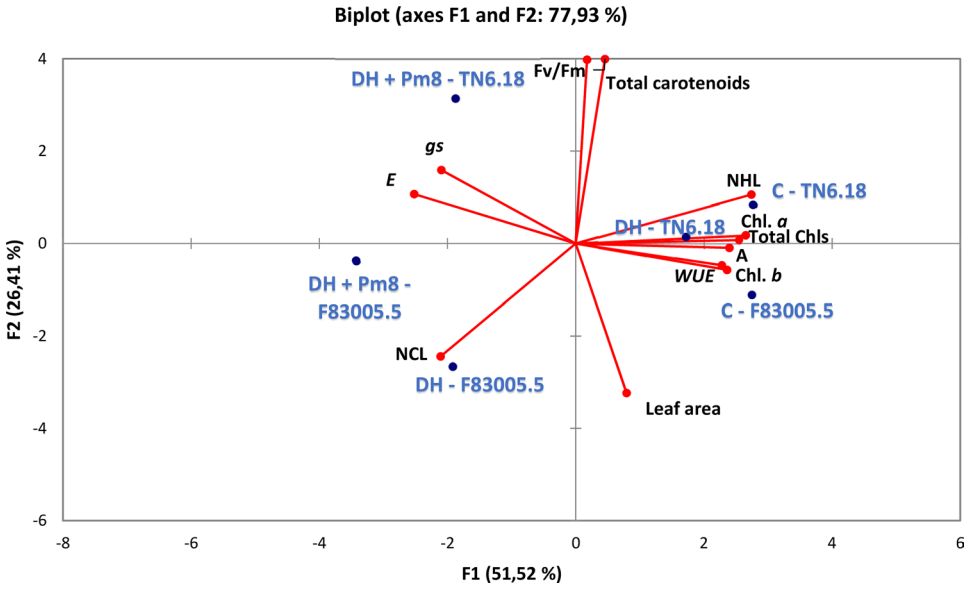

Figure 3. Two-dimensional plots generated by principal component Analysis (PCA) using all morphological and photosynthetic parameters in both lines of *Medicago truncatula*. C represents plants grown without drought stress and inoculation, DH represents plants grown under drought stress, and DH + Pm8 represents plants grown under drought conditions and infested with the *Phoma medicaginis* Pm8 strain.

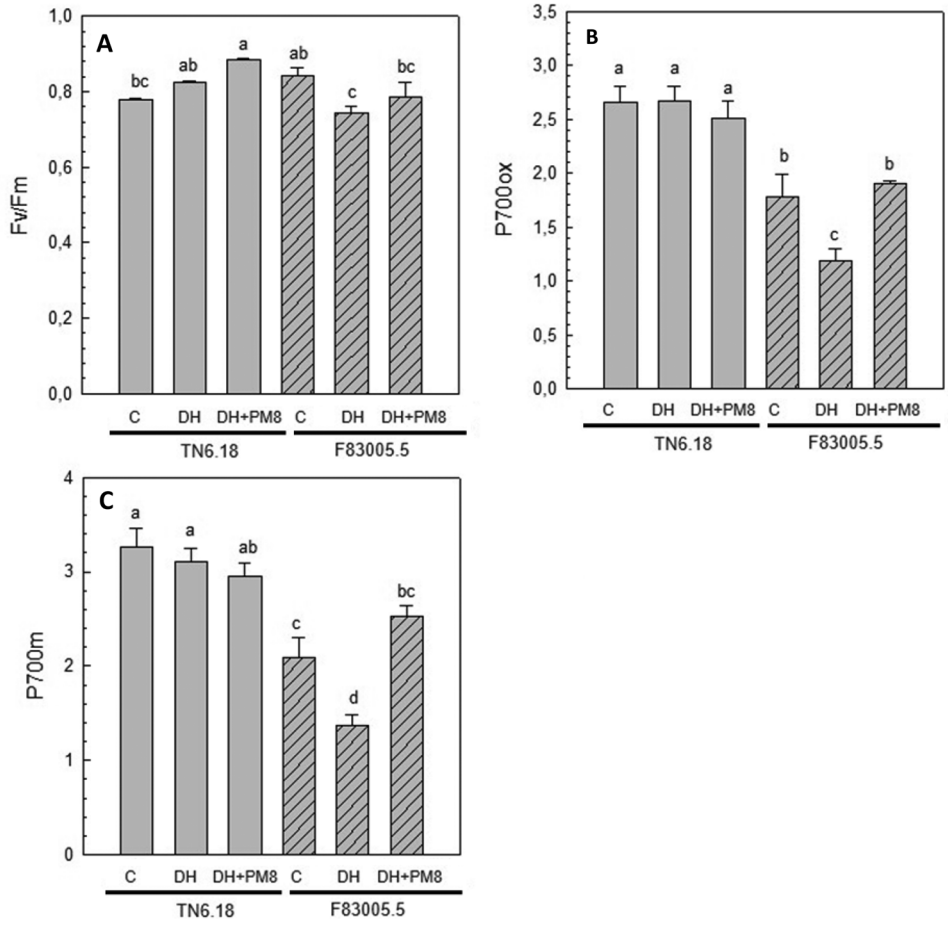

Figure 4. Effects of drought and combined drought and *Phoma medicaginis* infection on fluorescence parameters of PSII for dark-adapted leaves (dark test) in both lines of *Medicago truncatula*, TN6.18 and F83005.5. Fv/Fm represents the maximal quantum yield of PSII photochemistry (a), P700ox represents oxidized PSI (B), and P700m represents the maximal fluorescence yield of the dark-adapted sample with all PSI centers closed (C). C indicates plants grown without drought stress and without inoculation, DH indicates plants grown under drought stress, and DH + Pm8 indicates plants grown under drought conditions and infested with the *Phoma medicaginis* Pm8 strain. Values are averages of five replicates, and the error bars correspond to standard errors.

both PSI and PSII compared to control (Figures 5 and 6 (A), (B), (G), and (H)). The combined effects of drought and *P. medicaginis* were more evident in PSI and PSII across both lines. Notably, these results highlight that PSII was more adversely affected by both drought stress and combined drought and *P. medicaginis* infection.

The quantum yield of regulated non-photochemical energy dissipation [Y(NPQ)] increased proportionally to the rise in PAR. However, an antagonistic effect was observed under drought stress in both lines, with Y(NPQ) values being lower in TN6.18 and higher in F83005.5 than those of the control, respectively (Figure 5(C) (D)). Drought stress also elevated the quantum yield of non-regulated non-photochemical energy dissipation [Y(NO)] in TN6.18 (Figure 5(E)) and decreased it in F83005.5 (Figure 5(F)). When *P. medicaginis* infection coincided with drought, it further increased this unregulated energy loss, reaching values even higher than those of the control.

The quantum yield of non-photochemical energy dissipation in reaction centres limited by the acceptor side [Y(NA)] increased in both lines, whether subjected to drought stress alone or to combined stresses (Figure 6(E) (F)). In contrast, an increase in the quantum yield of non-photochemical energy dissipation in reaction centres limited by the donor side [Y(ND)] was recorded in F83005.5 under both treatments compared to the control. However, in the tolerant line TN6.18, a decrease in Y(ND) was observed under drought stress, with a subsequent lesser increase following *P. medicaginis* infection (Figure 6(C)). Drought stress adjusted the yields of both types of energy dissipation to levels even better in the tolerant TN6.18 [higher Y(NO) and lower Y(ND)] compared to the control. However, *P. medicaginis* infection in both drought-stressed lines resulted in higher energy dissipation, particularly more pronounced in the TN6.18 line than in F83005.5.

Drought and combined drought and *P. medicaginis* infection also induced a significant reduction in both P700ox (oxidized PSI) and P700m′ (maximal fluorescence yield of an illuminated sample with all PSI centres closed) in both lines compared to control and to drought-stressed plants. However, the decrease was more pronounced in TN6.18 (Figure 7(A) (C)) than in F83005.5 (Figure 7(B) (D)). This indicates that drought and combined stresses contributed to a reduction in PSI oxidation (Figure 7). Interestingly, an attenuation of this negative behavior was observed in the tolerant line TN6.18.

Discussion

Phoma medicaginis infection poses a significant constraint on various *Medicago* species cultivated in subarid conditions (Badri et al. 2023). Utilizing model plants like *M. truncatula* can enhance our comprehension of the effect of *P. medicaginis* infection on morphological and photosynthetic responses in two drought-stressed lines of *Medicago truncatula* known for their differing tolerance levels (Limami et al. 2014; Haddoudi et al. 2021; Maiza et al. 2021; Badri et al. 2023).

Our research indicates that the drought-tolerant TN6.18 line employs a morphological defense strategy under drought and combined drought and *P. medicaginis* infection characterized by a significant reduction in leaf area, a higher number of healthy leaves and a lower number of chlorotic leaves compared to the sensitive F83005.5. This aligns with studies demonstrating that under drought stress, plants exhibit a defense strategy by limiting the surface available for pathogen attachment and reproduction, ultimately protecting overall plant health and survival (Fang and Xiong 2015). Similar reductions in leaf size have been observed in various plant species, emphasizing its role as an indicator of resistance (Tribulato et al. 2019; Martins et al. 2020). In contrast, the sensitive F83005.5 line exhibits the higher leaf area compared to the tolerant TN6.18. These outcomes may be attributed to the decline in photosynthetic activity induced by drought stress and *P. medicaginis* infection, which disrupts leaf development, and leaf senescence (Pandey et al. 2017; Tribulato et al. 2019; Raza et al. 2023).

At the photosynthetic level, Plants respond to combined drought stress and *P. medicaginis* infection by adjusting water deficiency, directly influencing assimilation and transpiration leaves (Tribulato et al. 2019) to protect their organs from the negative effects of both water deficit stress and infections caused by *P. medicaginis* (Omidvari et al. 2022). Our results unveiled that drought stress doesn't affect the gas exchange parameters in the tolerant TN6.18. However, due to *P. medicaginis* infection, the drought-stressed TN6.18 line maintained the same level of photosynthesis rate (*A*) but showed an increased transpiration rate (*E*) and increased stomatal conductance (g_s), resulting in decreased water use efficiency (*WUE*). The increase in transpiration could be an adaptive mechanism to combat the pathogen and to increase the uptake of CO_2. However, this adaptation comes at the cost of using more water by opening its stomata, thereby decreasing water use efficiency. In fact, TN6.18 line managed to maintain its photosynthesis rate (*A*) despite the combined stresses of drought and fungal infection (Liu et al. 2015; Sinha et al. 2019).

The sensitive line F83005.5 demonstrated a similar decrease in photosynthetic rate (*A*), and water use efficiency (*WUE*) under drought stress and drought+ *P. medicaginis* infection compared to the control which may indicate that drought stress did not intensify the negative effects of dark stem pathogen (Pm8) on the gas-exchange parameters beyond the reductions seen in the drought-only treatment indicating no synergistic effects of drought and pathogen attack (Ghanbary et al. 2021). The decrease in photosynthesis rate *(A)* and water use efficiency *(WUE)* may be attributed to the accumulation of reactive oxygen species (ROS). According to Yang and Luo (2021), the inhibition of photosynthesis promotes ROS production and accumulation.

Leaf pigment content, including chlorophyll and carotenoids, serves as a crucial indicator of plant physiological condition and can be utilized to assess photosynthetic performance (Havaux 2014; Ramegowda and Senthil-Kumar 2015; Gómez-Sagasti et al. 2023). Both abiotic and biotic stresses frequently lead to a decrease in chloroplast numbers and the degradation of chlorophyll, resulting in chlorosis and necrosis in leaves, as observed in the present study (Dinis et al. 2011; Ramegowda and Senthil-Kumar 2015; Dikilitas et al. 2016; Martins et al. 2020; Ghanbary et al. 2021). Similar responses have been observed in different wheat varieties and *Pinus*

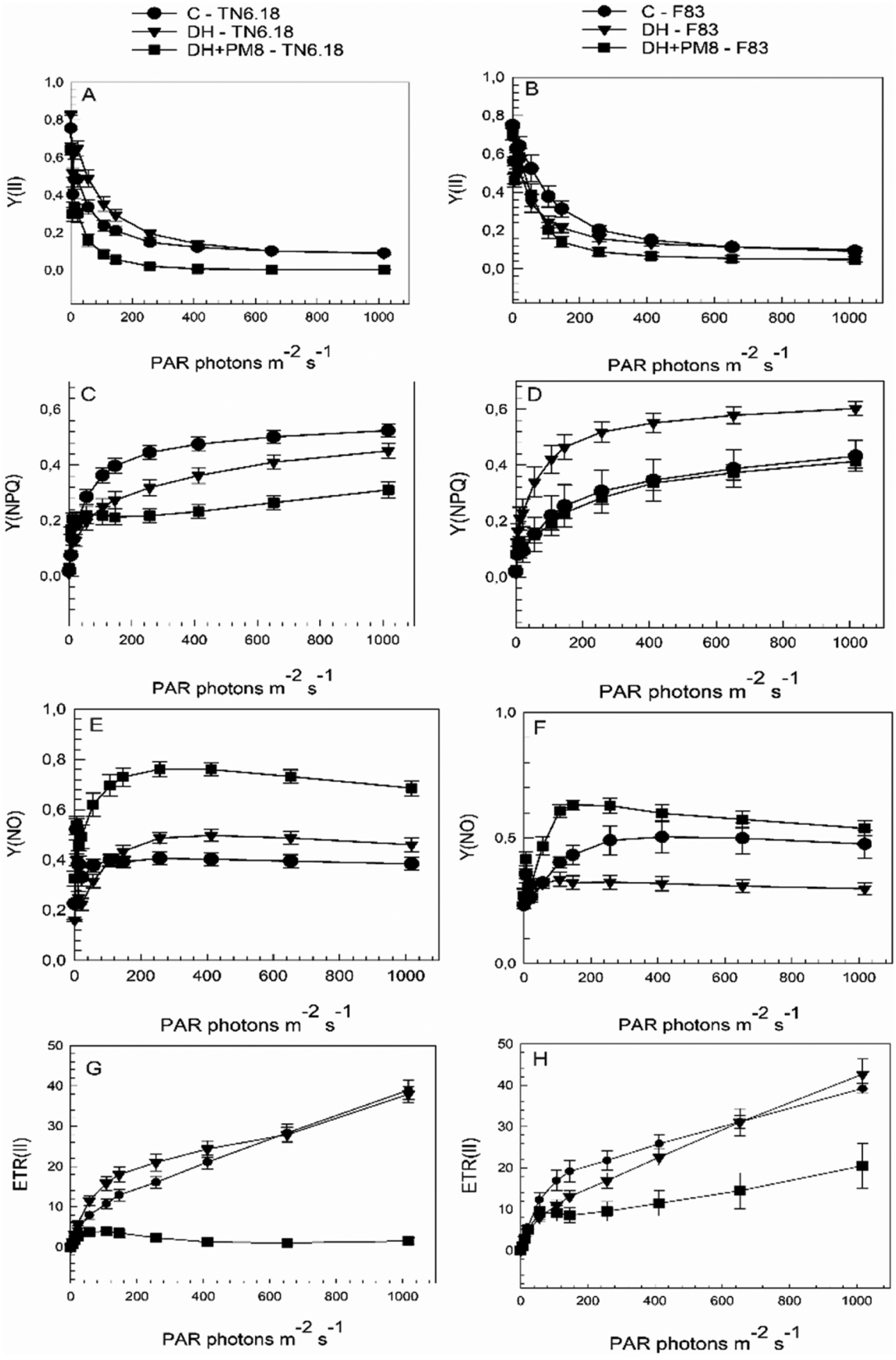

Figure 5. Means of fluorescence parameters of PSII from the light test for both lines of *Medicago truncatula*, TN6.18 and F83005.5 (F83), under control conditions (C), drought stress (DH), and a combination of drought and infection with the *Phoma medicaginis* Pm8 strain (DH + Pm8). Y(II) represents the quantum yield of photochemical energy conversion in PSII (A, B); Y(NPQ) represents the quantum yield of regulated non-photochemical energy dissipation in PSII (C, D); Y(NO) represents the quantum yield of non-regulated non-photochemical energy dissipation in PSII (E, F); and ETR represents the electron transfer rate (G, H) respectively in TN6.18 and F83005.5 (F83). PAR indicates photosynthetically active radiation. Values are averages of five replicates, and the error bars correspond to standard errors.

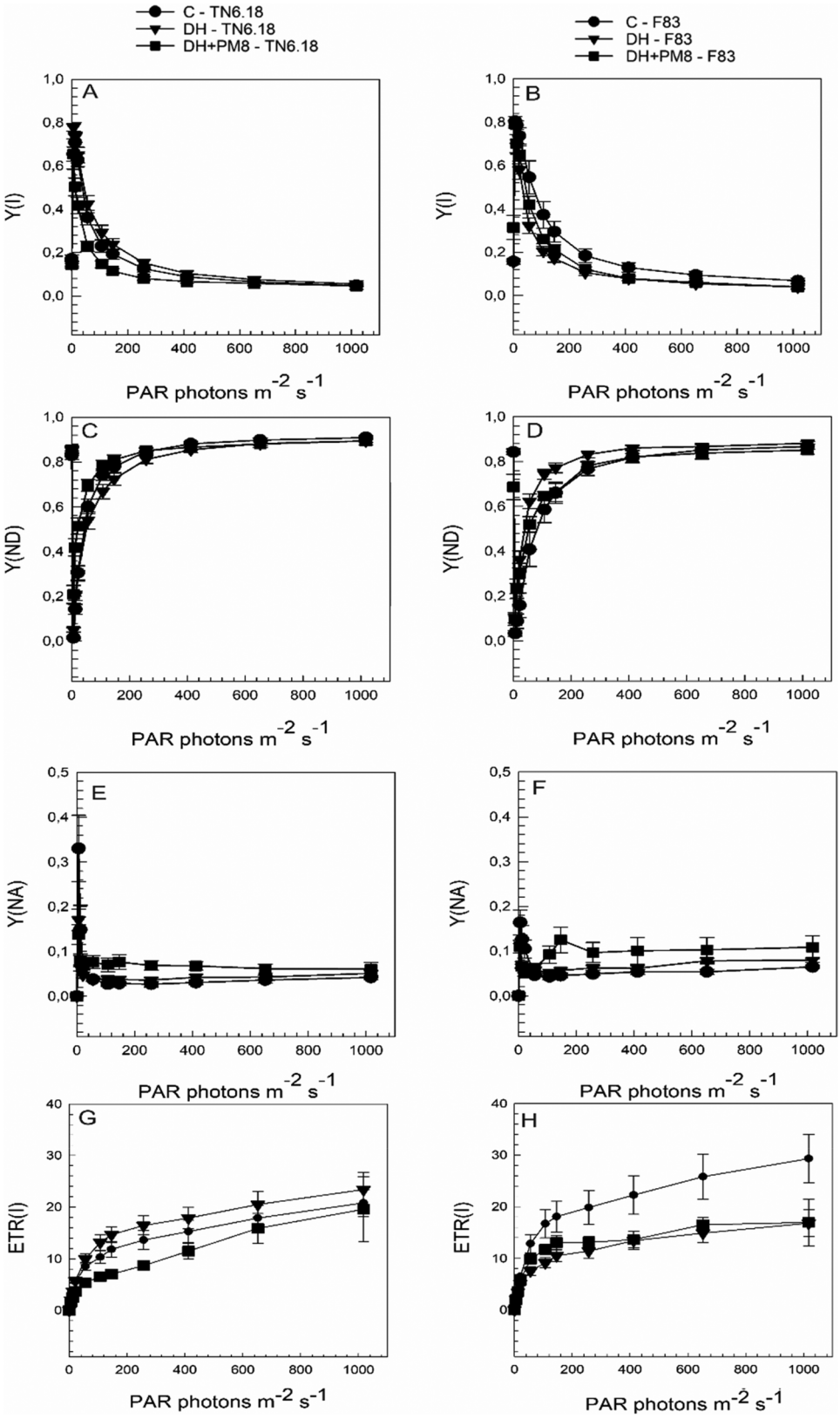

Figure 6. Means of fluorescence parameters of PSI for light-adapted leaves (light test) for both lines of *Medicago truncatula*, TN6.18 and F83005.5 (F83), under control conditions (C), drought stress (DH), and a combination of drought and infection with the *Phoma medicaginis* Pm8 strain (DH + Pm8). Y(I) represents the quantum yield of photochemical energy conversion in PSI (A, B); Y(ND) represents the quantum yield of non-photochemical energy dissipation in reaction centers limited by the donor side (C, D); Y(NA) represents the quantum yield of non-photochemical energy dissipation in reaction centers limited by the acceptor side (E, F); and ETR represents the electron transfer rate (G, H) respectively in TN6.18 and F83005.5 (F83). PAR indicates photosynthetically active radiation. Values are averages of five replicates, and the error bars correspond to standard errors.

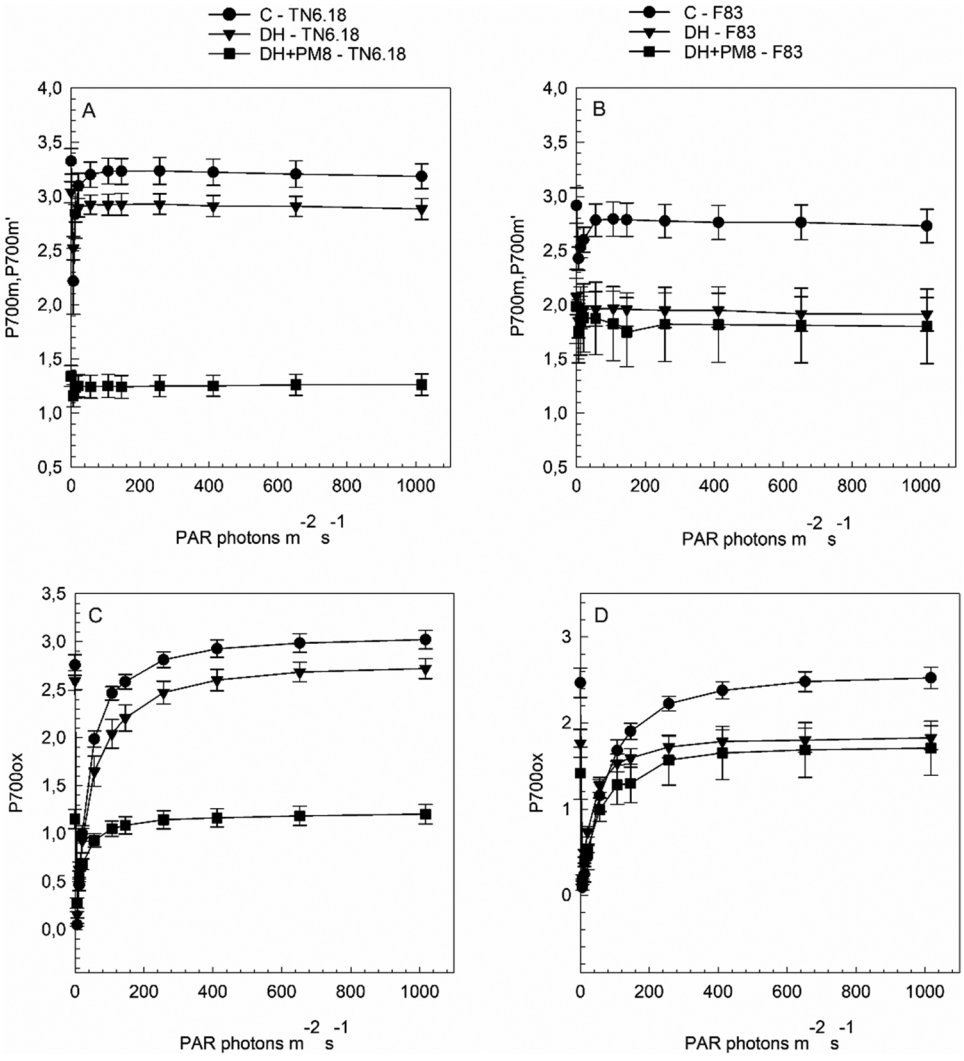

Figure 7. Means of fluorescence parameters of PSI for light-adapted leaves (light test) for both lines of *Medicago truncatula*, TN6.18 and F83005.5 (F83), under control conditions (C), drought stress (DH), and a combination of drought and infection with the *Phoma medicaginis* Pm8 strain (DH + Pm8). P700ox represents oxidized PSI (A, B); P700m represents the maximal fluorescence yield of the dark-adapted sample with all PSI centers closed; and P700m' represents the maximal fluorescence yield of the illuminated sample with all PSI centers closed (C, D). Values are averages of five replicates, and the error bars correspond to standard errors.

tabulaeformis Carr. under drought stress and pathogen infection (Mikaberidze and McDonald 2020; Zhou et al. 2021).

Conversely, the reduction in chlorophyll content was accompanied by an increase in carotenoid content in the tolerant TN6.18 line under combined drought and *P. medicaginis* infection compared to control. This increase may be linked to the plant's resistance to drought and pathogens, indicating an adaptive response to mitigate oxidative damage, protect the photosynthetic machinery, and maintain cellular homeostasis under adverse conditions (Havaux 2014; Ramegowda and Senthil-Kumar 2015; Martins et al. 2020; Gómez-Sagasti et al. 2023).

Our chlorophyll fluorescence results showed that the overall capacity of photosynthesis was significantly inhibited under combined drought and *P. medicaginis* infection compared to only-drought stress, impacting PSI and PSII activity (Nisar et al. 2015). The decrease in Y(I) and Y(II) in plants under combined drought and *P. medicaginis* infection indicate a more severe impact on photosynthetic activity compared to drought stress alone. The decrease in YII, ETR, and increase in NPQ were attributed to stomatal closure, reducing CO_2 supply, and protecting PSII from stress-induced photoinhibition. Chlorophyll fluorescence parameters, except Y(NPQ), were less affected in TN6.18, highlighting its resilience.

Moreover, the tolerant line TN6.18 exhibited a higher decrease of P700ox and P700m, P700m' under combined stresses compared to control and only-drought stress, suggesting a protective role against photo-oxidative damage to PSI. This reduction in PSI oxidation may contribute to safeguarding the photosynthetic apparatus in TN6.18 challenged with combined stresses (Shimakawa and Miyake 2018). Additionally, an increase in the Fv/Fm ratio suggests an improvement of the efficiency of photosystem II under stress conditions (Signorelli et al. 2013; Umar et al. 2019; Borisova-Mubarakshina et al. 2020). In our case, the tolerant line TN6.18 exhibits a higher Fv/Fm ratio when subjected to both drought stress and pathogen attack compared to control conditions. This could indicate that the tolerant line is better able to maintain the functionality of PSII and cope with the combined stresses, suggesting a more adaptive

response to the simultaneous challenges of drought and pathogen attack (Guidi et al. 2019; Pérez-Bueno et al. 2019). In contrast, the sensitive line F83005.5 demonstrated a reduction of photosystem II (PSII) efficiency, evidenced by lower levels of YII, ETRII, and FV/FM, along with increased Y(NPQ), which led to a reduced photosynthesis rate (Pérez-Bueno et al. 2019).

Divergent behavior between PSI and PSII implies that these two photosystems respond differently to the combined stress of drought and pathogen attack. It suggests that, under the influence of both drought stress and pathogen attack, one photosystem may be more affected to a lesser extent compared to the other (Borisova-Mubarakshina et al. 2020; Tan et al. 2020). In our results, PSII was more sensitive to combined drought and pathogen attack under the moderate light than PSI in *M. truncatula* lines because of a higher energy dissipation (loss) in PSII than in PSI.

Conclusion

In conclusion, our study reveals that drought stress adversely affects the water status, photosynthetic activity, and gas exchange parameters in the sensitive line of *M. truncatula* (F83005.5). These negative effects are even more pronounced when drought-stressed plants are inoculated with *P. medicaginis*. In contrast, the drought-stressed TN6.18 line shows superior physiological performance and a greater ability to mitigate fungal attacks. This resilience is likely due to morphological and physiological strategies such as reduced leaf area, chlorophyll degradation, increased carotenoid content, enhanced PSI and PSII performance, and lower oxidative damage.

The F83005.5 line is more affected by *P. medicaginis*, exhibiting a greater reduction in healthy leaves, an increase in chlorotic leaves, higher PSI oxidation, and higher energy dissipation in both PSI and PSII. Interestingly, PSI performance under combined drought and pathogen attack shows lower damage compared to PSII.

These findings highlight the potential of the TN6.18 line to withstand combined drought and fungal stress, making it a promising candidate for cultivation in Mediterranean regions. This could offer significant socio-economic benefits by enhancing crop resilience and yield under challenging environmental conditions.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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