



# The combination of abiotic stresses influences the physiological responses and production of *Macroptilium* genotypes

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## Abstract

Combined abiotic stress may harm forage yield due to the direct effects on physiological and production processes. Therefore, this study assessed the development of *Macroptilium* genotypes native to the Brazilian semi-arid region under the combination of water deficit and temperature in crop seasons. The experiments were conducted in 2021 and 2022, using five *Macroptilium* genotypes and combined water and temperature stress, in factorial  $5 \times 4 \times 2$  with five genotypes, four water availabilities (25, 50, 75, and 100% field capacity) and crop seasons (Season 1–high temperature and Season 2–temperature moderate) with four replications. Physiological analyses were performed 30 days after planting, aided by a portable infrared gas analyzer and Chlorophyllometer. Next, cutoffs were made for production assessments (leaf and shoot dry mass, leaf shoot dry mass content, plant dry mass, forage accumulation rate, and water use efficiency). The data were subjected to analysis of variance by the Scott-Knott test and regression for water availability. The interplay between water availability and crop season temperatures exerted a notable influence on the physiological, biometric, and production characteristics of *Macroptilium* genotypes. Opting for the warmest season for planting proved to be advantageous in enhancing the yield of the genotypes under scrutiny, thereby elevating water use efficiency. Notably, the A59 and L34 genotypes exhibited superior plant dry mass and forage accumulation when exposed to higher temperatures and less than 100% water availability. These findings suggest that A59 and L34 may hold promise as prospective cultivars of *Macroptilium* sp. with a heightened tolerance to combined abiotic stresses.

**Keywords** Crop physiology · Native plants · Climate change · Temperature · Deficit hydric · Forage legumes

## Introduction

Higher temperatures and alterations in precipitation patterns from climate changes may harm plant yield due to the direct effects on physiological processes and water use efficiency (Perdomo et al., 2015; Norton et al., 2016; Angelotti et al. 2022). The Intergovernmental Panel on

Climate Change—IPCC (2021) stated that these conditions may become more severe because scenarios indicate increases of up to 5.7 °C in average air temperature by 2081–2100 due to higher greenhouse gas concentrations. Furthermore, the occurrence of droughts combined with heat waves may increase under climate change scenarios (IPCC, 2021), highlighting the importance of investigating the metabolic response of plants to multiple stress factors to improve crop productivity (Zandalinas et al., 2022). Combinations of abiotic stresses cause damage to crop growth and yield, requiring plants to adapt at metabolic, physiological, and molecular levels (Suzuki et al. 2016; Priya et al., 2023). This is a complex challenge for scientific research, but an interactive platform with information about plant responses to combined stress, called Stress Combinations and their Interactions in Plants Database

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(<http://www.nipgr.ac.in/scipdb.php>) was developed to help understand this subject (Priya et al., 2023).

Climate changes in the Brazilian semi-arid region emerge as an additional concern because this area presents adverse environmental conditions with mean annual temperature between 23 and 27 °C, accentuated drought cycles, irregular rainfall, and water availability (annual precipitation) below 800 mm (Moura et al., 2019). These conditions are a warning for agricultural production and should be used for new technological developments (Angelotti et al., 2020).

Among the challenges from these events are the impacts of forage plant development for animal feed on livestock production. Thus, maintaining forage offering in climate change conditions by selecting adapted species that guarantee animal feed in periods of abiotic stress will be highly significant for livestock maintenance (Voltolini and Gois 2022). Native forage species become an alternative to forage offerings as an adaptation mechanism to abiotic stresses (Oliveira et al., 2022). *Macroptilium* plants stand out among these species, adapting to edaphoclimatic conditions of the Brazilian semi-arid region and serving as animal food supply due to their considerable protein content (Borges et al., 2018). Genetic diversity studies under conditions of increasing temperature have been carried out for *Macroptilium* (Nascimento et al., 2023). However, in the field stresses are not individualized and occur simultaneously (Obata et al., 2015). Therefore, to develop plants with better adaptation to field conditions, it is necessary to understand plant responses to combined stress conditions (Pandey et al., 2015; Priya et al., 2023; Zandalinas et al., 2022).

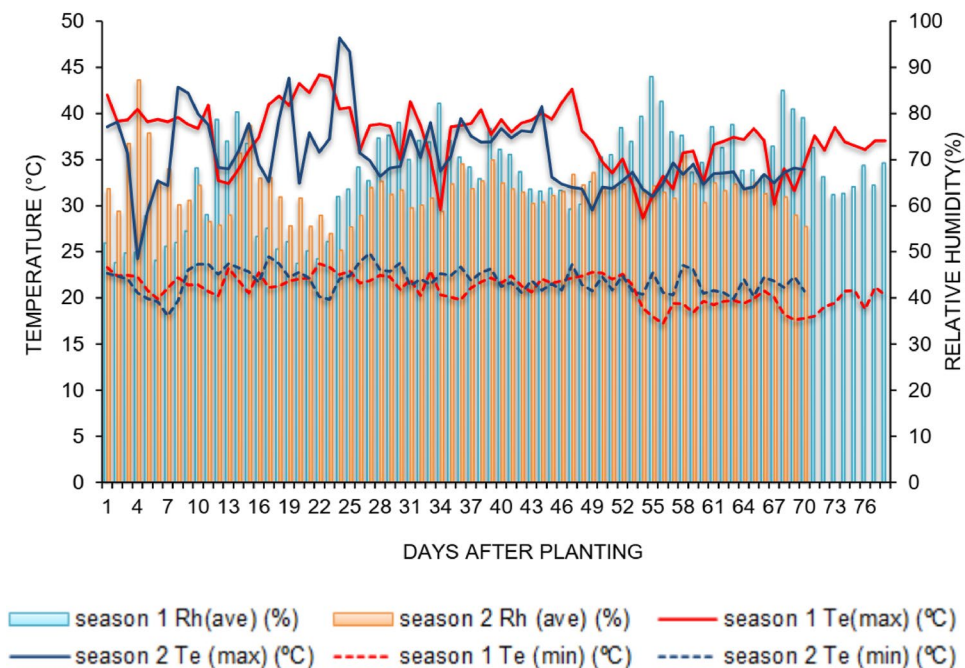
When different types of stress occur simultaneously, this leads to the implementation of stress adaptation strategies that can be distinct from, and sometimes opposite to, those observed in isolated stress situations (Pandey et al., 2015; Priya et al., 2023). This highlights the importance of investigating the physiological responses of plants to multiple stress conditions to understand the tolerance capacity of plants. Thus, the main objective of the current study was to assess the physiological and productive response of *Macroptilium* genotypes under combined abiotic stresses.

## Material and methods

The experiment was performed in a greenhouse located at Embrapa Semi-arid in Petrolina, PE, Brazil (latitude 9° 8' 8.9" S, longitude 40° 18' 3.6" W, altitude 373 m). Two experiments were planted at different times of the year, applying four levels of water availability. For planting in November 2021 (Season 1–high temperature), the maximum temperature was 37.45 °C, the minimum was 20.97 °C and the relative humidity was 86%. For planting in May 2022 (Season 2–temperature moderate), the maximum temperature was 35.36 °C, the minimum was 21.88 °C and the relative humidity was 78%. The climate was monitored by an automatic meteorological station installed at the experiment location (Fig. 1).

The tolerance of *Macroptilium* genotypes to abiotic stresses was analyzed based on the combination of thermal and water stresses. The experiment design was randomized blocks conducted in a 2×5×4 factorial arrangement, in two

**Fig. 1** Daily average values of maximum temperature ( $T_e$  (max) (°C)), minimum temperature ( $T_e$  (min) (°C)) and relative humidity (Rh (mean) (%)), in two crop seasons (Season 1: November 2021 (high temperature), Season 2: May 2022 (moderate temperature))



crop seasons (November 2021 and May 2022), and with five *Macroptilium* sp. genotypes (A15, A59, and A9 for *M. atropurpureum*; L34 for *M. lathyroides*; and S4 for *Macroptilium* sp.), and four water availabilities (25, 50, 75, and 100% soil water retention capacity), with four replications.

The genotypes were seeded in plastic trays containing a commercial substrate, and one seed was used per cell in the tray. Fifteen days after emergence, the seedlings of each genotype were transplanted into pots with a capacity of 5 L, with soil collected in the 0–20 cm layer, whose chemical and granulometric characteristics were determined according to the methodologies recommended by Donagemma et al. (2017).

Fertilization occurred three days before transplantation with the super simple, and ten days later, nitrogen fertilization was performed with 10 ml per pot A TDR (Time Domain Reflectometry) model TDR100, from Campbell assisted irrigation management. Coaxial probes with three rods were used, and the TDR was calibrated according to Batista et al. (2016). Irrigation system applications started 20 days after transplantation. Irrigation occurred every two days by replacing the evapotranspiration water volume to maintain the water availability of each treatment based on the generated TDR data.

The physiological assessments occurred 30 days after transplantation, between 9 a.m. and 11 a.m. in both crop seasons, using the third trifoliate leaf completely open. Gas exchanges were evaluated with a portable infrared gas analyzer (IRGA), model Li-6400, using an artificial light fixed at  $2500 \mu\text{mol m}^{-2} \text{s}^{-1}$ . The analyzed variables were photosynthesis rate ( $A$ ), stomatal conductance ( $g_s$ ), transpiration ( $E$ ), and leaf temperature ( $T_f$ ). Chlorophyll content was determined from the relative chlorophyll index aided by a portable chlorophyll meter, model CFL 1030 FALKER.

Production was evaluated when the plants started releasing pods by cutting the plant mass and separating the shoot from the leaves, which were packed in labeled paper bags and maintained in an oven at constant  $65^\circ\text{C}$  until stabilizing weight. After drying, the leaf and shoot dry mass was weighed with an analytical scale, and their dry mass content was determined with equations:

$$TLDM = LDM * \frac{100}{PDM} \quad TSDM = SDM * \frac{100}{PDM} \quad (1)$$

where  $LDM$  Leaf dry mass.  $SDM$  Shoot dry mass.  $PDM$  Plant dry mass.

Plant dry mass was determined with:

$$PDM = LDM + SDM \quad (2)$$

where:  $LDM$  Leaf dry mass.  $SDM$  Shoot dry mass.

These data allow establishing the forage accumulation rate (FAR) with:

$$FAR = \frac{PDM}{DAYS OF CYCLE \quad DURATION} \quad (3)$$

where  $PDM$  Plant dry mass.

Water use efficiency (WUE) of the tested materials for each soil water availability was determined with the equation:

$$WUE = \frac{PDM}{WC} \quad (4)$$

where  $PDM$  Plant dry mass.  $WC$  Water consumption of each genotype.

The results were subjected to an analysis of variance. When significance occurred, a regression analysis was performed to assess water availability, and the means of the other variables were compared by the Scott-Knott test using SISVAR software, version 5.6.

## Results

The interaction between combined abiotic stresses and genotypes was significant for the physiological variables of stomatal conductance, photosynthesis, and transpiration. Regarding stomatal conductance, water availability between 82 and 100% increased stomatal openings by 0.37, 0.24, 0.25, 0.42, and 0.37  $\text{mol H}_2\text{O m}^{-2} \text{s}^{-1}$  in A9, A15, A59, L34, and S4 genotypes, respectively, when combined with the planting season 1, with higher temperatures. S4 and L34 genotypes presented greater stomatal openings at 75% water availability, with mean values of 0.36 and 0.37  $\text{mol H}_2\text{O m}^{-2} \text{s}^{-1}$ , respectively. There were no regression model adjustments in the season from May to August 2022, and the means were represented in Table 1 and Supplementary Table S1.

As for photosynthesis, planting in 2021 combined with a water deficit of 25% negatively affected the photosynthetic activity of the evaluated genotypes (Table 1). At the planting time with higher temperatures (November), plants with greater stomatal opening showed greater photosynthetic activity (Table 1). It was observed that thermal stress (season 1) combined with water availability of 81, 53, 84, 83 and 79% of the water retention capacity in the soil contributed to the increase in the photosynthetic rate of genotypes A9, A15, A59, L34 and S4, respectively. A9, A59, L34, and S4 genotypes showed higher photosynthetic rates when the increase in temperature was combined with 50% water availability, with average values of 39, 38, 45, and 41 micromole  $\text{CO}_2 \text{m}^{-2} \text{s}^{-1}$ , respectively. L34 and S4 genotypes presented means of 52 and 49 micromole  $\text{CO}_2 \text{m}^{-2} \text{s}^{-1}$ , respectively, at 75% water availability and higher temperatures (Table 1).

The combined stresses, with water availability of 66.50% in season with temperature moderate (season 2), only the S4

**Table 1** Stomatal conductance ( $g_s$ ) and photosynthetic activity ( $A$ ) of *Macroptilium* genotypes as a function of the combination of thermal and water stress

		Stomatal conductance ( $g_s$ )					Photosynthesis ( $A$ )				
		Water availability (%)					Water availability (%)				
	Genotypes	25	50	75	100	$R^2$ (%)	25	50	75	100	$R^2$ (%)
Season 1 (high temperature)	A9	0.06Ab	0.29Aa	0.32Ab	0.37Ab	95.15**	23.04Aa	39.3Ab	40.65Ab	41.42Ab	95.53**
	A15	0.11Aa	0.27Aa	0.16Ac	0.26Ac	34.52**	26.79Aa	15.99Ac	26.83Ac	35.66Ac	85.61**
	A59	0.06Ab	0.24Aa	0.29Ab	0.33Ab	97.79**	29.46Aa	38.79Ab	40.28Ab	41.21Ab	97.01*
	L34	0.05Ab	0.27Aa	0.37Aa	0.42Aa	99.81**	25.06Aa	45.76Aa	52.08Aa	52.13Aa	99.33**
	S4	0.09Aa	0.28Aa	0.36Aa	0.34Ab	99.89**	27.48Aa	41.11Ab	49.65Aa	45.04Ab	98.81**
Season 2 (moderate temperature)	A9	0.03Aa	0.02Ba	0.04Ba	0.05Ba	–	4.43Ba	4.35Ba	5.73Ba	6.51Ba	–
	A15	0.02Ba	0.03Ba	0.02Ba	0.03Ba	–	1.87Ba	5.39Ba	4.02Ba	4.72Ba	–
	A59	0.03Aa	0.02Ba	0.02Ba	0.02Ba	–	4.84Ba	2.89Ba	4.76Ba	4.5Ba	–
	L34	0.03Aa	0.03Ba	0.02Ba	0.03Ba	–	0.99Ba	7.39Ba	3.96Ba	5.44Ba	–
	S4	0.02Ba	0.04Ba	0.04Ba	0.02Ba	–	1.51Ba	7.18Ba	7.21Ba	3.902Ba	98.85*

Means followed by the same upper-case letter in the column for crop season and lower-case letter in the column between crop seasons for genotypes do not differ by the Scott–Knott test at 5% probability. Significant regression coefficient when  $p < 0.01$  (\*\*) and  $p < 0.05$  (\*)

genotype showed a significant effect, with a higher photosynthetic rate. The other genotypes did not show adjustments in the regression model in this season and were represented by the means (Table 1).

The transpiration rate of A15 and L34 genotypes was higher when high temperature (season 1) was combined with 50% water availability, with mean values of 5.84 and 6.29 mmol H<sub>2</sub>O m<sup>2</sup> s<sup>−1</sup> respectively (Table 2). In L34 and S4 genotypes the higher transpiration rates occurred with 75% water availability combined with higher temperatures, with mean values of 6.31 and 6.29 mmol H<sub>2</sub>O m<sup>2</sup> s<sup>−1</sup>, respectively. The means did not adjust to regression models in season 2 and were represented by the mean value (Table 2).

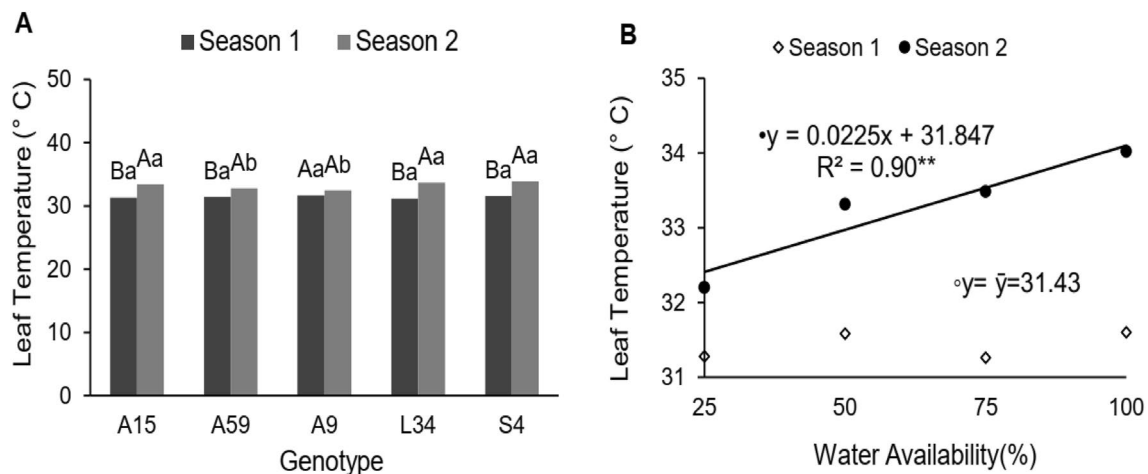
The combination of abiotic stresses and genotypes affected leaf temperature, which was higher in the May 2022 crop for all *Macroptilium* genotypes (Fig. 2A). As for water availability, with temperatures from the November crop (season 1), it did not show regression model adjustments, which were represented by the means. The combination of May crop temperatures (season 2) with the water availability detected a linear increase in leaf temperature is observed (Fig. 2B) due to the decrease in transpiration (Table 2).

The combination of abiotic stresses and genotypes was significant for the total chlorophyll index. The high temperature (season 1) did not interfere with the chlorophyll index of A15, A59 and A9 genotypes (Fig. 3A). The chlorophyll index

**Table 2** Transpiration rate of *Macroptilium* genotypes as a function of the combination of thermal and water stress

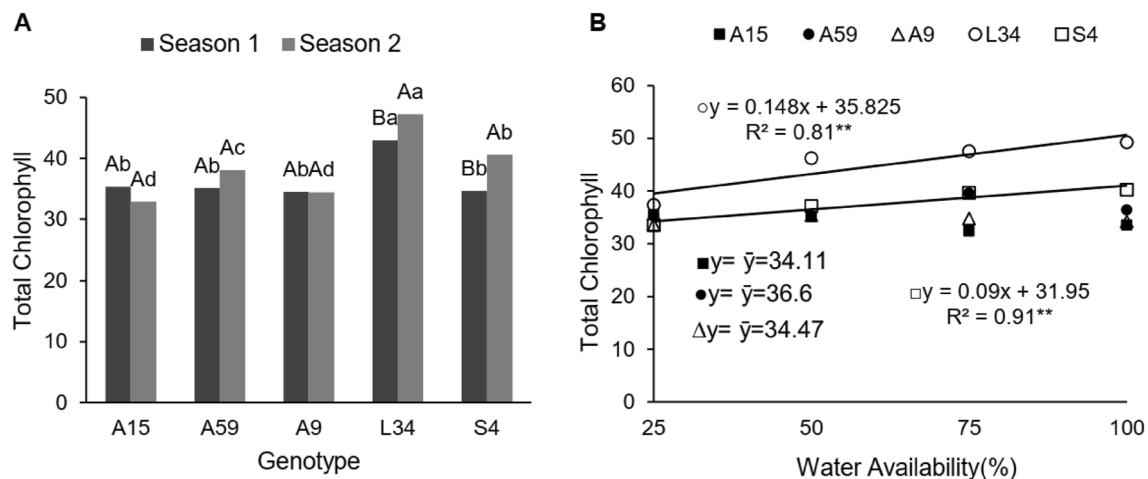
Water availability (%)						
Genotype	25	50	75	100	Equation	R <sup>2</sup> (%)
<i>Season 1 (High temperature)</i>						
A9	1.55Ab	5.16Ab	4.99Ab	6.34Ab	$y = -0.000906x^2 + 0.170x - 1.868$	89.09**
A15	2.38Aa	5.83Aa	2.52Ac	5.06Ac	$y = 0.018x + 2.777$	11.93**
A59	2.35Aa	4.40Ab	4.81Ab	6.11Ab	$y = 0.046x + 1.502$	93.52**
L34	1.00Ab	6.29Aa	6.31Aa	7.55Aa	$y = -0.001618x^2 + 0.281x - 4.686$	91.73**
S4	2.01Aa	4.91Ab	6.29Aa	6.34Ab	$y = -0.001140x^2 + 0.200x - 2.265$	99.98**
<i>Season 2 (Moderate temperature)</i>						
A9	0.67Aa	1.03Ba	0.91Ba	1.13Ba	$\bar{y} = 0.80$	–
A15	0.52Ba	0.9Ba	1.06Ba	1.07Ba	$\bar{y} = 0.89$	–
A59	0.65Ba	0.9Ba	1.05Ba	0.97Ba	$\bar{y} = 0.94$	–
L34	0.54Aa	0.59Ba	1.31Ba	0.75Ba	$\bar{y} = 0.98$	–
S4	0.69Ba	1.49Ba	0.93Ba	0.81Ba	$y = 0.90$	–

Means followed by the same upper-case letter in the column for crop season and lower-case letter in the column between crop seasons for genotypes do not differ by the Scott–Knott test at 5% probability. Significant regression coefficient when  $p < 0.01$  (\*\*) and  $p < 0.05$  (\*)



**Fig. 2** **A** Effect of thermal stress on leaf temperature of *Macropitium* genotypes, in which Season 1 (high temperature) and Season 2 (moderate temperature). The same lowercase (temperature) and uppercase letters (genotypes) belong to the same grouping, according to the Scott-Knott test ( $p < 0.05$ ). Values represent means of four biological

replicates. **B** Linear regression relationship between leaf temperature and water availability in two crop seasons, in which Season 1 (high temperature) and Season 2 (moderate temperature). Regression coefficient was significant at  $p < 0.01$  (\*\*). Values represent means of four biological replicates



**Fig. 3** **A** Effect of thermal stress on the total chlorophyll content of *Macropitium* genotypes, in which Season 1 (high temperature) and Season 2 (moderate temperature). The same lowercase (temperature) and uppercase letters (genotypes) belong to the same grouping, according to the Scott-Knott test ( $p < 0.05$ ). Values represent means

of four biological replicates. **B** Linear regression relationship between total chlorophyll and water availability of *Macropitium* genotypes. Regression coefficient was significant at  $p < 0.01$  (\*\*). Values represent means of four biological replicates

presented a linear increase according to water availability for L34 and S4 genotypes (Fig. 3B), and the remaining ones did not show regression adjustments and were represented by the means (Fig. 3B).

The combination of abiotic stresses and genotypes was significant for leaf, shoot and plant dry mass; leaf and shoot dry mass content (%); forage accumulation rate ( $\text{g day}^{-1}$ ); and water use efficiency.

Water restriction, with 25% availability, reduced leaf dry mass production in the 2021 and 2022 seasons (Table 3; Supplementary Table S3) A9, A59 and S4 genotypes had

higher leaf dry mass in response to 134, 91 and 88% water availability, respectively, when combined with temperatures in planting season 1 (November), with production means of 6.79, 5.80 and 4.43 g. The other genotypes showed a linear response for leaf dry mass production (Table 5). The combination of temperatures with the 70% water availability helped to increase leaf dry mass in the A9 genotype, with a mean peak value of 2.76 g (Table 5). The other genotypes presented linear behavior, and only S4 did not adjust to regression models and was represented by the means (Table 3).



**Table 3** Leaf and shoot dry mass (LDM and SDM) of *Macroptilium* genotypes as a function of the combination of thermal and water stress

	Genotypes	LDM					SDM				
		Water availability (%)					Water availability (%)				
		25	50	75	100	R <sup>2</sup> (%)	25	50	75	100	R <sup>2</sup> (%)
Season 1 (High temperature)	A9	0.38Ba	3.6Ab	4.42Ab	6.35Ab	96.74**	0.75Ac	3.7Ad	4.77Ac	6.29Ab	98.33**
	A15	0.78Aa	4.9Aa	3.64Ac	7.52Aa	76.43**	1.13Ab	4.45Ac	4.62Ac	6.18Ab	92.36**
	A59	0.62Aa	3.59Ab	5.63Aa	5.67Ac	99.66**	1.28Ab	5.50Ab	7.66Aa	6.75Ab	99.78**
	L34	0.96Aa	2.35Ac	2.45Ad	3.69Ac	92.00**	2.14Aa	7.18Aa	3.59Ad	4.80Ac	33.93**
	S4	0.51Aa	2.99Ac	4.24Ab	4.31Ac	100.00**	0.39Ac	3.39Ad	5.49Ab	7.42Aa	99.90**
Season 2 (Moderate temperature)	A9	1.22Aa	2.23Ba	2.96Ba	1.98Ba	93.35**	1.26Aa	2.23Ba	2.26Ba	1.51Bb	99.81**
	A15	0.76Aa	0.61Bb	2.15Bb	1.52Bb	47.65**	0.65Aa	0.39Bc	1.42Bb	1.45Bb	68.05**
	A59	0.99Aa	1.31Bb	2.13Bb	2.35Ba	95.01**	1.15Aa	2.6Ba	2.7Ba	2.47Ba	96.66**
	L34	1.18Aa	1.63Ba	2.71Aa	2.61Ba	85.62**	1.08Ba	1.34Bb	2.81Ba	2.97Ba	88.75**
	S4	0.69Aa	1.16Bb	1.01Bc	0.93Bb	–	0.78Aa	1.56Bb	0.7Bc	1.02Bb	–

Means followed by the same upper-case letter in the column for crop season and lower-case letter in the column between crop seasons for genotypes do not differ by the Scott-Knott test at 5% probability. Significant regression coefficient when  $p < 0.01$  (\*\*) and  $p < 0.05$  (\*)

For shoot dry mass in the November crop (season 1), L34 and A59 genotypes presented more mass at 50 and 75% water availability combined with higher temperatures, with means of 7.18 and 7.67 g, respectively. When season 1 temperatures were combined with 50% water availability, A9 and A59 genotypes showed more shoot dry mass, with mean values of 2.23 and 2.60 g, respectively. In the same season, A59 and L34 genotypes presented more shoot dry mass at 75% water availability, with mean values of 2.70 and 2.81 g (Table 3). The shoot dry mass in A9 and A59 genotypes increased due to the combination of temperatures in season 2 (May) with 64 and 74% water availability with means of 2.36 and 2.85 g, respectively. A15 and L34

genotypes presented linear growth and S4 did not adjust to regression models (Table 3).

The L34 genotype presented a higher dry mass of plants than the others, with the combination of temperatures from crop season 1 (November) with 25% water availability (Table 4). The 129, 148, 84, 78, and 114% water availability combined with the temperatures of the same season represented the peak of plant dry mass production for A9, A15, A59, L34 and S4 genotypes, respectively, meaning that A59 and L34 did not require 100% water availability to increase plant dry mass. The same occurred in season 2 (May crop) for A9 and A59 genotypes when temperatures were combined with 67 and 87% water availability favored

**Table 4** Plant dry mass of *Macroptilium* genotypes as a function of the combination of thermal and water stress

Genotype	Water availability (%)				Equation	R <sup>2</sup> (%)
	25	50	75	100		
<i>Season 1 (High temperature)</i>						
A9	1.13Ab	7.3Ab	9.2Ab	12.64Ab	$y = -0.001093 \times 2 + 0.282395x - 4.953125$	97.58**
A15	1.92Ab	9.35Aa	8.26Ac	13.7Aa	$y = -0.000798 \times 2 + 0.236710x - 2.738750$	84.03**
A59	1.90Ab	9.10Aa	13.30Aa	12.42Ab	$y = -0.003231 \times 2 + 0.546965x - 9.854375$	99.73**
L34	3.10Aa	9.53Aa	6.05Ad	8.50Ac	$y = -0.001590 \times 2 + 0.249610x - 1.346250$	48.91**
S4	0.90Ab	6.39Ab	9.73Ab	11.74Ab	$y = -0.001390 \times 2 + 0.317170x - 6.112500$	99.95**
<i>Season 2 (Moderate temperature)</i>						
A9	2.48Ba	4.46Ba	5.22Ba	3.49Bb	$y = -0.001485 \times 2 + 0.200875x - 1.676875$	98.13**
A15	1.41Aa	1.01Bc	3.57Bb	2.98Bb	$y = 0.029060x + 0.430000$	58.36**
A59	2.14Aa	3.91Ba	4.83Ba	4.82Ba	$y = -0.000714 \times 2 + 0.125110x - 0.542500$	99.99**
L34	2.27Aa	2.97Bb	5.52Aa	5.58Ba	$y = 0.050010x + 0.962500$	88.13**
S4	1.47Aa	2.72Bb	1.71Bc	1.96Bc	$\bar{y} = 3.23$	—

Means followed by the same upper-case letter in the column for crop season and lower-case letter in the column between crop seasons for genotypes do not differ by the Scott-Knott test at 5% probability. Significant regression coefficient when  $p < 0.01$  (\*\*) and  $p < 0.05$  (\*)

**Table 5** Leaf and shoot dry mass content (%) of *Macroptilium* genotypes as a function of the combination of thermal and water stress

		Leaf LDM (%)					Leaf SDM (%)				
		Water availability (%)					Water availability (%)				
	Genotypes	25	50	75	100	R <sup>2</sup> (%)	25	50	75	100	R <sup>2</sup> (%)
Season 1 (high temperature)	A9	31.02Bc	49.25Aa	48.9Aa	49.94Aa	92.12**	68.97Aa	50.74Ab	51.09Aa	50.05Ab	92.12**
	A15	43.21Ab	52.56Aa	43.81Ba	54.88Aa	–	56.78Ab	47.43Ab	56.18Aa	45.11Ab	–
	A59	32.96Bc	39.53Aa	42.22Aa	45.4Ab	95.43**	67.03Aa	60.46Ab	57.77Aa	54.59Aa	95.43**
	L34	30.45Bc	24.88Bb	40.48Aa	43.51Ab	66.54**	69.54Aa	75.11Aa	59.51Aa	56.48Aa	66.54**
	S4	60.93Aa	46.84Aa	43.57Ba	36.74Bb	92.45**	39.06Bc	53.15Ab	56.42Aa	63.25Aa	92.45**
Season 2 (moderate temperature)	A9	49.21Aa	50.04Aa	56.44Aa	56.22Aa	98.22**	50.78Ba	49.95Ab	43.55Ab	43.77Aa	–
	A15	52.5Aa	59.9Aa	61.27Aa	51.2Aa	98.13**	47.49Aa	40.09Ab	38.72Bb	48.79Aa	98.13**
	A59	45.87Aa	33.57Ab	44.05Ab	48.77Aa	69.02*	54.12Ba	66.42Aa	55.94Aa	51.22Aa	69.02*
	L34	50.8Aa	54.84Aa	49.96Ab	46.53Aa	–	49.19Ba	45.15Bb	50.03Aa	53.46Aa	–
	S4	46.91Ba	42.79Ab	58.73Aa	48.14Aa	–	53.08Aa	57.2Aa	41.26Bb	51.85Ba	–

Means followed by the same upper-case letter in the column for crop season and lower-case letter in the column between crop seasons for genotypes do not differ by the Scott-Knott test at 5% probability. Significant regression coefficient when  $p < 0.01$  (\*\*) and  $p < 0.05$  (\*)

biomass production, with 5.11 and 4.93 g, respectively (Table 4).

For leaf dry mass, the A9 genotype presented quadratic behavior, where the combination of higher temperatures in season 1, with 78% water availability, contributed to leaf dry mass content, with a mean value of 52%. Leaf dry mass content increased in A9, A15 and S4 genotypes at 50% water availability combined with high temperatures, with means of 49, 52 and 46%, respectively. The same variable also increased in all analyzed genotypes at 75% availability, with mean values from 40 to 48% (Table 5; Supplementary Table S5).

The combination of the 50% water availability with the moderate temperature in season 2 helped to raise the leaf dry mass content of the A15 genotype compared to the others, with a mean value of 59%. Leaf dry mass content increased in A9, S4, and A15 genotypes at 75% water availability, with mean values of 56, 58, and 61%, respectively (Table 5).

As for shoot dry mass content in season 1, A59, L34 and S4 genotypes presented linear behavior, A9 showed quadratic behavior, the combination of high temperatures and 78% water availability increased this variable, with a mean value of 47.99% (Table 5). In the same season, shoot dry mass content increased in the L34 genotype at 50 and 75% water availability, with means of 75 and 59%, respectively (Table 5). Shoot dry mass content increased in A15 and A59 genotypes in response to the combination of moderate temperature with 61 and 56% water availability, with mean values of 38.31 and 62.46%, respectively (Table 5).

The A59 genotype presented higher shoot dry mass content at 50% water availability compared to the others, with a mean value of 66%. The 25% water availability combined with high temperature drastically reduced the forage accumulation rate compared to the other availability in the soil

(Table 6; Supplementary Table S6). The high temperature in season 1, combined with 50% water availability, increased the forage accumulation rate in A59, A15 and L34 genotypes. The 75% availability favored a higher forage accumulation rate only in the A59 genotype, with a mean value of 0.31 g day<sup>-1</sup>. The 50% water availability combined with moderate temperature in season 2 promoted an increased forage accumulation rate in A9 and A59 genotypes, with means of 0.10 and 0.09 g day<sup>-1</sup>, respectively. The forage accumulation rate increased in A59, A9 and L34 genotypes at 75% water availability with means of 0.11, 0.12 and 0.13 g day<sup>-1</sup>, respectively (Table 6).

Water use efficiency (WUE) was higher in high temperature than in moderate temperature (Table 6), A9, A15, A59, L34 and S4 genotypes presented higher WUE when in high temperature were combined with at 97, 88, 83, 57 and 97% water availabilities, respectively, favoring a dry mass increase in these genotypes. The A59 genotype showed higher WUE than the others at 75% water availability, with a mean value of 1.33 gL<sup>-1</sup> (Table 6), A9 and A59 genotypes presented higher WUE when combined moderate temperature with 50% water availability, with means of 0.53 and 0.46 gL<sup>-1</sup>, respectively (Table 6). At 75% water availability, A15, A59, A9, and L34 genotypes showed higher WUE. With means of 0.36, 0.49, and 0.52 gL<sup>-1</sup>, respectively.

## Discussion

The increased photosynthetic rate of genotypes in season 1 (Table 1) contributed to a higher crop yield increasing plant dry mass (Table 4) because the photosynthetic rate is the primary source of carbohydrates used for plant growth and development (Ribeiro & Machado, 2007; Moller et al.

**Table 6** Forage accumulation rate (%) (FAR) and water use efficiency (WUE) of *Macroptilium* genotypes as a function of the combination of thermal and water stress

		FAR					WUE				
		Water availability (%)					Water availability (%)				
		Genotypes	25	50	75	100	R <sup>2</sup> (%)	25	50	75	100
Season 1 (high tem- perature)	A9	0.02Bb	0.17Ab	0.21Ab	0.30Ab	97.58**	0.21Bc	0.85Ab	0.84Ab	1.09Aa	90.36**
	A15	0.04Ab	0.22Aa	0.19Ac	0.32Aa	84.96**	0.35Ab	1.11Aa	0.86Ab	1.18Aa	71.16**
	A59	0.04Ab	0.21Aa	0.31Aa	0.29Ab	99.80**	0.35Ab	1.05Aa	1.33Aa	1.09Aa	99.90**
	L34	0.07Aa	0.22Aa	0.14Ad	0.20Ac	49.08**	0.57Aa	1.04Aa	0.48Ac	0.65Ab	15.02**
	S4	0.02Ab	0.15Ab	0.23Ab	0.27Ab	99.96**	0.16Ac	0.72Ab	0.84Ab	1.01Aa	96.86**
Season 2 (moderate tempera- ture)	A9	0.05Aa	0.10Ba	0.12Ba	0.08Bb	96.00**	0.39Aa	0.53Ba	0.52Ba	0.31Bb	99.87**
	A15	0.03Ab	0.02Bc	0.08Bb	0.07Bb	58.63**	0.22Ab	0.12Bc	0.36Ba	0.25Bb	–
	A59	0.05Aa	0.09Ba	0.115Ba	0.11Ba	99.89**	0.33Aa	0.46Ba	0.49Ba	0.43Ba	99.99*
	L34	0.05Aa	0.07Bb	0.13Aa	0.13Ba	90.11*	0.35Ba	0.33Bb	0.49Aa	0.47Ba	67.34*
	S4	0.03Ab	0.06Bb	0.04Bc	0.04Bc	–	0.23Ab	0.32Bb	0.17Bb	0.16Bb	–

Means followed by the same upper-case letter in the column for crop season and lower-case letter in the column between crop seasons for genotypes do not differ by the Scott-Knott test at 5% probability. Significant regression coefficient when  $p < 0.01$  (\*\*) and  $p < 0.05$  (\*)

2017). However, plants exposed to water deficit combined with high temperature may reduce the biomass production (Farooq et al., 2017) since the combination of stress causes greater damage when compared to isolated effects (Boeck et al., 2015; Keles & Oncel, 2002; Rizhsky et al., 2004; Zandalinas et al., 2016).

For example, Rizhsky et al. (2004) observed that *Arabidopsis thaliana* plants under combined drought and heat stress accumulate sucrose instead of proline. Furthermore, increased transpiration to cool the leaf surface during heat stress exacerbates the effects of simultaneous drought and salinity as increased transpiration rate leads to more water loss and increased salt absorption (Mittler, 2006; Rizhsky et al., 2004).

Reduced water availability combined with season 1 (high temperature) limited stomatal opening (Table 1) and decreased photosynthetic activity and transpiration (Tables 2 and 3), increasing leaf temperature (Fig. 1B) and dropping the total chlorophyll index (Fig. 2B). The adaptation strategy of plants to a combination of two stresses consists of general physiological adaptations of plants which can protect them against multiple stresses (Smekalova et al., 2014). In general, the physiological activity of plants is impacted when exposed to abiotic stresses, causing destabilization of Rubisco and damage to PSII (Nishiyama & Murata, 2014).

Changes in the stomatal opening are the plant's first response to environmental stresses, which aim to regulate the flow of CO<sub>2</sub> leaf temperature and water loss. However, stomatal responses to the combination of drought and heat represent a challenging situation, where plants must maintain a balance between avoiding water loss and protecting against overheating (Zandalinas et al., 2017). In most cases, heat causes increases in stomatal conductance as the

plant attempts to cool its leaves through transpiration while drought has the opposite effect to prevent water loss (Mittler, 2006).

According to Wang et al. (2010), a combination of drought and heat stress negatively affects photosynthetic rate to a more severe level than each of the different stresses applied individually. Plants subjected to a combination of drought and heat may have a lower photosynthetic rate, closure of stomata and increased leaf temperature and increased production of reactive oxygen species (Li et al., 2014). This is a function of changes in carbon assimilation rates, decreased turgor, increased oxidative damage, and alterations in leaf gas exchanges affecting growth (Kumar et al. 2016; Karim et al., 2018). That corroborates the physiological and production outcomes of *Macroptilium* at 25% water availability combined with high temperature in season 1 (Tables 1, 2, and 6 and Figs. 2 and 3). Conversely, plants growing under water availability of approximately 80% field capacity presented higher water and nutrient absorption by roots, favoring photosynthetic activity and contributing to biomass growth and accumulation (Chen et al., 2017).

Forage behavior depends on the interaction of its genetic potential with the environment. Thus, the different responses among genotypes allow the selection of tolerant materials to a combination of abiotic stresses. The A59 genotype stood out due to higher plant dry mass production (Table 4) and forage accumulation (Table 6) in crop season 1 in November, with a maximum temperature of 37.46 °C combined with 75% water availability.

The 100% water availability does not always represent higher production because water stress occurs in excess and deficit of water (Avozzani, 2021). Silva et al. (2019) found that cowpea had higher agricultural yield at approximately



92% water availability. Plants that increase their productive efficiency with less water are relevant measures for adapting to climate change. Selecting these genotypes tolerant to the combination of increased temperature and water deficit by assessing water use efficiency may represent an essential tool and contribute to decision-making for sustainable irrigation management, thus, saving water.

Besides water deficit, increased temperatures can affect forage production because they may reduce the photosynthetic rate and transpiration, harming osmotic adjustment, reducing the drought tolerance of plants and impairing and reducing agricultural yield (Blum, 2017). Plant adaptation strategies to combined stress may be different from those of two individual stresses (Mittler, 2006). The severity of the combination of stresses in plants largely depends on the age of the plant, whether the crop is tolerant or sensitive to stress, and the intensity of the two stresses involved (Pandey et al., 2015). Plant responses to combined stress are primarily determined by the most severe stress such that plant physiological processes resemble those observed under the most severe individual stress (Pandey et al., 2015).

Comparing both crop seasons, the additional 2 °C increased *Macroptilium* production, and A9 and S4 genotypes stood out for biomass production (Table 4). Therefore, the selected genotypes must be validated in the field. However, this research step was essential and will help define management strategies and tactics to face climate changes by planting tolerant native forage plants. The response of *Macroptilium* plants to the combination of different abiotic stresses is essential for the development of tolerant genotypes. According to Mittler (2006), these studies can significantly contribute to increasing the chances of developing crops with greater tolerance to combined stress conditions in the field.

## Conclusions

The interplay between water availability and crop season temperatures exerted a notable influence on the physiological, biometric and production characteristics of *Macroptilium* genotypes. Opting for the warmest season for planting proved to be advantageous in enhancing the yield of the genotypes under scrutiny thereby elevating water use efficiency. Notably, A59 and L34 genotypes exhibited superior plant dry mass and forage accumulation when exposed to higher temperatures and less than 100% water availability. These findings suggest that A59 and L34 may hold promise as prospective cultivars of *Macroptilium* sp. with a heightened tolerance to combined abiotic stresses.

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**Data availability** The data corroborating this study will be shared upon reasonable request to the corresponding author.

## Declarations

**Conflict of interest** The authors declare that they have no conflict of interest.

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