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THESIS

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Presented by

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Medic responses to water deficit alone or combined to salinity, evaluation of their yield and priming effects

PhD defence on 03 /06/ 2025 in front of the committee members:

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Dedication

This thesis is a reflection of not only my work but also the unwavering support and love of those closest to me.

To my mother, who once told a six-year-old girl with big dreams that there would be a university just for biology if I loved it enough. That simple encouragement planted the seed that grew into this journey. Thank you for always believing in me and for nurturing my dreams with your love and faith.

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Abstract : This thesis investigates the responses of two forage species, *M. ciliaris* and *M. polymorpha,* to water deficit, both alone and in combination with salinity. Additionally, it explores strategies to enhance their productivity under these challenging conditions, focusing on approaches such as seed priming and the incorporation of biochar into the culture medium.

In *Medicago ciliaris*, drought (36% water holding capacity) led to a significant reduction in plant growth and an increase in the root/shoot ratio. The growth response was accompanied by a decreased stomatal conductance and a reduction of the net CO_2 assimilation rate and water use efficiency. The associated higher risk of ROS production was indicated by a high level of lipid peroxidation, high antioxidant activities and high proline accumulation. Soil amendment with biochar (2%) enhanced the growth significantly and supported the photosynthetic apparatus of *Medicago ciliaris* species by boosting chlorophyll content and A_{net} both under well and insufficient watered plants and water use efficiency in case of water shortage. This increase of water use efficiency was correlated with the biochar-mediated decrease of the MDA and proline contents in the leaves buffering the impact of drought on photosynthetic apparatus by increasing the activity of enzymatic antioxidants SOD, APX, GPOX and GR and non-enzymatic antioxidants, such as AsA and DHAsA, giving the overall picture of a moderate stress response. These results confirmed the hypothesis that biochar application significantly reduces both the degree of stress and the negative impact of oxidative stress on *Medicago ciliaris* plants. These results implied that this species could be suitable as a cash pasture plant in the development of agriculture on dry wasteland in a future world of water shortages.

This study aimed to evaluate the extent to which seed priming with ascorbic acid (0.2 mM) enhances the performance of *M. polymorpha L.* under osmotic stress induced by 50 mM NaCl (salinity) or 100 g/L PEG. Parameters related to growth and photosynthesis were assessed to understand the impact. Both NaCl-induced salinity and PEG-mediated osmotic stress significantly reduced plant biomass (-30% and -40%, respectively), leaf and ramification numbers, stem length, net CO_2 assimilation, and leaf water content. However, both treatments, particularly PEG, increased the root/shoot ratio and leaf proline content.

Interestingly, seed priming with ascorbic acid alleviated these stress-induced effects, improving CO_2/H_2O gas-exchange and increasing plant biomass production. Water relations also improved, as indicated by reduced leaf osmotic potential, enhanced proline accumulation, and higher leaf water content. Additionally, ascorbic acid seed priming increased leaf carotenoid content by 65% and chlorophyll contents by 45%, thereby enhancing photosynthetic activity. These improvements collectively contributed to better plant performance, particularly under salinity stress.

Seed priming with ascorbic acid is an easy, cost-effective and promising approach to mitigate the impact of osmotic stresses like drought and salinity, especially by improving plant water relations and photosynthetic efficiency.

The effects of salinity, soil drying, and their interaction on the two forage species *Medicago ciliaris* and *M. polymorpha* were investigated. The results revealed that salinity and drought applied individually adversely affected growth, photosynthesis, and leaf water and potassium content in both species. However, the combined effects of both constraints were not significantly additive. Salinity affected the growth of both species to a similar extent.

The major advantage of *M. ciliaris* under dry or saline conditions seems to be its higher water use efficiency and its corresponding positive impact on the ROS risk. Compared to *M. polymorpha*, *M. ciliaris* demonstrated superior biomass production under drought, salinity, and combined stresses, making it a strong candidate for forage production and soil rehabilitation in arid and semi-arid salinized regions.

Keywords drought, salinity, *Medicago ciliaris, Medicago polymorpha,* biochar, growth; photosynthesis, antioxidant capacity, Seed priming, water use efficiency.

Résumé

Cette thèse a pour objectif l'étude de la réponse de deux espèces fourragères *M. ciliaris* et *M. polymorpha* au déficit hydrique seul ou combiné à la salinité, et d'améliorer leurs productivité en conditions de stress par différentes méthodes, y compris l'amorçage des graines (priming) et l'ajout de composé organique (Biochar 2%) dans le milieu de culture.

Chez *Medicago ciliaris*, le déficit hydrique (36% de la capacité au champ) a conduit à une réduction significative de la croissance des plantes et à une augmentation du rapport Racines/Parties aériennes. L'effet sur la croissance a été accompagné d'une diminution de la conductance stomatique et d'une réduction du taux d'assimilation net du CO_2 et de l'efficacité de l'utilisation de l'eau. Le risque plus élevé de production de ROS associé, a été indiqué par un niveau élevé de peroxydation lipidique, des activités anti-oxydantes élevées et une accumulation élevée de proline. L'amendement du sol avec du biochar (2%) a amélioré la croissance d'une manière significative et l'activité photosynthétique de l'espèce *Medicago ciliaris* en augmentant la teneur en chlorophylle et l'assimilation nette du CO_2 à la fois aux niveaux des plantes témoins et celles soumises au déficit hydrique et l'efficacité de l'utilisation de l'eau (EUE) en cas de déficit hydrique. Cette augmentation de l'EUE a été corrélée avec la diminution des teneurs en MDA et en proline dans les feuilles grâce au biochar, qui a allégé l'impact du déficit hydrique sur l'appareil photosynthétique en augmentant l'activité des enzymes anti-oxydantes SOD, APX, GPOX et GR et des antioxydants non enzymatiques, tels que l'AsA et le DHAsA, ce qui donne l'image générale d'une réponse modérée au stress. Ces résultats ont confirmé l'hypothèse selon laquelle l'application du biochar réduit significativement à la fois le degré de stress et l'impact négatif du stress oxydatif sur les plantes de *Medicago ciliaris*. Ces résultats montrent les effets bénéfiques du biochar sur l'amélioration des rendements de culture de *M. ciliaris* et la possibilité de son utilisation comme plante fourragère dans les zones arides ou semi aride

Afin d'évaluer dans quelle mesure l'amorçage des graines avec de l'acide ascorbique (0,2 mM) peut améliorer la performance de *Medicago polymorpha* L. en cas de stress osmotique induit par 50 mM NaCl ou 100g/L de PEG, des paramètres liés à la morphologie de la plante, aux échanges gazeux foliaires CO_2/H_2O, à l'ajustement osmotique, à la teneur en pigments et à l'accumulation de proline ont été spécifiquement déterminés. La salinité et le stress osmotique induit par le PEG ont réduit la biomasse des plantes (-30% et -40% respectivement), le nombre de feuilles et de ramifications, la longueur de la tige, l'assimilation nette de CO_2 et la teneur en eau des feuilles. Cependant, les deux traitements, et en particulier le PEG, ont entraîné une augmentation du rapport racines/parties aériennes et du contenu en proline des feuilles. Il est intéressant de noter que l'amorçage des graines par l'acide ascorbique a amélioré les échanges gazeux photosynthétiques et la production de biomasse (respectivement +66%, +100% et +92% au niveau des plantes témoins, traitées par NaCl et par PEG). Il a également amélioré les relations hydriques, comme le montre la diminution du potentiel osmotique des feuilles et l'augmentation de l'accumulation de proline et de la teneur en eau des feuilles. En outre, l'amorçage des graines avec l'acide ascorbique a augmenté les teneurs en caroténoïdes et en chlorophylle des feuilles (+65 et +45% respectivement pour le chl a et le chl b), contribuant ainsi à une meilleure activité photosynthétique, et donc à la performance des plantes sous salinité. L'amorçage des semences avec de l'acide ascorbique est une approche facile, rentable et prometteuse pour atténuer l'impact des stress osmotiques comme la sécheresse et la salinité, en améliorant notamment les relations hydriques et l'activité photosynthétique des plantes.

L'interaction entre le déficit hydrique et la salinité a été étudiée chez *M. polymorpha* et *M. ciliaris*. Nos résultats ont montré que la salinité et la sécheresse appliquées individuellement affectent négativement la croissance, la photosynthèse, la teneur en eau et en potassium des feuilles des deux espèces étudiées. Les effets des deux contraintes ne sont pas significativement additifs. La salinité affecte la croissance des deux espèces étudiées de la même manière. La supériorité de *M. ciliaris* dans des conditions sèches ou salines semble être liée à une plus grande efficacité d'utilisation de l'eau et son 'impact positif sur la réduction du stress oxydatif. *M. ciliaris* a produit plus de biomasse en condition de déficit hydrique, de la salinité et de la combinaison des deux stress, cette lignée pourrait être utilisée comme une espèce fourragère pour la réhabilitation des sols dans les régions arides et semi-arides salinisées.

Mots clés : déficit hydrique, salinité, *Medicago ciliaris, Medicago polymorpha,* biochar, croissance, photosynthèse, capacité anti-oxydante, prétraitements des semences, efficacité d'utilisation de l'eau.

Ziel dieser Arbeit ist es, die Reaktion von zwei Futterarten, *M. ciliaris* und *M. polymorpha*, auf Wasserdefizitstress allein oder in Kombination mit dem Salzgehalt zu untersuchen und ihre Produktivität unter diesen suboptimalen Bedingungen durch verschiedene Methoden zu verbessern, einschließlich Saatgut-Priming und der Zugabe von Pflanzenkohle in das Kulturmedium.

Bei *Medicago ciliaris* führte die Trockenheit (36 % Wasserspeicherkapazität) zu einer deutlichen Verringerung des Pflanzenwachstums und einer Erhöhung des Wurzel-Spross-Verhältnisses. Die Wachstumsreaktion ging einher mit einer verringerten stomatären Leitfähigkeit und einer Verringerung der Netto-CO2-Assimilationsrate und der Wassernutzungseffizienz. Das damit verbundene höhere Risiko einer ROS-Produktion wurde durch ein hohes Maß an Lipidperoxidation, hohe antioxidative Aktivitäten und eine hohe Prolinakkumulation angezeigt. Die Bodenverbesserung mit Pflanzenkohle (2%) förderte das Wachstum und den Photosyntheseapparat erheblich. Letzteres konnte bei gut als auch bei unzureichend bewässerten *Medicago ciliaris* Pflanzen durch Erhöhung des Chlorophyll- und A_{net}-Gehalts und der Wassernutzungseffizienz bei Wassermangel nachgewiesen werden. Diese Steigerung der Wassernutzungseffizienz korrelierte mit der durch Pflanzenkohle vermittelten Abnahme des MDA- und Prolingehalts in den Blättern. Letzteres pufferte die Auswirkungen von Trockenheit auf den Photosyntheseapparat, indem sie die Aktivität der enzymatischen Antioxidantien SOD, APX, GPOX und GR und nicht-enzymatischen Antioxidantien wie AsA und DHAsA erhöhte, was das Gesamtbild einer moderaten Stressreaktion ergab. Diese Ergebnisse bestätigten die Hypothese, dass die Anwendung von Pflanzenkohle sowohl den Grad des Stresses als auch die negativen Auswirkungen von oxidativem Stress auf *Medicago ciliaris* Pflanzen signifikant reduziert. Diese Ergebnisse deuten darauf hin, dass sich diese Art als Weidepflanze für die Entwicklung der Landwirtschaft auf trockenen Brachflächen eignen könnte.

Um zu beurteilen, in welchem Maße das *Seed Priming* mit Ascorbinsäure (0,2 mM) das Wachstum und die Photosynthese von *Medicago polymorpha* L unter osmotischem Stress beeinflussen kann, wurden Pflanzen mit 50 mM NaCl oder 100 g/L PEG kultiviert. Sowohl NaCl-Salzgehalt als auch PEG-vermittelter osmotischer Stress führten zu einer Reduktion der pflanzlichen Biomasse (-30 % bzw. -40 %), der Blatt- und Verzweigungszahl, der Stängellänge, der Netto-CO2-Assimilation und des Blattwassergehalts. Beide Stressoren und insbesondere PEG führten jedoch zu einem erhöhten Wurzel-Spross-Verhältnis und einem erhöhten Gehalt an Blattprolin. Interessanterweise verbesserte die Saatgutvorbereitung mit Ascorbinsäure den CO_2/H_2O-Gasaustausch und die Produktion pflanzlicher Biomasse. Es verbesserte auch den Wasserhaushalt, was sich in der Abnahme des osmotischen Potentials der Blätter und einer höheren Prolinakkumulation und einem höheren Wassergehalt der Blätter widerspiegelt. Darüber hinaus erhöhte das Priming der Samen mit Ascorbinsäure die Caroteoind- und Chlorophyll-Gehalte der Blätter (+65 bzw. +45 % für chl a und chl b) und trug damit zu einer besseren photosynthetischen Aktivität und verbesserten Leistung der Pflanzen unter salinen Bedingungen bei. Es wird resumiert, das *Seed Priming* mit Ascorbinsäure ein einfacher, kostengünstiger und vielversprechender Ansatz ist, um die Auswirkungen von osmotischen Belastungen wie Trockenheit und Salzgehalt zu mildern, indem insbesondere die Wasserverhältnisse der Pflanzen und die photosynthetische Aktivität verbessert werden.

Weiterhin wurden die Auswirkungen von Salzgehalt und Trockenheit und deren Wechselwirkung bei den beiden Futterarten *Medicago ciliaris* und *M. polymorpha* untersucht. Unsere Ergebnisse zeigten, dass sich Salzgehalt und Trockenheit individuell auf das Wachstum, die Photosynthese, den Wasser- und Kaliumgehalt der Blätter bei beiden untersuchten Arten negativ auswirken. Die gemeinsamen Auswirkungen beider Stressoren waren nicht signifikant additiv. Der Salzgehalt beeinflusst das Wachstum der beiden untersuchten Arten auf die gleiche Weise. Der große Vorteil von *M. ciliaris* unter trockenen oder salzhaltigen Bedingungen scheint die allgemein hohe Wassernutzungseffizienz und der damit verbundene positive Einfluss auf das ROS-Risiko zu sein.

Im Vergleich zu *M. polymorpha* produzierte *M. ciliaris* mehr Biomasse bei Trockenheit, Salzgehalt und kombinierten Belastungen, so dass diese Art als Futterkandidat für die Sanierung von Böden in ariden und semiariden versalzenen Regionen empfohlen werden kann.

Schlüsselwörter Dürre, Salzgehalt, *Medicago ciliaris, Medicago polymorpha,* Pflanzenkohle, Wachstum; Photosynthese, antioxidative Kapazität, Saatgutvorbereitung, Wassernutzungseffizienz.

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C3: **Jihed Gharred**, Ons talbi, Mouna Ben Farhat, Walid Zorrig, Mounawer Badri, Arafat Manaa, Chedly Abdelly, Hans-Werner Koyro, Inès Slama. NaCl seed priming improves tolerance to drought in *Medicago polymorpha.* BMAT, 2019.

C4: Jihed Gharred, Walid Derbali, Imed Derbali, Mounawer Badri, Chedly Abdelly, Inès Slama, and Hans-Werner Koyro. Biochar-improved growth and physiology of *Medicago ciliaris* under water-deficit condition, The Tunisian-South African-Algerian Workshop, oral presentation, Tunisia ,October 2023.

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LIST OF ABBREVIATIONS :

(A)	CO_2 assimilation
(C)	Control plants
(D)	Plants subjected to water deficit
(D+S)	Plants subjected to water deficit and 50 mM NaCl salinity
(FC)	Field capacity
(S)	Plants subjected to 50 mM NaCl salinity
A/E	Ratio of net CO_2 assimilation rate and transpiration
ABA	Abscisic acid
ADP	Adenosine diphosphate
A_{gross}	Gross CO_2 assimilation
A_{net}	Net CO_2 assimilation rate
ANOVA	Analysis of variance
APX	Ascorbate Peroxidase
AsA	Ascorbic acid
AsA	Reduced ascorbate
AsA-GSH	Ascorbate-glutathione cycle
AsA_{tot}	Oxidized and reduced ascorbate
ATP	Adenosine triphosphate
Bc	Biochar
BSA	Bovine serum albumin.
Ca CO_2	Atmosphere CO_2 concentration within the leaf chamber
$CaCO_3$	Calcium carbonate
CAT	Catalase
CBB	Coomassie Brilliant Blue
Chl a	Chlorophyll a
Chl b	Chlorophyll b
Chl	Chlorophyll
Ci	Intercellular CO_2 concentration
Ci/Ca	Ratio of intercellular and atmospheric CO_2 concentration
CO_2	Carbon Dioxide
DHA	Oxidized ascorbate
DHAsA	Oxidized ascorbate
DNA	Deoxyribonucleic acid
DO	Optic Density
DTT	Dithiothreitol

DW	Dry Weight
E	Transpiration rate
e^-	Electron
EC	Electrical conductivity
EDTA	Ethylenediaminetetraacetic acid
ETR	Electron Transport Rate
F'm	Maximal level of Chl fluorescence in light
F0	Basal level of chlorophyll fluorescence
Fm	Maximal level of Chl fluorescence in dark
Fv	Variable fluorescence
Fv/Fm	Maximum quantum yield of PS II photochemistry
FW	Fresh Weight
GCC	Guard cell chloroplast
GPOX	Guaiacol peroxidase
GPX	Glutathione peroxidase
GR	Glutathione reductase
GRP	Glycine-rich proteins
Gs	Stomatal conductance
GSH	Reduced glutathione
GSSG	Oxidized glutathione
H_2O	Water
H_2O_2	Hydrogen peroxide
HRGP	Hydroxyproline-rich glycoproteins)
MDA	Malondialdehyde
$MgCl_2$	Magnesium chloride
NaCl	Sodium Chloride
LWC	Leaf water content
MAPK	Mitogen-activated protein kinase
MDA	Malondialdehyde
MDHA	Monodehydroascorbate
MDHAR	Monodehydroascorbate reductase
NADH	Nicotinamide adenine dinucleotide
NBT	Nitroblue tetrazolium chloride
NP	Non-primed
P	Primed
P5CS	D1-pyrroline- 5-carboxylate synthetase
PAM	Pulse-amplitude modulation

PEG	Polyethylene glycol
PGA	3-Phosphoglyceric acid
PPFD	Photosynthetic photon flux density
PQ	Plastoquinone
ProDH	Proline dehydrogenase
PRP	Proline-rich proteins
PSI	Photosystem I
PSII	Photosystem II
PVPP	Polyvinylpolypyrrolidone
R_D	Dark respiration
R_L	Photo-respiration
ROS	Reactive oxygen species
RWC	Relative water content
Sc	Stomatal conductance
SE	Standard Error
SI	Sensitivity index
SOD	Superoxide dismutase
SP	Saturation pulse
SPAD	Sensitive Single Photon Avalanche Diode
TBA	Thiobarbituric acid
TCA	Trichloroacetic acid
Vc	Photosynthetic efficiency
WHC	Water holding capacity
WPh	Water potential high
WPl	Water potential low
WUE	Water use efficiency
Y(II)	Photochemical quantum yield of photosystem II
Y(NO)	Quantum yield of non-regulated non-photochemical energy loss in PS II
Y(NPQ)	Quantum yield of regulated non-photochemical energy loss in PS II

INTRODUCTION

Climate fluctuations are very important factors affecting agriculture and food production around the world (Pörtner et al., 2022). Global earth temperature is dangerously warming up and experts are predicting an increase of 1.5 to 2◦C in the near future (IPCC 2021). Warmer temperatures enhance evaporation, which reduces surface water and dries out soils and vegetation. Nevertheless, the intensity of drought usually depends on many factors, such as the occurrence and distribution of rainfall, shifting seasons, evaporative demands, and moisture storing capacity of the soil, especially in semiarid climates (Chakraborty and Pradhan, 2012, Zubieta et al., 2021).

Facing fluctuating and unstable environmental conditions, plants need to adjust themselves to these changes by relying on their resilience (exposure, sensitivity, adaptive capacity) and vulnerability (Volaire, 2018). This includes a response to water and nutrient shortage, temperature variations, UV radiation, fungal pathogens, and insects, as well as other biotic and abiotic factors (Singh and Bainsla, 2015, Ramazan et al., 2021). These facts confirmed the contribution of climate change on the increasing severity of drought adverse effects on many agricultural areas around the world and more particularly in Africa (Pörtner et al., 2022). This is scary, given that by 2080 its projected that the ever-growing population will reach 10.8 billions people (FAO 2018) equivalent to an increase of 47 % of the global population and the competition for scarce natural resources, especially in countries with low incomes and large marginal lands, will get more intensified (Kathleen Hermans 2021). Indeed, water shortage in particular threaten food security in the world. It affects plant performance, reduces crop production, amplifies environmental pollution, and accentuates soil and fresh water salinization resulting in the disruption of agriculture. When faced with increasing water demands for agriculture, the selection of ecological sustainable plants, resistant to water deficit and able to grow on wasteland has high priority.

To ensure sustainability and environmental compatibility on wasteland, endemic plants should be chosen, such as representatives of the family Fabaceae, with a low demand on nutrients, a distinct resistance to saline conditions and the ability to grow on poor sandy soils (Araújo et al., 2015). Particularly, the identification of physiological and morphological proprieties to cope with drought or with low quality water resources (such as saline or sewage water) are of paramount importance (Vasconcelos et al., 2020).

In such context, annual *Medicago* commonly known as medics belong to the family of Fabaceae, originally native to regions surrounding the Mediterranean can be a good candidate to increase crop production and avoid desertification thanks to their capacity to fix atmospheric nitrogen thus contribute to the improvement of the overall soil conditions (Slama et al., 2022).

Since the Mediterranean region is mostly arid and semi-arid areas, the climate conditions of these lands are not optimum for crop production especially since environmental constrains such as drought, salinity and extreme temperatures cause crucial losses on legume growth and productivity(Al-Tawaha et al., 2022). Thus, plants in arid or saline regions are constantly exposed to a significant severe water deficit, against which they must develop adaptive mechanisms for their survival, growth and development (Hossain et al., 2020).

In Tunisia, water deficit is very acute in the arid and semi-arid regions of the country where plants are subject to various environmental constraints, particularly salinity, drought and nutritional deficiencies (Ben Gaied et al., 2024). At the same time, pastural farming is becoming the only more or less profitable alternative in these regions, given the excessive salinization of the soil, which has led to the abandonment of several crops (Devkota et al., 2022). Annual medics should be a good option to optimize the rehabilitation and exploitation of such lands and improve productivity since they can grow in saline marginal areas (Slama et al., 2022). *Medicago* is already in use because of its high biomass production as a potential species for pasture improvement in arid Mediterranean regions. *M. ciliaris* alone, produce up to 40% of the vegetative cover in rainy years at the edges of salt flats along arid coasts in Tunisia (Abdelly et al., 1995).

Even though many reviews reported that these species are relatively resistant to salinity, limited literature is available about *M. polymorpha* and M. *ciliaris* performances in the drought conditions. Further adjustments have been tested in this study to optimize plant response to the drought conditions and ensure sustainable agriculture practices by improving the complex interacting system of the plant–soil continuum.

For instance, It may be a good strategy to optimize environmental conditions by improving soil properties with the application of organic amendments such as biochar (Yu et al., 2019). Biochar as a soil conditioner, has the potential to improve soil physio-chemical properties, increase fertility by improving nutrient retention, and boost the soil water holding capacity (WHC). It also improves soil moisture contents, stability of soil texture, and aggregate formation, and porosity which is a good strategy to improve water infiltration, organic matter status, nutrient and soil aeration and respiration particularly under low soil water content (Koyro and Huchzermeyer, 2022).

Moreover, one effective approach to optimize plant performance under abiotic stress is by enhancing seed quality. Seed priming is a strategy used to improve seed performance and enhance synchronized uniform germination, seedling growth and ultimately increase crop yield (Marthandan et al., 2020). This technique involves briefly exposing seeds to specific environmental stimuli, which reduces their sensitivity to adverse external factors. As a result, seed priming fosters the development of vigorous seedlings capable of thriving in diverse agro-climatic conditions. By preparing seeds to respond more quickly and effectively to environmental challenges, this method enhances a plant's ability to tolerate various stresses, thereby boosting crop productivity, particularly under extreme conditions (Mustafa et al., 2017, Raj and Raj, 2019).

Based on recent studies and current reviews of two *Medicago* varieties and their ability to grow under saline conditions, the thesis aims to improve the productivity of these alternative crops, increase their resistance to extreme conditions through various methods, and encourage farmers to cultivate these species on dry or saline wastelands, transforming unproductive areas into fertile agricultural land.

In the first part of our study , we studied the impact of biochar on drought resistance of crop plant *M. ciliaris* based on physiological and biochemical approaches.

In the second part of our study, we aimed to assess the effectiveness of ascorbic acid (0.2 mM) in enhancing the drought and NaCl resistance of *M. polymorpha L*. Our objective was to enhance the response of *Medicago* to water deficit, either alone or in combination with salinity, through priming methods.

In the third part of our study, we delved into the interactive effects of salinity and drought stress on two forage species: *M. ciliaris* and *M. polymorpha*. Our fundamental inquiry aimed to compare how each species responded to drought and salinity individually, as well as in combination. It was our aim to discern the impact on growth, photosynthesis, water, and nutrient status, with the overarching goal of identifying the most tolerant species suitable for rehabilitating arid and semi-arid regions.

Chapter 1

Literature Review

Introduction

Global population is increasing continually and expected to attend 9.7 billion by 2050 (Hossain et al., 2020). Consequently, crop demands are growing as well and scientists are urged to develop new strategies to meet these growing needs not just for humans but also for animals. In accordance, Searchinger (2019) reported that global crop demands should be increased 70% by the year 2050 (Searchinger et al., 2019). To make this happen, farmable lands and the productivity of existing farmland need to be multiplied (Fahad et al., 2017). It had been estimated that farmland area needed to meet crop demands and pasture yields worldwide is around 593 million hectares (Searchinger et al., 2019).

Legumes can be promoted as a key components of the Climate Smart Agriculture concept thanks to the many advantages that these plants have like for example, the ability of atmospheric nitrogen fixation (Martins et al., 2020). Due to this property, legumes have high overall crop productivity on nitrogen-poor soils while saving costs and resources by reducing the need for chemical fertilizers (Nadeem et al., 2019a). Moreover, over the past decades, groundwater has been contaminated with nitrate because of the excessive use of inorganic nitrogen fertilizers (Lv et al., 2019). This agricultural strategy caused a very serious environmental problem as leaching pollutants such as NO_3^- in ground-water (Zheng et al., 2019).

Thus, a proper exploitation of legumes in agricultural and crop rotation systems may be a good eco-friendly strategy to improve soil quality with inorganic nitrogen (Ma et al., 2018).

The strategic use of legumes, along with efficient rhizobia and plant growth-promoting bacteria, offers a promising approach to sustainable agriculture, particularly in arid and semi-arid regions (Swarnalakshmi et al., 2020). Researchers are emphasizing the use of plant growth-promoting bacteria (PGPB) due to their ability to increase plants' resistance to extreme conditions in arid and semi-arid regions by (i) phytohormone secretion as PGPB can produce hormones like auxins, gibberellins, and cytokinin, which promote root growth and improve plant resilience to stress (Orozco-Mosqueda et al., 2023)., (ii) mineral solubilization as PGPB can solubilize essential minerals like phosphorus and potassium, making them more available to plants (Bargaz et al., 2021)., and (iii) enhancing atmospheric nitrogen fixation since nitrogen fixation by legumes increases soil nitrogen content, benefiting subsequent crops in rotation systems. As mentioned before, legumes form symbiotic relationships with nitrogen-fixing bacteria, primarily Rhizobium species, these bacteria reside in root nodules of legumes and convert atmospheric nitrogen (N_2) into ammonia (NH_3), a form of nitrogen that plants can utilize (Chellem et al., 2024). This natural process enriches the soil with nitrogen, reducing the need for synthetic fertilizers.

Indeed, the introduction of efficient rhizobia in legume agriculture is often suggested in areas with low or incompatible native rhizobia populations. This ensures effective nodulation and nitrogen fixation, even in challenging soil conditions (Clúa et al., 2018). Some published studies confirmed that rhizobium, in combination with other beneficial microbes like plant growth-promoting bacteria (PGPB) and arbuscular mycorrhizal fungi (AMF), can be an effective method for enhancing the cultivation of legumes, such as alfalfa, faba beans, and cowpea, in arid conditions (Ben Gaied et al., 2024).

Nitrogen fixation is not the only beneficial effect of legumes on agriculture. It was reported that the use of legumes as an intercrop or in crop rotation in farming systems has more advantages reducing weed populations, insect pests and diseases (Daryanto et al., 2015). Moreover, legumes protect the soil from erosion and optimize the soil quality by reducing the incidence of soil pathogens, as well as, improving the soil nutritional profile, when grown in rotation with cereals (Khatun et al., 2021b).

In terms of food production, Legumes provide 27% of primary crop production worldwide, almost as important as other crops like cereals, they are a nourishing low-cost source of protein, suppling 33% of global protein requirements (Pradhan et al., 2019).

The economics of legumes in the developing countries is actually very promising as it has been reported that they are a major cash crop for over 700 million smallholders in developing countries, with an annual value of about 31 billion USD (Meena et al., 2018).

I. The genus *Medicago :*

1. Special interest in *Medicago spp:* (Environmental and Agricultural impact):

Because of its geographical location, researchers are warning that climate change will especially affect equatorial regions such as sub-Saharan Africa (Durodola, 2019). Livestock in North Africa, was already suffering from a chronic shortage in food fodder, especially during dry periods in the summer and autumn (Schilling et al., 2020). Now, these already water shorted countries with poor resources are challenged even harder to act urgently and elaborate a strategy about how to coexist with these constantly changing climate fluctuations by adapting to climate-smart agriculture to increase crop production (Hermans and McLeman, 2021).

The introduction of medic species in agriculture system (Medicago spp.), is a reasonable and promising way for a sustainable and ecological feasible agriculture (Vasconcelos et al., 2020). As a matter of fact, farmers had already started getting interested in these species very early in the past (1930s) and started growing cultures of *M. truncatula* on calcareous soils of southwestern Australia thanks to the recommendation of Trumble and Donald (1938) who promoted the potential of these species in these areas (Badri et al., 2016, Trumble and Donald, 1938). Later on the incorporation of annual medics in agricultural systems in Australia was carried on particularly in regions with arid and semi-arid conditions where it was employed in soil management strategies (Nichols et al., 2012, Cocks and Bennett, 1999).

Indeed, the genus *Medicago* offers distinct advantages that further amplify the benefits provided by legumes. They can play a crucial agronomic role in the dryland agriculture regions in the Mediterranean area, especially in North Africa (Slama et al., 2022) where drought is getting more drastic, soil moisture declined >10% across entire region north of the Sahara desert (Hermans and McLeman, 2021).

Moreover, climatic change is increasing earth temperature which will potentially increase heat impact on vegetation, causing land degradation in forested and agricultural environment soil erosion by wind and rain, soil salinization, changes in soil moisture availability and an overall decline in soil nutrient vegetation and biomass (Talukder et al., 2021).

Medicago species, such as *M. polymorpha* and *M. ciliaris*, are particularly noteworthy for their unique attributes that address specific environmental challenges. These species offer several critical advantages for mitigating the impacts of global climate change described below thanks to their capacity to produce organic nitrogen thus contribute to soil fertility. *Medicago* species also exhibit exceptional adaptability to poor and saline soils. Their extensive root systems enhance soil structure, promote water infiltration and retention as well as nutrients uptake, which helps prevent soil erosion and degradation caused by extreme weather events (Phillips et al., 2006).

In addition, the biomass production during periods of low production from traditional pasture species make them invaluable for maintaining agricultural productivity in arid and semi-arid regions. (Brummer et al., 1995). In fact, annual medics (*Medicago* spp.) are able to form root nodules in symbiosis with soil bacteria that can fix important amount of atmospheric nitrogen (N_2) that can compensate for the chemical nitrogen fertilizer supply , leading to lower greenhouse gas emissions and promoting sustainable practices (Ben Salah et al., 2018, Snyder et al., 2014). These species also stimulate beneficial soil microbial communities, fostering nutrient cycling and boosting soil health, which is crucial for maintaining agricultural productivity amid climate fluctuations (Dollete, 2024).

Moreover, in areas of low fertility and/or high salinity, by enriching the soil with nitrogenous compounds *Medicago* species promote growth of other halophytes which contribute periodically to the preservation of low saline soil (Abdelly et al., 1995). It should be noted that in these areas, halophytes are not very appreciated as meadows because they are loaded with salt that can reach 15 to 30% of dry matter, opposite to medics who contain 90% less NaCl (Abdelly et al., 1995). Furthermore, *Medicago* ability to grow in saline soils makes it valuable for reclaiming lands affected by rising sea levels and increased evaporation, thus mitigating the impacts of soil salinization (Cuevas et al., 2019). Additionally, the diverse growth forms of *Medicago* species, such as their prostrate habit, also provide effective ground cover, aiding in soil erosion control and stabilization, which is vital in areas prone to climate-induced erosion (Deepika et al., 2023). Indeed, *Medicago* can be used to optimize the rehabilitation and proper utilization of marginal lands and contribute in the establishment of a diversified and rich ecosystem alongside halophytes that may be used as grazing land (Cherifi et al., 2016). They are therefore a good candidate for the improvement of marginal lands with low fertility and/or high salinity (Ben Salah et al., 2018).

In North Africa, these plants are mainly used as pasture species in commercial grain agriculture systems, essentially to optimize the poor quality of natural pastures (Monjardino et al., 2022). Thanks to their rich concentration in proteins, minerals and vitamin contents and their low proportion of cell wall, medics are considered as a high feeding crop (Crespo, 1991). The promising agronomic potential of these plants, due to their low production cost and high nutritive value (Mbarki et al., 2020) is further supported by evidence of the high nutrient quality of medic fodder (Badri et al., 2016). These plants used for hay production especially for sheep can be grazed by cattle as well (Badri et al., 2016). Besides, Annual medic pastures can produce 6 to 10 tons of dry matter per hectare in suited conditions (Abdelly et al., 1995). It had been reported that *M. ciliaris* alone, can produce up to 40% of the vegetative cover in rainy years at the edges of salt flats along arid coasts in Tunisia (Abdelly et al., 1995). This interesting species has the potential to be a mother plant in row crop production, where they could muffle weeds and contribute to the proliferation of grassland supporting livestock productivity and food security as climate change impacts forage availability (Slama et al., 2022).

In addition to these benefits, *Medicago* species support biodiversity by enhancing plant diversity in cropping systems, which in turn supports a range of beneficial organisms and improves ecosystem resilience (Islam and Ashilenje, 2018). Their adaptability to various cropping systems, including conservation tillage and agroforestry, offers flexible management options for farmers facing changing climatic conditions, further contributing to soil health and reducing environmental impacts (Jena et al., 2022, Rahman et al., 2022).

Annual medics combine traits such as vigorous seedling growth and prolific seed production. Moreover, these species proved to be resistant to pests and morphological diversity (Yousfi et al., 2010b). However, medics seeds are covered with an impermeable coat which can play an important role in the initiation of germination pattern (Lodge and Whalley, 2002). This coat is double edged, while it can impose dormancy, known as ''hardseededness'' and affect germination rate especially when it's highly required. It has also a role of protection as it emphasize seed germinate only when environmental conditions can support seedling growth (Lodge and Whalley, 2002).

By leveraging these attributes, *Medicago* species significantly advance the goals of sustainable land management and climate adaptation, highlighting their essential role within the broader context of leguminous plants.

2. Classification:

The genus *Medicago* is one of most important genera of forage plants (Tucak et al., 2018). These genus is classified as glycophyte (Abdelly et al., 1995). It comprises approximately 85 species in which two are shrubby (*M. arborea* and *M. strasseri*), one-third are perennial herbaceous, and two-thirds are annual predominantly autogamous (Small and Jomphe, 1989). Most species have a basic chromosome number of $x = 8$, but there are some species with $x = 7$ (Small and Jomphe, 1989). *M. ciliaris* and *M. polymorpha.* for example, both are autogamous species (Cherifi et al., 2016) but *M. ciliaris* is a diploid with ($2n = 16$), It could be intercrossed readily with *M. intertexta* and also with *M. muricoleptis* (Badri et al., 2016), and *M. polymorpha* is a diploid with ($2n = 14$) (Badri et al., 2016).

3. Medicago habitat and diversity:

While perennial species are commonly grown in Central and Western Asia and East of the Mediterranean (see Figure 1.1) (Yang et al., 2022, Djedid et al., 2021). Annual *Medicago* commonly known as medics originally native to regions surrounding the Mediterranean Sea (North Africa, South Europe, and the Middle East) (Sheaffer et al., 1997) got widely distributed throughout the world as weed during the different streams of human colonization (Mbarki et al., 2020). Around the 19th century, some varieties were introduced to North Europe, North America and Australia (Prosperi et al., 2001). Namely, *M. ciliaris* has a broad geographical distribution, which plays a key role in its adaptability to various environmental conditions. Native to the Mediterranean Basin, it is found in North African countries such as Morocco, Tunisia, and Algeria, as well as parts of the Middle East, where it thrives in typically arid and semi-arid climates (Rejili et al., 2019, Prosperi et al., 2001). Its natural habitats often include sandy and saline soils, these regions, characterized by low rainfall and high temperatures, which support its resilience to relatively harsh conditions (Khalil et al., 2011, Mbarki et al., 2020). The species is also present in southern Europe, particularly in Greece, Spain and Italy, where it is valued as a fodder while contributing to soil fertility (Thanopoulos, 2007, Laouar and Abdelguerfi, 2014).

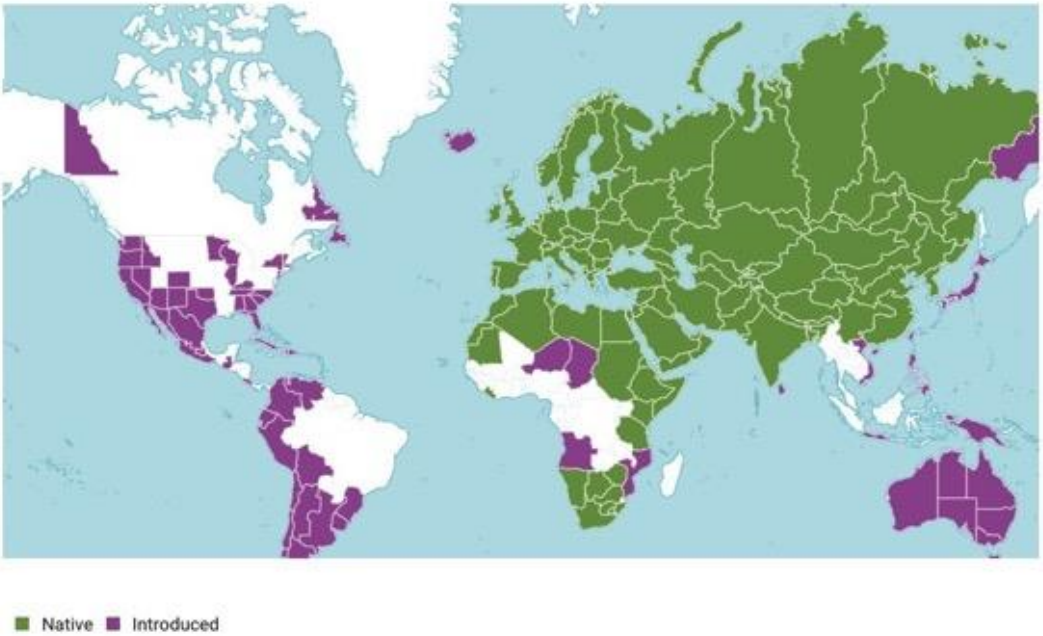

Figure 1.1 Geographical distribution of *Medicago* in the world. The green color represent the native origin of this genus and the purple color represent the areas where it was introduced (World, 2005).

In Tunisia *Medicago* species are considered as a precious genetic inheritance usually found in sabkha edges in association with perennial halophytes where they largely contribute to the primary productivity (Ferchichi et al., 2016). They grow spontaneously and represent an important proportion of the native flora (Abdelly et al., 1995). They can be found in different climates and soil conditions from humid to

Saharan (Slama et al., 2022). Some of them are present along the country, while others are more endemic (Yousfi et al., 2010a).

M. truncatula and *M. polymorpha* for example, exhibit a broad ecological range, thriving in various bioclimatic stages from humid to semi-arid environments. These species are considered ubiquitous. In contrast, *M. laciniata* and *M. minima* have a more restricted range, growing from semi-arid to Saharan conditions. (Prosperi et al., 2001, Prosperi et al., 1993, Cherifi et al., 1993).

Moreover, Medicago can adapt to different kinds of soil. It is possible to find various ecotypes of one species in different kind of soil/climatic conditions (Innes et al., 2021). *M ciliaris* for example, can grow naturally in calcareous, sodic and saline-sodic soils which are characterized by high pH values (Mbarki et al., 2020). It can be located in humid to lower semiarid climates (Mbarki et al., 2020). On the other hand, *M. polymorpha* is well adapted to neutral and slightly acid soils (Kotula et al., 2019). It spreads from humid to higher arid climates, opposite to *M. truncatula* witch is omnipresent in all bioclimates (Badri et al., 2016). Additionally, *M. polymorpha* growing in Tunisian soils forms a symbiotic relationship with *Sinorhizobium medicae* similar to *M. ciliaris* which also prefers nodulation with this bacterium (Zribi et al., 2007, Dourado et al., 2009, Ben Salah et al., 2018).

Unlike *M. sativa*, which is well-known for its high forage quality but demands significant water resources, and *M. truncatula*, a model species for general stress research, *M. ciliaris* and *M. polymorpha* have not been extensively studied under drought conditions. This makes them valuable alternatives for uncovering novel drought resistance mechanisms. As these species are well-adapted to various soil types, from sandy loam to clay, their integration into dryland farming systems offers potential solutions to critical agricultural challenges. Incorporating these species into such systems could help slow or even stop the spread of dryland salinity, as well as, enhancing soil sustainability. Furthermore, selecting salt-tolerant populations and developing near-isogenic lines provides valuable genetic resources that can contribute to green farming systems. By addressing this research gap, researchers need to provide new strategies for boosting crop productivity and resilience amid climate change, offering vital insights into adapting agriculture to increasingly arid conditions.

II. Environmental constraints:

Abiotic constraints such as extreme temperature, drought and flood, salinity, heavy metals, and nutritional imbalances affect plant development ranging from germination to the maturity stage and limit its productivity (Gull et al., 2019). Land degradation is a great challenge for agriculture as crop productivity is continually declining due to environmental constraints (Koyro and Huchzermeyer, 2022). As a matter of fact, there is no practical and economical way to overcome drought and salinity. Along with the continued decline in soil fertility, poor organic and inorganic soil resources, low water holding capacity (WHC), higher evaporation and drainage rate causing shortage of water availability to roots (Koyro and Huchzermeyer, 2022, Zainul et al., 2017). As a consequence, agricultural food production on global scale risk to be jeopardized (Teklić et al., 2021).

It should be mentioned that climate changes like heatwaves or heavy rainfall may occur in short and long terms (Hermans and McLeman, 2021). Precisely, these long term events extending over periods of several weeks of heatwaves or heavy rainfall risk to impact the environment as a whole and threaten to affect food security in the world (Lichtenthaler, 1998). The frequency of the occurrence of these extreme abiotic constraints could even lead to an increase of the probability of the occurrence of these unusual climate fluctuations (Koyro and Huchzermeyer, 2022). From an agricultural point of view, cropping systems that are able to adapt to these increasingly harsh environmental conditions are urgently needed (Hasanuzzaman et al., 2020a). Indeed, plants responses to abiotic constraints and the generation of plants that are resistant to environmental stress have been the topics of researchers worldwide in recent years (Teklić et al., 2021).

As a matter of fact, growing under non optimum conditions (water and energy recourses), affect plants growth and reduce its fertility and may even interrupt their life cycle leading to death (Billah et al., 2021). To survive under adverse environmental conditions, plants can adapt themselves by adjusting their metabolic activities and adopt a new homeostasis. The range of this flexibility is determined in the genome of a plant species (Koyro and Huchzermeyer, 2018). For instance, stress-resistant plants can initiate multiple changes at the physiological, cellular, and molecular traits as well as signal transduction pathways (Mareri et al., 2022). It is very important for stress-resistant plants to be able to produce metabolites that protect them from ionic and osmotic stresses or eventually from toxic compounds (Koyro and Huchzermeyer, 2018). Actually, researchers had already succeeded to identify and characterize most of the genes involved in the essential steps that regulate the molecular mechanism as well as stress-responsive genes thanks to the advanced genomics and sequence technologies (Kumar et al., 2021). The identification of stress signaling molecules, transduction pathways, and discovering of ABA receptors offer the opportunity to understand the functions as well as transcriptional and post-transcriptional regulation of stress-responsive gene expression (Koyro and Huchzermeyer, 2022). This knowledge may help to select plants which are more resistant to extreme environmental conditions and introduce them in green farming systems (Riyazuddin et al., 2021).

1. Drought constraints:

Drought is one of the most serious adverse environmental constrains, affecting plant growth, development, and survival (Zhang et al., 2018). Future climate scenarios predict in many regions around the world a decrease of total water storage from 3% to 8% over the 21st century (Masson-Delmotte et al., 2021). The shortage of global water resources, will amplify environmental pollution, and accentuate soil and fresh water salinization (Shahbaz and Ashraf, 2013).

Along with the expected rises in temperature and decreased water availability, drought stress risks are projected to increase faster and with greater intensity over the 21st century as a constraint factor of future agricultural production (Zhang et al., 2018). Indeed, climate fluctuations have contributed to the increased severity of the impact of drought in agriculture in many regions. The IPCC reported that 75% of the global harvested areas (~454 million hectares) experienced harvest losses caused by water limitation, with the cumulative production losses corresponding to the US $166 billion between 1983 and 2009 (Arias et al., 2021). Water scarcity caused up to 21 and 40% yield reductions in wheat and maize, respectively between 1980 and 2015 worldwide (Daryanto et al., 2016). There is an increasing need to look for strategies about how to increase global food production in the next decades to meet the increasing world food demand and energy needs of the population (Tilman et al., 2002).

1.1 Perception of water deficit:

When plants are subjected to water deficit, receptors in the cytoplasmic membrane of roots initiate cellular signals (Priya et al., 2019), which would either regulate cell volume or change the concentration of mineral and/or organic solutes. Signal perception can also induce changes in the cell surface or lead to loss of cell integrity (Suprasanna and Ghag, 2019, Bray, 1997). Dehydration induces the expression of two groups of genes: the first one encodes proteins directly involved in protection against water deficit and the second one controls the establishment of proteins involved in signal regulation and transduction (Abbas et al., 2022, Mundree et al., 2002). In soybean (*Glycine max*), Creelman and Mullet (1991) showed the accumulation of transcripts corresponding to GRP (glycine-rich proteins), PRP (proline-rich proteins) or expensin (HRGP: hydroxyproline-rich glycoproteins) (Li et al., 2019, Creelman and Mullet, 1991). In *Craterostigma plantagineum*, an increase in mRNAs corresponding to the ELIP (early light-inducible protein) genes has been observed (Mihailova et al., 2022). These genes are induced by light and play a role in the assembly of the photosystems. Under drought conditions, chloroplasts undergo morphological changes during which ELIP proteins maintain the assembly of the photosystems and pigments, structures that are essential for the functioning of the photosynthetic machinery (Challabathula et al., 2018). Identical results have been observed in *Sporobolus stapfianus* (Challabathula et al., 2018). At the cellular level, signal conversion induces the synthesis of messengers causing changes in intracellular Ca^{2+} concentrations that initiate a protein phosphorylation cascade which would activate specific genes that would eventually trigger the signaling mechanism (Cheng et al., 2022). These regulatory molecules, such as abscisic acid (ABA) and salicylic acid, are involved in the initiation of other signaling cascades (Kumar et al., 2019).

Abscisic acid (ABA) acts on certain tissues or on isolated cells such as guard cells leading to the decrease of their turgor as a consequence of the limitation of cation and anion influx (Zhang et al., 2020). In addition to its involvement in stomatal closure, ABA as a stress hormone, causes leaf drop and dormancy (Parwez et al., 2022). It is also involved in the regulation of membrane channel permeability to ions (Singh et al., 2021). Under drought conditions, ABA has a beneficial effect on hydraulic conductivity of roots as it plays an important role in increased water uptake and transport in plants (Nadeem et al., 2019a). ABA is actually synthesized in the root cap and transported by the sap to the leaves mainly to the guard cells to enable stomatal closure (Singh et al., 2021). As, the opening and closing of stomates is controlled by guard cell chloroplasts (GCC), since these later are responsible for the movement of osmolytes that influence cell water potential (Wise, 2016).

Moreover, this plant stress hormone stimulates superoxide radicals and H_2O_2 which will eventually stimulate the activities of antioxidant enzymes such as glutathione reductase (GR). In this way ABA can improve plants drought resistance as a stimulus of gene overexpression (Nadeem et al., 2019a).

1.2 Plant defense strategies to water shortage :

Plants resistance to drought conditions is usually correlated to its ability to minimize water loses. Which is why traits relative to water uptake, storage, and efficient use of captured water have a big influence on plants drought resistance (Sletvold and Ågren, 2012, Ludlow, 1989). Plants respond to drought stress by inducing multiple morphological, physiological and biochemical responses to enable them to adapt better to soil water availability (Kapoor et al., 2020). They have evolved several sophisticated and complex adaptive mechanisms and strategies to survive extreme conditions like drought. Indeed, adaptation conferring drought resistance have historically been divided into three major strategies: that include drought escape, drought avoidance, and drought tolerance (Batool et al., 2020, Nadeem et al., 2019a, Ludlow, 1989). Depending on the severity of the stress, these strategies may generate an increase in growth rate under mild drought conditions (drought avoidance) or a low growth rate with high survival rate (drought tolerance) under severe drought conditions (Keep et al., 2021). Plants may also develop rapidly and reproduce before drought conditions become severe which is also a very smart strategy to survive adverse cues (drought escape) (Kooyers, 2015). Indeed, it's beneficial or even necessary to understand how plants behave and witch strategy and mechanism they use to survive adverse environmental conditions to increase biomass production and optimize plant performance under drought conditions in arid and semi-arid environments (Kumar et al., 2021).

1.2.1 Drought Avoidance and Escape

Stressed plants try to avoid drought conditions by undergoing some physiological changes that enable them to minimize water loss, increase water uptake and maintain their osmotic adjustment (Seleiman et al., 2021). These changes include increasing sugar content of roots and leaves, in addition to closing stomata, and delaying leaf senescence (Hanly et al., 2020). Plants can also enhance biosynthesis and transport of soluble secondary metabolites and inorganic compounds, known as osmolytes (sugars, amino acids, and other organic molecules), to maintain cell turgor and hydrostatic pressure at lower soil water potential (Khatun et al., 2021a). It should be mentioned that even though these strategies can help the plant avoid stress, they also affect plant growth, biomass production and reproduction (Wasaya et al., 2018).
Some plants tend to speed up their growth and development and shorten their life span before the beginning of the hot season to escape adverse drought conditions (Álvarez et al., 2018). It's harder for annuals plants with shallow-root systems to survive low soil moisture than perennial plants with deep rooting system (Nadeem et al., 2019a). The phenological development of plants should be coordinated with a periods of soil moisture availability including early flowering. Usually flowering starts when rainy seasons are over, which is an adaptive approach. This strategy is

very common trait in legumes and cereals (Kumar, 2017). However, like avoidance mechanisms, escape mechanisms also have a subside effect on plant growth and productivity (Khatun et al., 2021a).

1.2.2 Tolerance mechanisms :

Plant's level of tolerance to environmental constraints is very different depending on the species and the severity of the applied constraint (Riyazuddin et al., 2021). It has been observed in some earlier experiments that the degree of tolerance and plant growth are two correlated factors. Plants with high tolerance to extreme conditions show a reduced growth rate, opposite to , plants with low tolerance may produce much biomass but express low survival rate (Koyro and Huchzermeyer, 2022).
Another intelligent strategy applied by plants with high tolerance to adapt to water shortage is by limiting photosynthetic activity by reducing plant leaf area and reducing the number of new leaves. Nevertheless, the increase of root, length compared to shoots, and the expansion of its size and density is by far the optimum strategy for drought-tolerant plants to cope against the adverse effects of water shortage (Seleiman et al., 2021).

1.3 Influence of water shortage on plant performance:

1.3.1 Germination and reproductive stage:

The adverse effects of water limitations have frequently affected several aspects of plant growth (Nadeem et al., 2019a). As it was reported, drought often lead to a severe reduction in germination rate and reproductivity , which are critical factors in the determination of plant productivity (Zhang et al., 2018). These statements match to some findings in which water scarcity significantly reduced the germination rate in soybean and chickpea in addition to the significant reduction in yield in faba bean (Nadeem et al., 2019a). It should be mentioned that some studies reported that studying the germination stage of plants could be very informative as it helps to elucidate the sensitivity of plants to water stress as it proved that moisture contents can influence enzyme activation during germination (Khatun et al., 2021a).

1.3.2 Growth:

Due to global climate change, drought has become one of the most significant abiotic constraints limiting crop yields and productivity and has adverse effects on legume crops all over the world (Nadeem et al., 2019a). To survive under these circumstances plants had to undergo several changes in their morphological, physiological, biochemical and molecular processes to reduce the impact on water relation, gas exchange, photosynthesis, gene expressions (Ahanger et al., 2017, Mostofa et al., 2018), the metabolism of organic compounds (Osakabe et al., 2014) in addition to the regulation of the membrane system (Krasensky and Jonak, 2012). These changes resulted in the alteration of plant growth, thereby inhibiting the plant nutrient uptake leaf senescence and reductions in yield (Elemike et al., 2019) leading to substantial losses in agricultural productivity (Staudinger et al., 2016). Undoubtedly, drought is known as the most limiting constraint in agriculture which is why the changes in plant growth and response mechanisms under drought constraints have always been important scientific research subjects (Seleiman et al., 2021, He et al., 2020).

Plant production in terms of biomass and water content are found to be positively correlated (Khatun et al., 2021a). Indeed, water deficit affects the physiology of the plant in several ways, and results in a reduction or inhibition of its growth (Sadiqi et al., 2022). Depending on the species and the stage of development (germination, growth and reproduction), water deficit can lead to a stronger reduction in root growth, than that of the aerial parts (Khatun et al., 2021a), which is manifested by a reduction in leaf surface, leaf curling, and even an acceleration in senescence (Slama et al., 2007). The purpose of these morphological changes is to limit water loss, which influences the rate of carbon assimilation by the leaves and consequently the photosynthetic functioning of the plant (Seleiman et al., 2021).

1.3.3 Water relations and osmotic adjustment:

Water deficit often leads to internal water imbalances (Kapoor et al., 2020). There is a strong correlation between water availability and plant growth as cell enlargement is more affected by water shortage than cell division (Humplík et al., 2017). It can also lead to decreases in cell water potential, and turgor pressure, cell dehydration, shoot and leaf wilting and senescence, growth inhibition, and eventually plant death (Seleiman et al., 2019).

It should be mentioned, that plants can only prevent water loss by transpiration in the leaves if the water potential in the plant is lower than that in the soil (Koyro and Huchzermeyer, 2018). The plant performance in drought conditions is strongly dependent on the intensity of this constraint and the other related environmental constraints (Seleiman et al., 2021). Moreover, a gradient of decreasing water potential (Ψ) must be established for water to flow through the soil–plant–atmosphere continuum (Koyro and Huchzermeyer, 2018). To compensate for the water loss by transpiration, roots tend to grow deeper in the soil to absorb more water (Goche et al., 2020).

It should be mentioned that drought initiate a decrease in cell water potential and consequently reduce essential minerals absorption like potassium phosphorous and nitrogen. In addition, reduced minerals absorption induces nutrients imbalances and as it affects the efficiency of their translocation to the target sites in plants growing in drought conditions (Ahanger et al., 2016). Therefore, vital mechanisms would be affected, such as photosynthetic mechanisms. Indeed, insufficient nutrient uptake can have negative impact on the regulation of stomatal conductance and carbon dioxide diffusion (Iqbal et al., 2020)

One of the fundamental strategies implied by plants to survive adverse environmental constrains is the accumulation of compatible solutes (Koyro and Huchzermeyer, 2018). These compatible solutes are either nitrogen-containing compounds such as polyamines and ammonium compounds, amino acids and proline, or hydroxyl compounds like polyhydric alcohols, sucrose and oligosaccharides, (Nadeem et al., 2019a). They facilitate both water absorption (by reducing cytoplasmic osmotic potential) and nutrient uptake (Seleiman et al., 2021). They participate also in proteins stabilization, excess ROS scavenging as well as the maintenance of cellular redox balance (Tani et al., 2019).

Drought can induce a decrease in the cytosolic volume (Ozturk et al., 2021). The increased production and storage of compatible solutes can also help to decrease the osmotic potential in the cytosol (Koyro and Huchzermeyer, 2018, Al-Yasi et al., 2020).

Plants accumulate proline as the first response to water stress (Khatun et al., 2021a). This solute plays an important role as it acts as a signaling molecule to modify cell organelle function. It regulates mitochondria function, stabilizes subcellular structures, and promotes cell proliferation by regulating the activation of specific stress responsive genes, which are essential for stress recovery (Nadeem et al., 2019a). Moreover, proline contributes in the anti-oxidant defense, it reduces oxidation of lipids and maintains membrane integrity (Ozturk et al., 2021). Also, it contributes in free radicals scavenging and buffering of cellular redox potential (Khatun et al., 2021a). It should be noted that Bhaskara (2015) reported that proline synthesis and degradation activities is what ensure resistance in plants not proline storage (Bhaskara et al., 2015). As a matter of fact, proline synthesis and proline degradation are regulated by ABA- responsive genes (Koyro and Huchzermeyer, 2018, Ozturk et al., 2021).

1.4 Photosynthesis performance under drought constraint:

Water limitation can lead to an impairment of photosynthesis in all its phases mainly due to the reduction of CO_2 diffusion to the chloroplast and to metabolic constraints (Tani et al., 2019). This is a key phenomenon of water deficiency (Pinheiro and Chaves, 2011). There is a strong correlation between photosynthesis, transpiration, plant growth and development (Gharred et al., 2022). In this field of tension, crop yield has always been seen in connection with CO_2/H_2O gas exchange (Koyro and Huchzermeyer, 2018).

The reduction in photosynthetic activity due to water shortage can be induced by both stomatal and non-stomatal limitations depending on the intensity of the applied constraints in addition to the differences among species, as well as other environmental constraints, may also play a role in the relative importance of stomatal *vs*. nonstomatal limitations (Dias and Brüggemann, 2010). In case of moderate stress, photosynthesis is usually regulated by stomatal limitations. However when the stress is intensified, the regulation of photosynthesis switch to non-stomatal limitation in chloroplast CO_2 fixation ability, rather than CO_2 diffusion resistance (He et al., 2020). In the same way, it was observed in some publications that mild stress may increase the respiration rate on plants (Seleiman et al., 2021).

It's important to mention that coordinated regulation of photosynthesis in plants can increase biomass production and resistance (Gharred et al., 2022). When faced with water limitation, usually plants reduce transpiration rate by decreasing stomatal conductance so they can prevent leaf water loss and increase water use efficiency. The decrease of photosynthesis at limited water supply is mainly caused by the reduction of intercellular CO_2 concentration (Ci) caused by stomatal closure which would restricts rates of CO_2 diffusion into the leaf and reduced mesophyll conductance (Dias and Brüggemann, 2010). Reduced intercellular CO_2 concentration causes metabolic impairment, especially decreases in RuBisCO activity and synthesis resulting in inhibition of Calvin cycle activity, hence, reduced electrons transport to PSII and reduction of ATP production resulting in photoinhibition, these measures would eventually affect net CO_2 assimilation. (Ahanger et al., 2021).

The regulation of photosynthesis machinery by stomatal and non-stomatal limitations under drought conditions was always a controversy subject between scientists. Studying the deviations in the intercellular CO_2 concentration (Ci) levels and stomatal limitation rates enable us to better understand whether the changes in net CO_2 assimilation are regulated by stomatal or non-stomatal limitations (He et al., 2020).

1.4.1 Impact on Gas Exchange (dark reaction):

RuBisCO (ribulose bisphosphate carboxygenase /oxygenase) is the most abundant protein in leaves (Koyro and Huchzermeyer, 2018). It's a key metabolic enzyme in plants photosynthetic carbon assimilation, and it determines the efficiency of photosynthesis (Lyu et al., 2016). It's capable of catalyzing both a carboxylation and an oxygenation reaction depending on the physiological conditions of the plant. RuBisCO initiate the first stage of the Calvin cycle by catalyzing the carboxylation of ribulose-1,5-bisphosphate and CO_2 to form two molecules of 3 phosphoglyceric acid (3-PGA) (Zhang et al., 2021) and in the same time it can use O_2 to oxygenate substrates, which ultimately results in ATP/NADPH consumption and release of fixed CO_2 (Benedetti et al., 2018a). Thus CO_2 and O_2 are constantly competing at the catalytic site of RuBisCO (Koyro and Huchzermeyer, 2018). In non-stress conditions RuBisCO has a high affinity to CO_2. However, under drought conditions, the closure of stomates limits CO_2 diffusion into the leaf. A critical point for plant physiology is reached when CO_2 fixation (apparent photosynthesis) falls below CO_2 production (compensation point (see Fig. 2)) (Koyro and Huchzermeyer, 2018). CO_2 availability to RuBisCO will be affected resulting in the inhibition of Calvin cycle activity, hence, decreased demand for NADPH and ATP (Vanlerberghe et al., 2016).

The inhibition of photosynthetic activity due to low soil water potential can lead to energy imbalances in the chloroplast (Dahal and Vanlerberghe, 2017) including the inhibition of photosynthetic electron transport (Koyro and Huchzermeyer, 2022).

Another consequence of gas exchange limitation is the increase of CO_2/O_2 partial pressure ratio resulting in the inhibition of the water-splitting system (Koyro and Huchzermeyer, 2018). As oxygen is produced by the water-splitting system located adjacent to PSII, ROS formation is a real risk. Therefore, the degree of ROS damage and the efficiency of ROS detoxification system in the chloroplasts are of crucial importance for plant survival (Gharred et al., 2022).

1.4.2 Impact on Chlorophyll fluorescence (light reaction):

Drought constraint reduces photosynthesis and photosynthetic capacity (Ahluwalia et al., 2021). As a result, plants subjected to water shortage require lower light intensity to saturate photosynthesis. Under these conditions, it's unlikely for plants to use all the excess light energy for photosynthesis. The excess light energy in PSII, which was neither used nor dissipated is defined as photoinhibition (de Oliveira Maia Júnior et al., 2020). As a way to protect its photosynthetic machinery damage from excess light, the susceptibility of stressed plants to photoinhibition is much higher compared to plants grown in optimal conditions (Dias and Brüggemann, 2010, Siddique et al., 2016).

Generally and independently of the stress, a typical photosynthesis light saturation curve displays 3 phases: first, photosynthetic rate is linear when light intensity is low. At increasing light intensity photosynthetic rate increases as a function of light. However, because of the limiting capacity to fix CO_2, and when the irradiation is too high to overcome the rate of downstream biochemical reactions, plants subjected to high light intensity for a long time exceeding a critical threshold which reduce the photosynthetic activity. This phenomena is identified as heat dissipation or photoinhibition (see photo 1) (Koyro and Huchzermeyer, 2022, Benedetti et al., 2018b).

Drought is usually associated with increased photoinhibition, particularly affecting Photosystem II (PSII). This is because PSII is more sensitive to excess light than Photosystem I (PSI), especially during drought when plants close their stomates to minimize water loss through transpiration. The resulting decrease in internal CO_2 concentration (Ci) leads to reduced RuBisCO synthesis and activity, along with a decline in electron transport rate (ETR) through PSII. Additionally, the reduction in photochemical quenching of chlorophyll fluorescence contributes to photoinhibition (Ahanger et al., 2021).

Non-photochemical quenching (NPQ) mechanism as indicators of excess light energy dissipation to heat in the PSII antennae are meant to protect plants from excess light by balancing the match between the excitation energy being transferred to PSII and that being used in photochemistry. They ensure the de-excitation of chloroplasts that operates in the PSII mainly to prevent over-excitation of reaction centers (Benedetti et al., 2018a).

Drought is associated with the increased engagement of chloroplast NPQ mechanisms (Dahal and Vanlerberghe, 2017).

The measurement of chlorophyll fluorescence enables to study the solar energy distribution in Photosystem II (PSII) without damage to the plant leaf especially when plants are subjected to environmental constraints (Abideen et al., 2022). Pulse-amplitude modulation (PAM) fluorometry and the saturation pulse (SP) are the most frequently used methods to analyze the photosynthetic performance of green plants (Cruz and Avenson, 2021, Baker, 2008). Modulated fluorescence and SP offer the possibility to do quenching analysis and to study various fluorescence parameters (Brestic and Zivcak, 2013a). Indeed, effective Quantum Yield of Photochemical Energy Conversion in PSII proved to be very useful for the analysis of the photosynthetic performance of plants as it allow deep insights into the plant's capacity to cope with excess excitation energy.

Florescence yield is actually the result of competition for excitation energy among several pathways. It was described in different forms in many papers but the simplest one was presented by Genty et al. (1996). He divided the absorbed excitation energy (1 = 100%) in PSII in three fundamental pathways expressed in terms of the complementary quantum yields of Y(II), Y(NPQ) and Y(NO) (Klughammer and Schreiber, 2008, Genty and Harbinson, 1996).

Y(II) value corresponds to the effective quantum yield of PSII that is converted into chemical energy by the photochemical charge separation in PSII active reaction centers (Malambane et al., 2023). It's the most useful parameter derived from quenching analysis as it measures the fraction of photons absorbed in P680 antennae and utilized in the PSII photochemistry. It quantifies as well the efficiency of the linear electron transport rate. Therefore, Y(II) analysis are usually correlated with photorespiration rate and the quantum yield of carbon fixation (Osório et al., 2011).

On the other hand, Y(NPQ), the non-photochemical quenching parameter, represents the quantum yield of downregulated energy dissipation in PSII antenna in form of heat via the regulated photoprotective NPQ mechanism (Malambane et al., 2023). Open reaction centers affect Y(NPQ) parameters. When Y(NPQ) values are high, It is a proof that the plant is dealing with high light intensity and it's trying to protect itself by rapid and reversible harmless thermal dissipation to avoid ROS formation which would cause irreversible damage (Osório et al., 2011, Gorbunov and Falkowski, 2022, Li et al., 2020).

Y(NO) corresponds to the combined pathways of the quantum yields of fluorescence and constitutive thermal loss, leading to non-photochemical losses that do not involve the non-photochemical quenching (NPQ) mechanisms, primarily due to closed PSII reaction centers (Klughammer and Schreiber, 2008). It should be mentioned that an increase in Y(NO) proves the inefficiency of both photochemical energy conversion and protective regulatory mechanisms in shielding the plant from incident radiations, which may lead to photodamage (Malambane et al., 2023).

Maximum quantum yield of PSII photochemistry (Fv/Fm) is considered as an indicator of photoinhibition, it is also an index of photosynthesis efficiency. It measures the maximum of photochemical efficiency of open PSII reaction centers. In optimum conditions its value for most plants is equal to 0,832. When plants are subjected to environmental constraints its value decreases (Brestic and Zivcak, 2013b).

It should be mentioned that the monitoring of chlorophyll fluorescence enables to evaluate light compensation point deviations in stressed and non-stressed conditions. Previous studies showed that in most crops (C3 plants), an increase in the intensity of applied drought stress would induce an increase in the light compensation point (Dias and Brüggemann, 2010, Koyro and Huchzermeyer, 2022)

In C3 plants, under a given light intensity, both Fv/Fm, the potential of maximum quantum yield of PSII photochemistry and Y(II), the actual quantum yield of PSII don’t usually show a big variation when plants are submitted to a mild water deficit (Koyro and Huchzermeyer, 2018). Nevertheless, a severe applied constraint can significantly reduce both of them when photoinhibition occurs. In the same way, a mild drought constraint may not affect electron transport rate and can even act as preadaptation to the stress (Dias and Brüggemann, 2010).

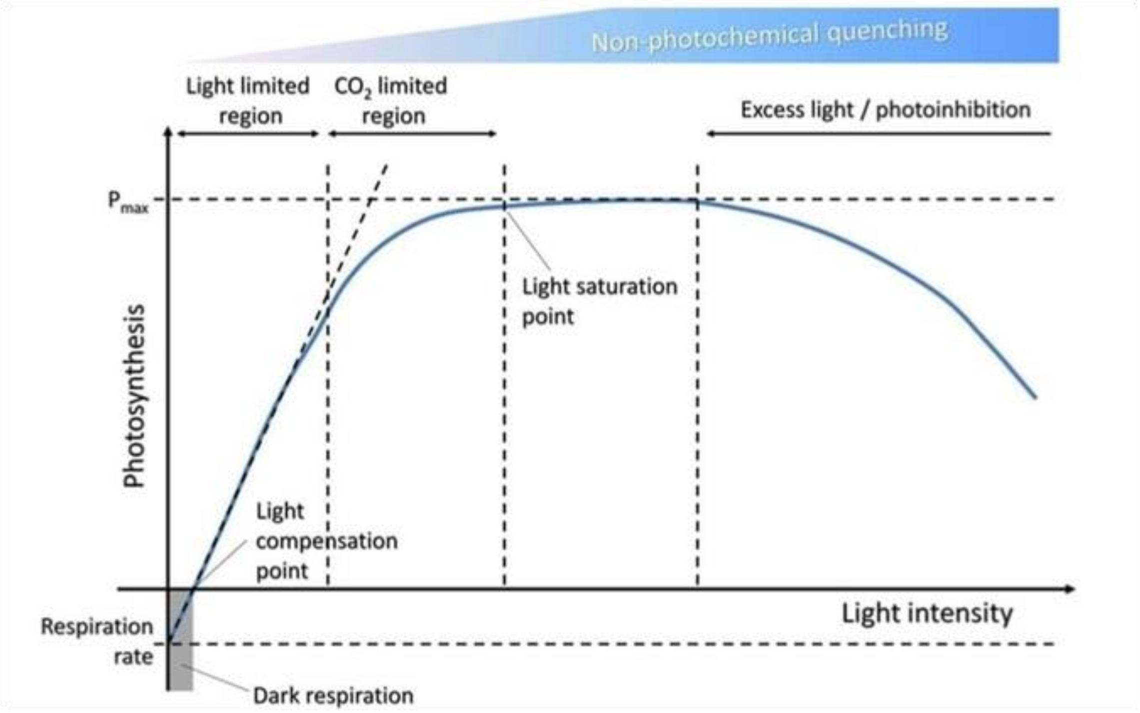

Figure 1.2: Typical curve of photosynthesis increase at increasing light intensity until saturation (light saturation point). Finally photoinhibition occurs and the efficiency of net CO_2 assimilation decline (Benedetti et al., 2018a)

1.4.3 Impact on Photorespiration :

Usually, when photoinhibition increases the rate of photorespiration (thermal dissipation) increases too, as photorespiration works as an alternative sink to get rid of excess light energy (Siddique et al., 2016). The decrease of photosynthetic activity in plants subjected to environmental constraints is partially buffered by the increase of photorespiration (Koyro and Huchzermeyer, 2022). However the sum of both is usually not sufficient to maintain homeostasis of cells leading to the production of ROS in mitochondria, chloroplasts, and peroxisomes, as well as, the plasma membrane and the apoplast. This later is the main factory for ROS production in response to endogenous signals and exogenous environmental constraints (Billah et al., 2021).

Photorespiration is one of the mechanisms plant use to get rid of solar energy by heat dissipation. It’s actually a good strategy to avoid photosystem II damage under drought conditions (Abideen et al., 2022). As it was mentioned before, plants close stomates in response to drought stress, reduce transpiration rate and gas exchange which will consequently increase the O_2/CO_2 concentration ratio and $NADP^+/ATP$ ratio in plant leaves.

Photorespiration uses energy provided by photosynthetic electron transport to consume O_2 and release previously fixed carbon CO_2 in mitochondria (Timm and Hagemann, 2020). It starts with the binding of molecular oxygen to the acceptor ribulose-1,5-bisphosphate catalyzed by RuBisCO. Contrary to the carboxylation reaction previously described that releases two 3-phosphoglycerate used in the Calvin–Benson cycle, 2-phosphoglycolate are synthesized through ribulose-1,5-bisphosphate oxygenation (see photo 3). 2-phosphoglycolate must be processed through photorespiration subsequent reactions at the expense of energy supplied by photosynthetic electron transport (Kalaji et al., 2017).

As an alternative sink, photorespiration pathway requires energy (ATP) to release CO_2 that can be refixed again. When $NADP^+/ATP$ ratio is high due to the high electron pressure occurring on PSI acceptors usually happening on stress conditions, photorespiration plays its role of photoprotection and uses the surplus energy to reduce $NADP^+$ and produce CO_2 (Koyro and Huchzermeyer, 2022).

It should be mentioned that photorespiration (heat dissipation) is mainly an issue for C3 plants, in C4 plants photorespiration is negligible. It's actually a good strategy that C3 plants use to get rid of excess light energy and in the same time produce their own internal CO_2 (Kalaji et al., 2017). It occurs simultaneously with respiration and regular net CO_2 assimilation therefore photosynthesis and photorespiration are constantly competing for absorbed light energy (Timm and Hagemann, 2020, Koyro and Huchzermeyer, 2022).

Indeed, the reactions of photosynthesis, chlorophyll fluorescence, heat release and ROS formation are constantly competing for energy from light-activated chlorophyll (Foyer and Harbinson, 2019). While chlorophyll fluorescence consumes less than 5% of light energy and heat release has almost constant rate. Photosynthetic electron transport significantly depends on the availability of light harvesting complex (electron acceptors). ROS formation varies with the partial pressure of oxygen (Koyro and Huchzermeyer, 2018). Actually, due to the decrease of CO_2/O_2 partial pressure ratio, light activated chlorophyll can direct more electrons to produce ROS by transferring an electron to an oxygen molecule to form an oxygen radical (Koyro and Huchzermeyer, 2022).

By channeling its energy to release CO_2 which is actually a way to open up an additional chloroplast electron sinks, photorespiration contributes to the prevention of ROS production and supports plant defense system (Vanlerberghe et al., 2016).

Moreover, photorespiration contributes in photosynthetic quenching, it is usually accompanied by harvesting complexes, involving the xanthophyll cycle, lutein cycle, water-to-water cycle that involves the Mehler reaction and synthesis of photoprotective compounds such as carotenoids that control plant thermal dissipation (Abideen et al., 2022). These mechanisms are very important to prevent PSII damage during drought (Abideen et al., 2022).

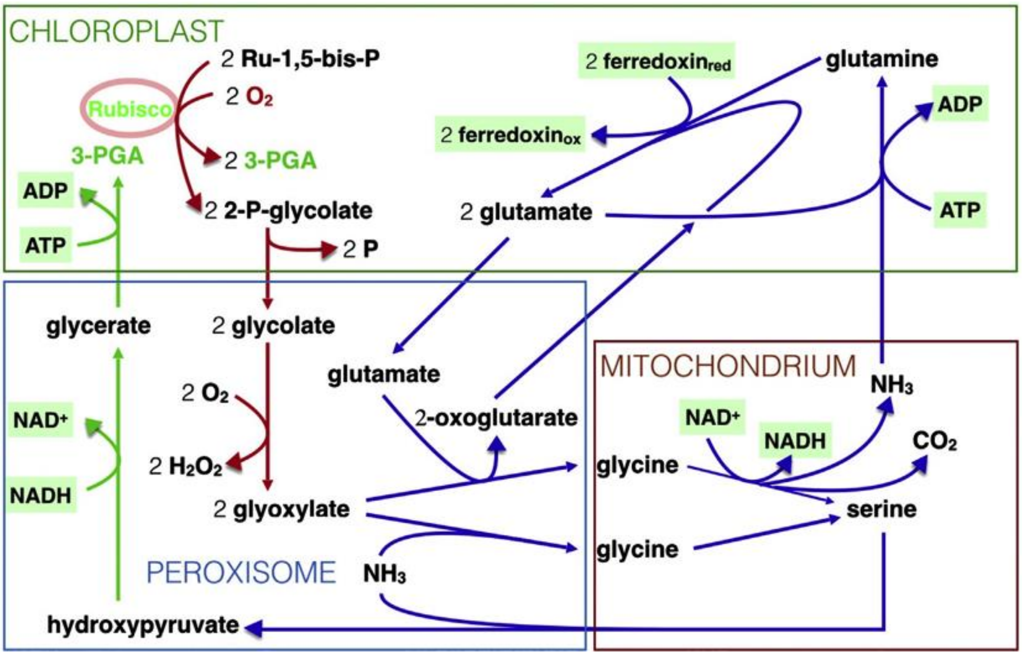

Figure 1.3 Major photorespiration pathway: It occurs mainly in three interacting subcellular compartments which are chloroplasts, peroxisomes and mitochondria. Photorespiration uses energy provided by photosynthetic electron transport to reduce O_2 and release previously fixed carbon CO_2 in mitochondria reactions at the expense of energy supplied by photosynthetic electron transport (more details are included in the text) (Koyro and Huchzermeyer, 2018)

1.4.4 Impact on the Anti-oxidant response :

The limitation in stomatal conductance by water deficiency can induce a decline in

carbon fixation and also in the flow of electrons to Photosystem II (Y (ll)) (Abideen et al., 2022). This is very critical for the plant, because when the export of carbohydrates from the chloroplast decrease, the Calvin Benson cycle mechanism will be inhibited too and the recycling of $NADP^+$ and ADP would be altered (see photo 4). As a consequence, they won't be functional as primary acceptors of photosynthetic energy flow (Dahal and Vanlerberghe, 2017). Consequently, the quantum yield of PSII (Y(II)) needs to be reduced (Koyro and Huchzermeyer, 2022) and the plant has to get rid of the excessive light excitation energy through heat release (Y(NPQ)) and chlorophyll fluorescence (Y(NO)) to prevent the production of reactive oxygen species (ROS) (Vanlerberghe et al., 2016) .
There is a competition between photosynthetic electron transport and ROS formation for electrons from the light-activated chlorophyll. Because of stomatal limitation under drought conditions, ROS production can exceed ADP and $NADP^+$ recycling, in this case, chloroplast won't be able to detoxify itself and may reach a toxic level (Koyro and Huchzermeyer, 2022). Therefore, a good functioning of photosynthetic electron transport and reduction of ROS production can be assured if the plant can keep light energy balanced by permanent recycling of the two energy acceptors $NADP^+$ and ADP (see photo 4) (McCormick et al., 2006).

Some drought resistant plants are able to regulate the photosynthetic machinery and photoprotective mechanism to maintain optimum ATP synthesis, and NADP formation and reduce the toxic effects of ROS (Abideen et al., 2022). On the other hand, Drought-sensitive plants can ultimately succumb to oxidative stress due to the accumulation of ROS including free radicals and nonradical molecules that cause changes in signaling cascades and transcription factors and finally cell apoptosis (Hanly et al., 2020, Hasanuzzaman et al., 2020a). This later, will disrupt the growth of plants (Zhang et al., 2021).

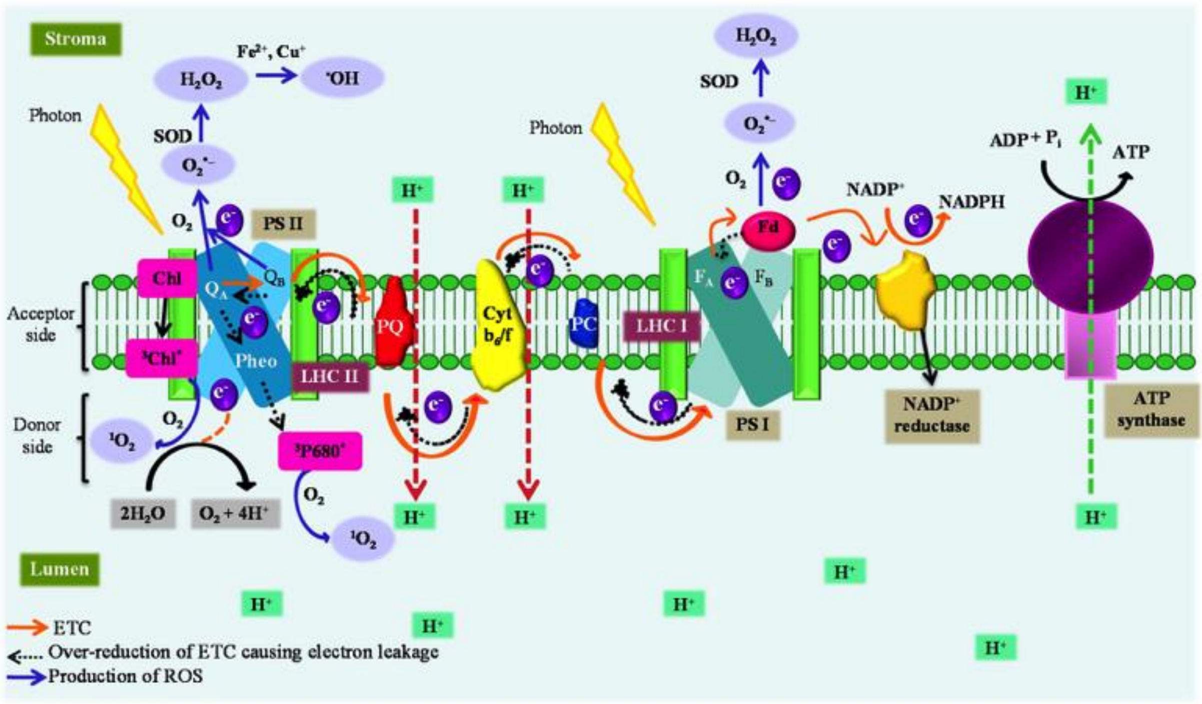

Figure 1.4 Photosynthetic electron transport rate (ETR): ATP and NADPH synthesis. When plants are subjected to abiotic stress, photosynthetic electron transport chain triggers the ROS formation (Sachdev et al., 2021). Photosystem I (PSI), Photosystem II (PSII), Cytochrome b6/f complex (Cyt b6f), plastocyanin (PC), Ferredoxin (Fd), light-harvesting complex I and II (LHC I and II)

2. The Oxidative Stress:

As mentioned by many reviews, reactive oxygen species (ROS) are the production of an initial response of drought-stressed plants. ROS include hydrogen peroxide (H_2O_2), superoxide anion (O_2^-), hydroxyl radical ($\cdot OH$), alkoxy radicals (RO) and singlet oxygen ($^1O_2$), each showing a characteristic half-life and oxidizing potential and acts as a messenger to activate defense mechanisms in plant (Tanwir et al., 2022). It has been discovered recently that the reactive oxygen species can be identified as universal signaling metabolites and their role is actually the induction of redox signals, which regulate a diverse array of cellular responses needed for cellular redox homeostasis (Farooq et al., 2019). ROS have a crucial dual role in plants which can be constructive or destructive depending on plant growth conditions. For instance, in low concentration, ROS production is involved in the plant cell regulation as they help to maintain and control cell cycle and growth (including cellular proliferation and differentiation) as well as cell apoptosis (Xie et al., 2019). Moreover, they play an important role in hormone signaling pathway, plant responses to biotic and abiotic constrains, and development of tissues (Laus et al., 2021). Actually, ROS play a key signaling role in the adaptation process of plants to abiotic constraints, they act as a secondary messengers using mitogen-activated protein kinase (MAPK) pathway to transport signals to the nucleus through redox reactions to ameliorate plant performance under environmental stress (Singh et al., 2019). However, it's very critical for plants if ROS production is higher than ROS scavenging as it may cause oxidative damage to the cell's enzyme by protein oxidation, membrane lipids and genetic material witch will affect the impairment of cellular integrity and functionality and promote cell apoptosis (Khatun et al., 2021a). This condition is known as oxidative stress and it's characterized by a rapid leakage of excess ROS into other parts of the plant (Laus et al., 2021).

2.1. Lipid peroxidation :

When plants are subjected to water deficit, The increase of ROS production is usually correlated with the increase of lipids peroxidation in the cell membrane which has been linked to oxidative damage (Khatun et al., 2021a). ROS will eventually increase the extent of lipid peroxidation and the level of the highly reactive molecules of malondialdehyde (MDA one of the final products of lipid peroxidation) and as a consequence destroy the membranes completely (see photo 5) (Kosar et al., 2021)

The increase of MDA content in leaves under drought conditions is actually linked to an enzymatic breakdown in cells. This phenomenon is considered as an indicator of oxidative stress (Yildirim et al., 2021). For example, water deficiency lead to an increased lipid peroxidation in pea leaves four times compared to plants grown in the control conditions (Khatun et al., 2021a). Similar responses were shown in other plants like for example *Dendrocalamus minor var. amoenus* (ghost Bamboo), cabbage seedlings and *Helianthus annuus L.* (He et al., 2020, Kosar et al., 2021, Yildirim et al., 2021).

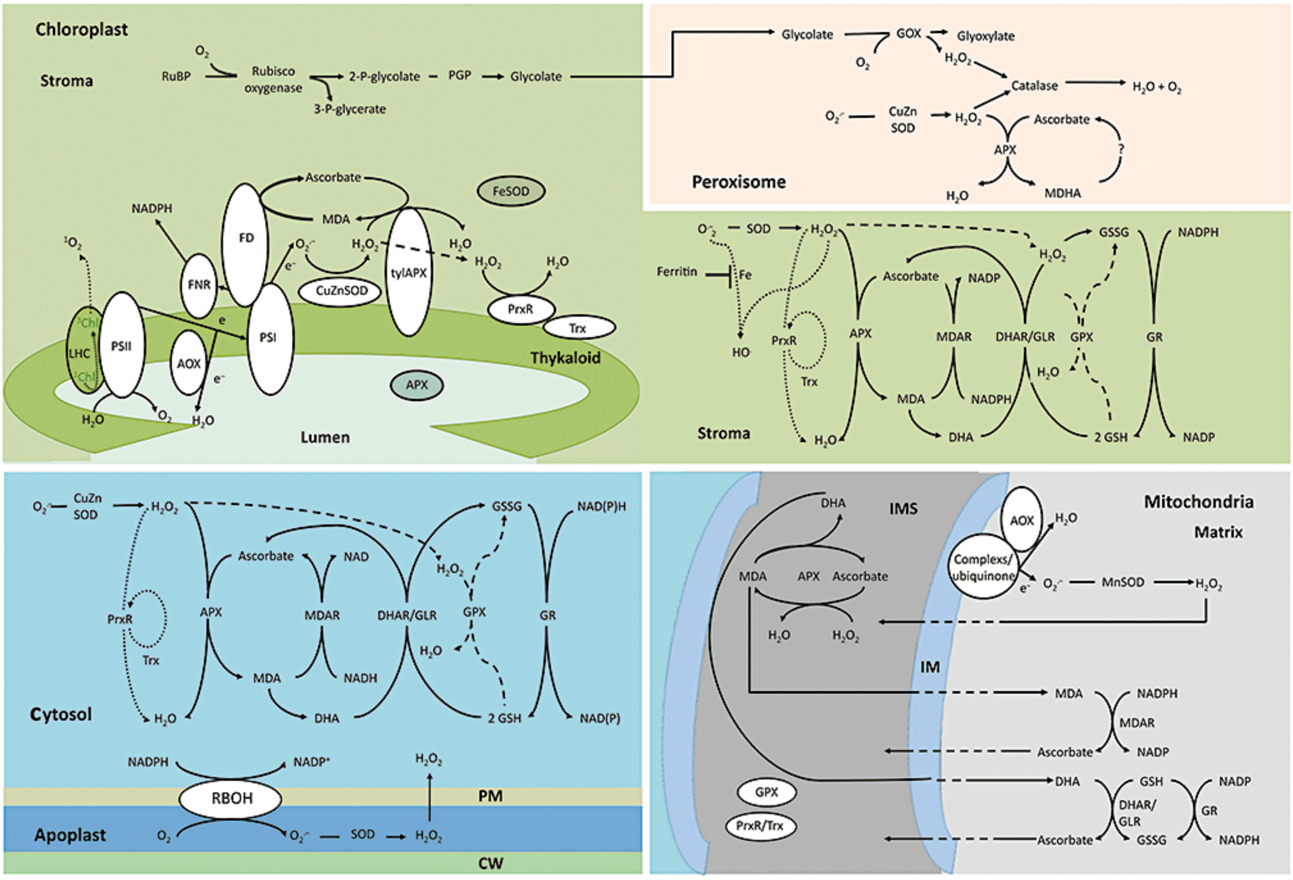

Figure 1.5 ROS scavenging in different compartments in plant cells (Miller et al., 2010).

2.2. The anti-oxidant defense:

Experiencing drought, plants undergo a series of responses, including the development of a high capacity system to scavenge ROS (Xie et al., 2019). A set of active oxygen species detoxification systems has been formed in plants to combat oxidative damage in which ROS-scavenging enzymatic antioxidants and nonenzymic antioxidants working together to regulate the defensive mechanisms of ROS in the cells (Garcia-Caparros et al., 2021). They are located in different sites of plant cells, and they directly or indirectly play a key role in ROS homeostasis by minimizing, buffering, and efficiently scavenging ROS to avoid oxidative damage (Billah et al., 2021). Plant resistance to water stress is evaluated based on stress-induced endogenous antioxidants contents along with enzymatic activities (Billah et al., 2021). In plants, the foremost relevant enzymatic antioxidants comprise catalase (CAT), superoxide dismutase (SOD), ascorbate peroxide (APX), glutathione peroxidase

(GPX), guaiacol peroxidase (GPOX), glutathione reductase (GR) and monodehydroascorbate reductase (MDHAR) (Nadeem et al., 2019a). The efficiency of ROS removal is much higher in reactions mediated by enzymatic systems (Laus et al., 2021). They are directly engaged in catalyzing ROS degradation reactions by directly scavenging ROS and indirectly reducing membrane lipid peroxidation and alleviating the damage in PSII structure and function (see photo 5 (Miller et al., 2010).) (Gharred et al., 2022).

Besides enzymatic antioxidants, the foremost prominent non-enzymatic antioxidants include lipophile compounds such as tocopherols and carotenoids as well as hydrophile molecules like glutathione (GSH), reduced ascorbate (AsA), oxidized ascorbate (dehydroascorbate (DHA)) and phenolics (Nadeem et al., 2019a). These molecules can play a role in the antioxidant system in two ways, either directly interacting with ROS and detoxify them thus playing an important role in plant protection from oxidative stress and in optimizing plant stress resistance (Laus et al., 2021) or functioning as substrates in enzyme-catalyzed ROS-degrading reactions.

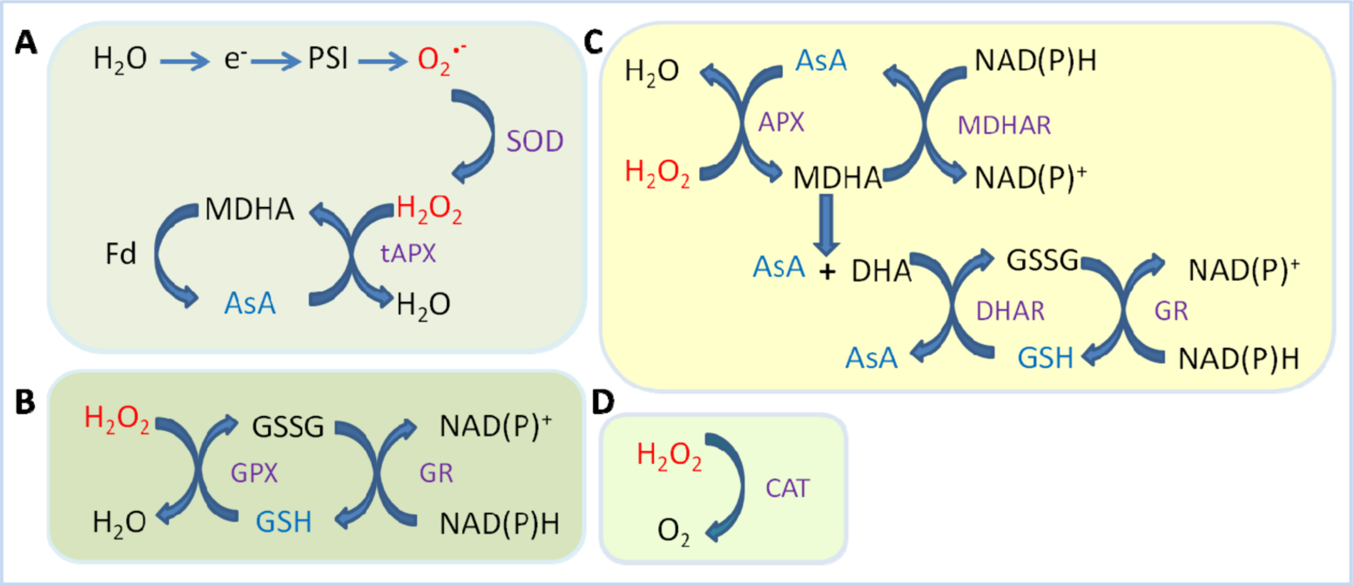

Figure 1.6 Reactive oxygen species (ROS) detoxification mechanisms in plants cells: (A) The Mehler reaction (water-water cycle), (B) The glutathione peroxidase (GPX) cycle, (C) The ascorbate-glutathione (AsA-GSH) cycle, (D) The catalase reaction. (Racchi, 2013) .

The increased production of ROS accumulation due to water scarcity affects the enzymatic activities of ascorbate-glutathione AsA-GSH (C) pathway for ROS detoxification (Hasanuzzaman et al., 2020b) known also as Halliwell-Asada pathway (Kumari et al., 2021). It is the major pathway of antioxidant defense and it takes place in the chloroplast where it provides photo-protection by detoxification of H_2O_2 (Panda et al., 2021). Depending on the plant species, age, drought intensity, and duration, plants cells can regulate the enzymatic antioxidant activities of AsA-GSH pathways in more than one way. In addition to AsA and GSH, its key four enzymes are APX, MDHAR, DHAR, and GR each have a significant roles for buffering stress effect by scavenging of ROS (Hasanuzzaman et al., 2020b).

Usually, the first enzyme in the antioxidant pathway of protection against ROS is SOD (EC 1.15.1.1). SOD is a metallo-enzyme found in almost all cellular components of all organisms involved in scavenging $O_2{}^{\cdot -}$ produced by Mehler reaction by dismutation to H_2O_2 and O_2 (see photo 6). SOD activity is closely correlated with other antioxidant enzymes, such as CAT and APX. It should be mentioned that, increased level of SOD in plants subjected to water deficit was observed in many plants such as sweet potato pea, rice, wheat, bean and sunflower (Panda et al., 2021). Plants have multiple choices about how to scavenge H_2O_2. Whether it continue with GPX pathway or AsA-GSH pathway or other ways depends on each species (see photo 5 and 6) (Haider et al., 2017). It should be mentioned that H_2O_2 as an abundant radical in plant cells could mediate plant defense mechanisms by signaling cascades of enzymatic responses as it will be discussed later and interactions with cellulose in the cell wall, amelioration cell wall stability during oxidative stress (He et al., 2020).

The AsA-GSH cycle starts when APX converts H_2O_2 into H_2O with the help of AsA acting as an electron donor (Li et al., 2020). As a matter of fact, APX is a widely distributed enzyme operating in H_2O_2 scavenging mainly in chloroplasts, where CAT is absent, in addition to other cellular compartments. It should be mentioned that APX has a better affinity for H_2O_2 than CAT (Laus et al., 2021).

Later on, the oxidized ascorbate (DHA) will be regenerated to reduced ascorbate (AsA) in two steps using NADPH and glutathione (GSH), respectively (Li et al., 2020).

First of all AsA by donating an electron is converted into monodehydroascorbate (MDHA). Then, MDHA use monodehydroascorbate reductase (DHAR) to regenerates AsA and DHA. Subsequently, DHA is reduced to AsA again by the help of reduced glutathione (GSH) which results in its oxidation to produce oxidized glutathione (GSSG) (Nadeem et al., 2019a, Hasanuzzaman et al., 2019). Finally, GR plays a vital role in reducing GSSG to GSH in the expense of NADPH as an the electron donor (Laus et al., 2021)

Both AsA and GSH are strong antioxidants and play an important role in the improvement of the physiological performance of plants. The regulation of their redox state is important to ameliorate the antioxidant defense and consequently the overall resistance of plants against abiotic constraints which mostly depends on the activities of the enzymes that are associated with the AsA-GSH cycle pathway (Hasanuzzaman et al., 2020b).

In the CAT pathway, CAT (EC 1.11.1.6) will successfully detoxify the resulting H_2O_2 to H_2O and O_2 and prevent the formation of other toxic ROS (Nadeem et al., 2019a, Laus et al., 2021). It is located mostly in peroxisomes (see photo 5), where it can detoxify H_2O_2 during photorespiration and β-oxidation of fatty acids (Li et al., 2020). Nevertheless, traces of CAT have been also found in mitochondria, cytosol and other cellular components, but not in chloroplasts (Laus et al., 2021). Depending on the intensity of the applied stress, CAT activity can increase or decrease. However, it's more common in stressed plant cells to reduce CAT activity most probably due to the suppression of the enzyme active sites (Qureshi et al., 2018). Nevertheless, plants response to water shortage can be heterogenous when it comes to CAT activity, as it can increase, decrease or remain unchanged depending on the species and other exogenous and endogenous factors (Panda et al., 2021).

Similar to APX, The down-regulation of Glutathione peroxidase (E.C. 1.11.1.7) (GPX), uses GSH as a reducing agent to scavenge H_2O_2 to H_2O (Li et al., 2020). GPX is a great scavenger of ROS. GPX has affinity toward aromatic compounds like lignin, guaiacol and pyrogallol as electron donors for scavenging H_2O_2 both intracellular (cytosol, vacuole), and extra-cellular (in the cell wall) (Banerjee and Roychoudhury, 2017). It's very common in plants subjected to water deficit to increase GPX production such as wheat and rice (Panda et al., 2021).

$H_2O_2 + 2\ GSH \rightarrow 2\ H_2O + 2\ GSSG$

Glutathione Synthase (GSH) is a very strong oxidant playing an important role in both AsA-GSH and GPX pathways. It's a highly efficient scavenger of ROS over-production as many researchers reported that GSH increase in response to abiotic stress. Moreover, Hanly et al (2020) suggested that the reduction of GSH content may help in determining or screening plants for drought stress resistance.

To maintain a normal physiological state of plants in response to oxidative stress induced by plant exposure to abiotic stress for sure anti-oxidant enzymes are the major defense system of plant cells to scavenge ROS. Thus, It's very important for plants to maintain a high level of anti-oxidative enzyme activity to tolerate stress and produce high biomass production with good quality (Yildirim et al., 2021).

The enzymatic anti-oxidant activity in plants can be diversified depending on many factors like the species and the intensity of the applied stress. Some previous experiments reported that the activity of anti-oxidant enzymes initially increased when plants are subjected to water deficit, but, if the stress continues for long time or gets intensified they reduce their activities (Zhang et al., 2021). For example, the anti-oxidant defense mechanism of *A. lancea* seedlings subjected to water deficit increased and had successfully reduced oxidative damage. However, *A. lancea* seedlings responded differently in adult stage as water deficit downregulated the anti-oxidant enzymes in this species (Zhang et al., 2021).

In a different study, Muhammad Nadeem (2019) mentioned that the activity of enzymatic anti-oxidants increased more in the recovery phase rather than in the stress phase of oxidative stress as it was observed in some varieties of *Leguminosae* like chickpea, pea, green bean, and soybean (Nadeem et al., 2019a).

It was also reported that the activity of anti-oxidant enzymes increased in some resistant cultivars such as common bean and horse gram, which emphasizes the hypothesis that drought resistance in plants is strongly correlated with enhanced activity of antioxidant enzymes which is a successful adaptive mechanism implied by plants to resist extreme environmental constraints (Nadeem et al., 2019a, Laus et al., 2021, Bilska et al., 2019)).

3. Salinity constraint

3.1 Plant response to salinity

The salinization of soil constitutes a widespread environmental problem and an important factor limiting plant growth and productivity. The yield of most crops is significantly reduced when the electrical conductivity (EC) of the soil reaches 4 dS.m^{-1} (equivalent to 40 mM NaCl) (Munns et al., 2008). Salinity affects 932 million hectares of land worldwide (Rao et al., 2008). Moreover, salinity is responsible for the deterioration of almost 20% of total cultivated and 33% of irrigated agricultural land globally (Abobatta 2020, Amin et al., 2020).

In recent years, the problem of salinity is increasing rapidly worldwide and it has been estimated that more than 50% of the cultivated land will be salinized by the year 2050 (Wang, Vinocur and Altman, 2003). Increasing salinity is due to low precipitation, high surface evaporation, weathering of native rocks, irrigation with saline water, and poor cultural practices. Adverse effects of salinity on crop productivity and agricultural yields may be due to osmotic changes, cellular ion imbalance, photosynthetic inhibition, reduced nutrient acquisition, membrane disorganization, oxidative stress, metabolic toxicity, and membrane leakage (Munns and Tester, 2008, Khan *et al*. 2019). Different adaptations were applied to allow certain crop cultivars to grow and produce a harvestable yield under soil salinity (Munns et al., 2015).

3.2 Effects of salinity on growth and photosynthesis

Salinity threatens the sustainability of global agricultural production around the world by negatively affecting plant growth and development of agricultural crops. Consequently, the yield and quality of crops would be hampered too (Laus et al., 2021). In extreme conditions if an important amount of salt infiltrates plant cells and reach toxic levels it can even lead to plant death (Hasanuzzaman et al., 2021).

Salinity affects almost all aspects of plant development as it can permanently alter the physical and chemical properties of the soil which would affect the osmotic properties of seeds and initiate toxicity mechanism which would eventually hampers seed germination by inhibiting the water uptake, seedling development and embryonic tissue development (Massange-Sánchez et al., 2021).

Moreover, increased NaCl levels is usually accompanied by impaired plant vegetative growth, and reproductive development as well as less growth hormones as salt exposure hinder plant's cell prolongation in growing tissues which would consequently reduce the leaf growth biomass as well as root and shoot prolongation (Ondrasek et al., 2022).

It should be mentioned that plants capacity to grow in saline conditions is different, depending on many factors like the species, severity and time scale of the stress and whether it was gradually or abruptly applied (Shahid et al., 2020). Usually salinity affects plant growth in two levels, it causes osmotic imbalances because of the accumulated salt around the roots in the soil in addition to the ion toxicity of salt accumulation inside the plant cells (Laus et al., 2021). It was reported that salinity had a drastic effect on legumes by reducing its nitrogen and carbon fixation, number of flowers and pods. It caused as well hormonal deregulation and nutritional imbalances and as a consequence hampered crop yield (Nadeem et al., 2019b). In case of tomatoes, increasing salinity was accompanied by significant reductions in shoot weight, plant height, number of leaves per plant, root length, and root surface area per plant in tomatoes (Mohammad et al., 1998).

It's well established that plant growth and development are deeply correlated with photosynthetic efficiency as it represents the main supply of needed energy (Yang et al., 2020). Studies have shown that salinity hampers the relative water content of plant leaves, in addition to the osmotic potential, water potential, sap flow, transpiration rate, stomatal conductance, leaf temperature, as well as photosynthesis alteration (Hao et al., 2021). Salt-induced photosynthetic reduction can be due to stomatal and / or non-stomatal limitation (Pan et al., 2021). Stomatal limitation is the consequence of stomatal closure which limits leaf gas exchange and affects ribulose biphosphate carboxylase-oxygenase activity (Zahra et al., 2022). Chloroplasts that are the primary source of salt-induced ROS accumulation are themselves targets of damages caused by ROS, which leads to photoinhibition as a nonstomatal limitation of photosynthesis (Mittler 2002, Falouti et al., 2022). The harmful effects of salinity are mainly associated with toxic accumulations of Na^+ and Cl^- which could affect metabolic processes that limit photosynthesis. Indeed, hyperosmotic salinity decreases photosynthetic electron transport activities and inhibits PSII causing an increase of ROS production which would eventually alters photosynthetic proteins and the photosystem assembly (Falouti et al., 2022). In the same way, Shin et al. (2020) confirmed that high NaCl concentrations decrease the efficiency of PSI and PSII.

Moreover, it has been proved that exposing plants to a high concentration of NaCl for a short time affects chloroplast ultrastructure as it causes starch accumulation as well as thylakoid swelling and can reach a high severity level with the prolongation of stress (Hasanuzzaman et al., 2021). Indeed, the concentration and time line of stress exposure is very important as low NaCl concentration lower photosynthetic rates, and moderate to high NaCl concentration can induce severe photosynthetic apparatus and chloroplast damage (Balasubramaniam et al., 2023). This was shown on many plants species such as *Jatropha curcas, L. culinaris Portulaca oleracea, Solanum melongena,* and *Oryza sativa* (Balasubramaniam et al., 2023).

Salt-induced photosynthetic reduction is usually correlated with a decline in chlorophyll a, b, and carotenoid contents in salt susceptible plants such as *Solanum tuberosum*, *Pisum sativum*, *Phaseolus vulgaris, Triticum aestivum and, Solanum lycopersicum* (Hasanuzzaman et al., 2021). However, in salt-bearing plants such as pearl millet, mustard, and wheat Chlorophyll content increased (Sudhir and Murthy, 2004).

Hyperosmotic salinity adversely affects photosynthesis and chlorophyll content from two aspects. Firstly, it alters the enzymatic activity and its expression involved in photosynthesis mechanisms and induce an impairment in chlorophyll biosynthesis. Secondly, it indirectly affects the the regulation pathways of antioxidant enzyme systems (Yang et al., 2020). Balasubramaniam (2023) reported that salt-induced chlorophyll content degradation happens mainly because of the increase of ROS level in plant cells which would eventually initiate chlorophyll oxidation. Also, he mentioned that the reduction in chlorophyll content is correlated with the increase of the severity of salinity in the soil (Balasubramaniam et al., 2023).

On the other hand, Srinieng et al. (2015) linked chlorophyll a and b decrease to a nutritional deficiency caused by salt that inhibits the absorption of essential nutrients (Srinieng et al., 2015). In addition, Santos (2004) suggested that the drop in Chlorophyll contents could be due to a suppression of their synthesis as well as an increase in the activity of the enzymes involved in their degradation (Santos, 2004).

3.3 Effects of salinity on water relations, and ion levels

The deleterious impacts of salinity on plants are manifested through two mechanisms. The first mechanism is determined by osmotic stress which occurs very shortly after exposure to salinity (within minutes to days) due to the incremental uptake of salts and subsequent reduction of water potential around the root zone, which reduces water absorption in plant cells leading to stomatal closure, reduction of cell expansion and division and eventually the inhibition of plant growth (Pan et al., 2021, Hao et al., 2021). Indeed, salinity alters plant water relations by decreasing plants water potential. As a consequence, plant water uptake would be reduced too, as water absorption and circulation between the plant and the soil is dependent to the osmotic gradient and because of the high NaCl concentration in the soil water would be circulated from the plant to the soil (Betzen et al., 2019). In the normal conditions, water responds to the hydrostatic pressure gradient and circulates from the soil to the root xylem via an apoplast pathway. In the saline conditions, plants respond to osmotic stress by adjusting their water balance typically by reducing transpiration rate and accumulating compatible solutes (Yang et al., 2020).

Water uptake and tissue hydration are important aspects to assess salt resistance in plants (Shi et al., 2013). It should be mentioned that relative water content (RWC) in plant cells may not be affected in moderate salinity conditions but at high salinity conditions plants RWC is greatly reduced especially in the shoots, and a turgor loss at the cellular level (Hasanuzzaman et al., 2021).

The second mechanism called also ion toxicity is a long-term accumulation of phytotoxic ions (Pan et al., 2021). It occurs days to weeks after first exposure to soil salinity causing nutrient imbalance and ion imbalance because of the increase in endogenous sodium (Na^+) and chloride (Cl^-) contents which would negatively affect the cytosolic enzymes activities in addition to the impairment of ions homeostasis, cellular functions, membrane lipid peroxidation, photosynthesis alteration, premature senescence and ultimately affecting plant growth (Laus et al., 2021, Nadeem et al., 2019b). Jointly the deleterious effect of osmotic and ionic stress induce oxidative stress which can create life-threatening conditions for plants subjected to salt stress (Massange-Sánchez et al., 2021).

Mineral elements usually existing as ions in plant cells, play an important role in the regulation of enzymatic activity, osmotic regulation, charge neutralization in addition to the colloidal stabilization which is why it's very important for plant cell membrane to maintain its ion metabolism stable (Hao et al., 2021). As mentioned before, Ionic imbalance occurs in plant cells due to excessive accumulation of endogenous Na^+ and Cl^- in the expense of other useful mineral nutrients such as K^+, Ca^{2+}, and Mg^{2+} (Zahra et al., 2022).

The accumulation of toxic amounts of Na^+ in the root cells would depolarize the plasma membrane and promote the absorption of Cl^- under a chemical gradient (Hasanuzzaman et al., 2021). In the same way, high concentration of Na^+ would increase the Na^+ / K^+ ratio as Na^+ and K^+ compete for the same root uptake sites (Balasubramaniam et al., 2023). Usually, in plant cells cytoplasm, the concentration of K^+ is kept higher than that of Na^+ for the purpose to ensure ion homeostasis, not disturb plant metabolic activity and ultimately avoid ionic stress (Hao et al., 2021). Otherwise, a large amount of external Na^+ influx would humper mineral nutrient acquisition and translocation causing nutrient deficiency not only for K^+ but also Ca^{2+}, and Mg^{2+} (Balasubramaniam et al., 2023). Moreover, the accumulation of toxic amounts of Na^+ in the leaf apoplast is very harmful to plant cell metabolism activity as it alters the osmotic balance of the cells leading to its dehydration and turgor loss in addition to membrane dysfunction and enzymes inactivation by replacing K^+ content from the enzymatic components of the cell (Hasanuzzaman et al., 2021, Massange-Sánchez et al., 2021). As a consequence, ROS accumulation increases which would humper cell division and growth leading to death of leaf cells and tissues and ultimately reducing crop production (Zahra et al., 2022). In the same way, the accumulation of toxic amounts of Cl^- in the leaf apoplast leads to a disturbance of thylakoid membranes and organelle structure, a reduction in chlorophyll content, which would inhibit PSII activity and eventually impedes plant growth (Pan et al., 2021). Moreover, excess Cl^- caused by hyperosmotic salinity reduces the uptake, accumulation, and transport of nitrogen in the shoots because of the Cl^-/NO_3^- antagonism which would affect the nutrient balance of the plant as nitrogen is very important mineral for cell metabolism (Balasubramaniam et al., 2023). Legumes are known for their ability to fixate nitrogen in their nodules. Previous studies reported that salinity affected legumes nodulation and induced their premature senescence, by altering nitrogen uptake and fixation in several species such as faba bean and pigeon pea (Nadeem et al., 2019b).

The reduction in plant phosphorus (P) concentration by salinity results from reduced activity of P in the soil solution due to the high ionic strength of the media and low solubility of Ca ± P minerals . It should be mentioned that P is very essential for photosynthesis metabolism and energy transfer (Dey et al., 2021).

To survive hyperosmotic salinity plants need to maintain relatively higher concentrations of K^+ and Ca^{2+} (Greenway and Munns, 1980). Potassium plays an important role in the photosynthetic activity and light signal transduction of plant (Pan et al., 2021). It participates as well in protein synthesis and water relations (Balasubramaniam et al., 2023). In saline conditions, the decrease in potassium contents might be explained by either a decrease in the transpiration rate, since potassium transport is reliant on that of water, or a decrease in its absorption.

The decrease in absorption could be due to the decline in the activity of potassium transporters in the presence of Na^+. Alternatively, it could result from the competition between potassium and sodium at these transport sites, as their molecular similarity allows Na^+ to replace K^+ at uptake sites, though Na^+ cannot fulfill potassium's role in cellular processes (Balasubramaniam et al., 2023).

Increased Na^+/K^+ ratio was observed in several plants undergoing hyperosmotic salinity such as *S. lycopersicum, Lens culinaris, Vigna radiata, O. sativa seedlings, Glycine max, Vigna radiata* and *Cicer arietinum* (Hasanuzzaman et al., 2021, Nadeem et al., 2019b). These plants also experienced a significant decrease in Ca^{2+} due to competitive uptake of Na^+ and Ca^+ ion flux.

Ca^{2+} plays a vital role in signal transduction of metabolic processes in plant cell. Calcium deficiency caused by excess endogenous Na^+ concentration disturbs intracellular ion homeostasis is leaf mesophyll and induce membrane instability (Hao et al., 2021). Moreover, in saline conditions, the decrease in Ca^{2+} concentration contributes to the regulation of osmotic pressure in plant cells (Hao et al., 2021).

3.4 Ion regulation and compartmentalization

It's very crucial for plant survival growing in hyperosmotic salinity to maintain ion homeostasis stable by maintaining lower concentrations of Na^+ and higher concentrations of K^+ (Yang and Guo, 2018). Since ion imbalance can disturb plant growth and development in many ways like hampering photosynthesis, enzymatic activity, the regulation of cell volume and maintenance of membrane potential (Yokoi et al., 2002), plants adapt to hyperosmotic salinity by regulating ion influx of intracellular Na^+ and K^+, enhancing Na^+ exclusion out of the cell, and its compartmentalization into the vacuole. It's actually a basic dynamic process in which plants eliminate toxic ions from the cytosol by primary active transport along with secondary transport according to an energetically costly gradient that balance the ratio of Na^+/K^+ and ultimately improve plant growth (Nadeem et al., 2019b).

Whether they were glycophytes or halophytes, plants cannot tolerate large amounts of Na^+ in the cytoplasm, therefore, they cope with excess intracellular Na^+ centration in two ways, either by accumulating the excess salts into the vacuole of the root parenchyma and suppress its transportation up to the shoots, this phenomena is called salt exclusion and was observed in many plants such as rice, maize, and sweet sorghum or compartmentalize the ions in different tissues to facilitate their metabolic functions (Yang et al., 2020, Torabi, 2014).

The high-affinity potassium transporters (HKTs) in addition to the nonselective cation channels existing in the roots such as glutamate receptors (GLRs) and cyclic nucleotide-gated channels (CNGCs) play an important roles in retrieving Na^+ transport from the roots to the shoots through the apoplastic pathway and reduce the content of Na^+ in shoot and maintain K^+ homeostasis in vivo (Balasubramaniam et al., 2023).

Plants who can exclude salt from the cytosol into the vacuole of the roots have apoplastic barriers in their roots consisted of casparian bands and suberin lamellae which are able to restrict Na^+ flow to the shoots and accumulate it into the roots. The plant than will try to get rid of this accumulated Na^+ out of root cells by the SOS1 Na^+/H^+ antiporters in the plasma membrane or it can also transport it to the vacuoles through the overexpression of genes NHXs encoding proteins for K^+/H^+ exchangers related to salt stress response (Yang et al., 2020). It should be mentioned that it's very important for plant cell survival to exclude Na^+ from vacuole to avoid the lethal effects of Na^+ in the cytosol of the cell (Pan et al., 2021).

Indeed, plants cope with hyperosmotic salinity by the regulation of Na^+ compartmentation from the transpiration stream into the xylem by exchange with K^+ at the xylem/symplast boundary of the roots, which is accomplished through the action of Na^+/H^+ and K^+/H^+ antiporters located in both the plasmalemma and in the tonoplast (Hao et al., 2021). These antiporters ensure the absorption, long-distance transportation, and redistribution of Na^+ and K^+, they play key roles in maintaining Na^+, K^+, and Ca^{2+} homeostasis under hyperosmotic salinity and they operate in conjunction with the same H^+-ATPase pumps that function to establish the internal negative membrane potential (Nadeem et al., 2019b). ATPase pumps functioning depends on anion permeability and magnified by higher concentrations of apoplastic K^+ (Balasubramaniam et al., 2023). Evidence suggests that the overexpression of genes related to proton pump activity like SOS1 and NHXs increase salt resistance in plants and that the responsiveness of the membrane pump could be a measure of salt resistance as was shown in many plants like *Arabidopsis thaliana, Leptochloa fusca, Iris halophila, and Halostachys caspica* (Hao et al., 2021).

3. 5 Osmotic adjustment and compatible solutes accumulation

A strong osmotic gradient was created across the vacuolar membrane after the transfer of salt from the cytoplasm into the vacuole. Plants respond to this imbalance often by an accumulation of suited organic solutes in the cytoplasm (Yang et al., 2020). Osmotic adjustment is considered as an important adaptation of plants to salinity as it maintains cell turgor and volume desired for crop growth (Dourado et al., 2022).

The process of osmoregulation, can be realized in two ways, solutes can either stabilize the structure of biological macromolecules or they can themselves take on the role of osmolytes (Hao et al., 2021). Osmotic adjustment occurs through the accumulation of numerous organic solutes and inorganic ions (Farouk and Al-Huqail, 2022). Compatible organic solutes usually include free proline, soluble sugars, glycine betaine, amides, amino acids, quaternary ammonium compounds proteins and polyols in the expense of ions uptake (Nadeem et al., 2019b). Compatible solutes are not toxic even at higher concentration. They are hydrophilic compounds that do not interfere with normal biochemical reactions, they have a low polar charge, high solubility, low molecular weight, and in addition to their participation in osmotic adjustment they contribute as well in ROS scavenging while protecting the membrane structure (Balasubramaniam et al., 2023). Moreover, compatible solutes are also termed as osmoprotectants and involved in the stabilization of the active conformation of cytoplasmic enzymes, thereby protecting them against inactivation by inorganic ions (Bhattacharya and Kundu, 2020). It's said that the increased concentration of compatible solutes in plant cells is often claimed as a sign of stress resistance provoked by osmoregulation (Farouk and Al-Huqail, 2022). It should be mentioned that organic solutes production is metabolically expensive as it consumes an important quantities of carbon that could be used for plant growth (Khalid and Ahmed, 2017). Nonetheless, even at small concentrations organic solutes can efficiently regulate and stabilize osmotic potential if partitioned entirely to the cytoplasm (Munns and Shavrukov, 2023).

3.5.1 Proline

Many plants accumulate proline as a nontoxic and protective osmolyte under hyperosmotic salinity. Proline is classified as a free amino acid that has a strong hydration ability which is very convenient for plants undergoing osmotic stress (Massange-Sánchez et al., 2021).

Proline is commonly biosynthesized in large amounts in response to abiotic and biotic stresses, with its concentration being higher in stress-resistant plants compared to stress-sensitive ones (Masouleh et al., 2019). The synthesis and degradation of proline are dynamic processes. During stress, proline biosynthesis can be upregulated by two different pathways: via glutamic acid or via ornithine. The glutamic acid pathway is more frequent. It occurs in the cytosol and partly in the chloroplast. It starts through the activation of the enzyme D1-pyrroline- 5-carboxylate synthetase (P5CS), which converts glutamate to proline (Soares et al., 2019). As a matter of fact, proline possesses antioxidant property enabling it to play the role of redox-buffering agent. Proline is synthesized at the expense of NADPH, which is used to reduce glutamate. By acting as an electron sink, proline promotes an increased $NADP^+$/NADPH ratio, thereby preventing reactive oxygen species (ROS) generation by suppressing free radical production and/or scavenging singlet oxygen ($^1O_2$) from PSI (K. Kishor et al., 2014, J. Bose et al., 2014). Under adverse conditions, the increase in $NADP^+$ is essential to reduce excessive PSI activity, making proline accumulation a potential mechanism to prevent photoinhibition (Soares et al., 2019, M. Meena et al., 2019). In its role as an antioxidant, proline not only prevents the production of reactive oxygen species (ROS) and scavenges them but also enhances antioxidant enzyme capacity. Additionally, proline aids in regenerating other antioxidants, such as glutathione, thereby strengthening the plant's overall antioxidant defense system, reducing osmotic stress, and minimizing cellular damage (Masouleh et al., 2019). Upon the alleviation of stress, proline is degraded by proline dehydrogenase (ProDH) into glutamate, which can be redirected into metabolic pathways, providing energy and building blocks for growth and recovery (K. Kishor et al., 2014).

Additionally, proline acts as a molecular chaperone, stabilizing proteins and protecting them from denaturation and degradation, ensuring that essential enzymatic activities remain functional even under severe stress conditions (Massange-Sánchez et al., 2021).

Moreover, proline plays a significant role in maintaining cellular homeostasis via osmotic regulation. Under hyperosmotic salinity, the external environment becomes hyperosmotic, leading to water loss from plant cells. Proline helps in retaining water by lowering the osmotic potential inside the cells, allowing plants to sustain cell turgor and prevent wilting (Alhasnawi, 2019, M. Meena et al., 2019).

Furthermore, Proline plays a vital role in stabilizing membranes by interacting with phospholipids, which helps maintain membrane integrity and fluidity, particularly under high salinity. Additionally, it reduces lipid peroxidation, thereby protecting membrane structures from damage caused by stressful statuses, further ensuring stability in stressed plants (K. Kishor et al., 2014).

Interestingly, proline is not only a protective molecule but also acts as a signaling molecule. It regulates the expression of stress-responsive genes, modulating various physiological pathways that enable plants to cope with saline environments. Proline accumulation is closely linked with stress-related hormonal pathways, such as abscisic acid (ABA), which further enhances stress tolerance by regulating stomatal closure, thus reducing water loss (Masouleh et al., 2019).

3.5.2 Glycine betaine

Glycine betaine is identified as is a quaternary ammonium compound. It's synthesized in the chloroplast where it has the role of an osmoprotectant and maintains the osmotic balance of the thylakoid membrane, thus ensuring the efficiency of photosynthesis mechanism (Balasubramaniam et al., 2023).

These compounds have hydrogen- bonding characteristics that allow them to protect macromolecules from the adverse effects of increasing ionic strength in the surrounding media (Klähn and Hagemann, 2011).

Beyond its role as an osmoprotectant, glycine-betaine can also play a key role in ROS homeostasis. Although glycine-betaine is not a direct ROS scavenger, it indirectly protect the plant from oxidative injury during environmental stresses by optimizing the performance of anti-oxidant enzymes such as SOD, CAT, and APX (J. Bose et al., 2014). Glycine betaine content increases by hyperosmotic salinity in a number of plants such as *Cajanus cajan (L)* (pigeon pea) and *Vigna radiata L.* (mung bean) (Nadeem et al., 2019b, Fareen et al., 2016)

3.5.3 Soluble sugars

Soluble sugars are essential components of metabolic processes and structural constituents of plant cells. Water-soluble sugars, such as glucose and sucrose, along with sucrose-derived carbohydrates like oligosaccharides and fructans, play multifaceted roles in helping plants cope with various abiotic stresses, including drought, salinity, extreme temperatures, and oxidative stress (Fareen et al., 2016).

Mannitol, sorbitol and trehalose in particular, play a crucial role in plant metabolic processes during their life cycle. The concentration of soluble sugars typically increases under adverse conditions (Khan et al., 2020a). Their functions extend beyond serving as metabolic resources, acting as osmoprotectants and signaling molecules that integrate stress pathways, forming a complex network to regulate plant metabolism (Soares et al., 2019). This includes processes like photosynthesis, mitochondrial respiration, and the oxidative pentose phosphate pathway, where sugars modulate both ROS production and scavenging systems. Indeed, these compounds are recognized as some of the most effective osmolytes for protecting plants during stress (Masouleh et al., 2019).

Moreover, thanks to their special characteristics like hydrogen bonding, glassy appearance and strong hydrophilicity, soluble sugars play a significant role in mitigating oxidative stress by directly scavenging reactive oxygen species (ROS) and enhancing the plant's antioxidant defense system. Soluble sugars like sucrose and trehalose can interact with ROS, including singlet oxygen and hydroxyl radicals, neutralizing these damaging species (Hassan et al., 2023). Additionally, sugar accumulation induces the expression of genes involved in detoxification of reactive oxygen species, leading to heightened activity of critical enzymes such SOD, CAT, APX, DHAR, and MDHAR. These enzymes, in conjunction with other antioxidants like glutathione and proline, create a robust system that effectively counters oxidative damage, allowing the plant to maintain cellular homeostasis even under severe stress (Nawaz et al., 2022).

In addition to structural protection, soluble sugars serve as signaling molecules that regulate various stress-response pathways. Through sugar sensing and signaling, they influence the expression of stress-responsive genes, integrating with other hormonal signaling like abscisic acid , gibberellins, auxins and ethylene to modulate plant metabolism and growth under stress conditions (Hassan et al., 2023). These sugars regulate key metabolic processes, such as photosynthesis, by protecting the photosynthetic apparatus and stabilizing thylakoid membranes in chloroplasts.

In parallel, they are involved in mitochondrial respiration and the oxidative pentose phosphate pathway, both of which are critical for energy production and the generation of reducing power (NADPH) during stress conditions (Masouleh et al., 2019). It had been shown that the rate of photosynthesis increased with the overexpression of trehalose in *Oryza sativa*, which protected photosystem II (PSII) against photooxidation (Garg et al., 2002).

Furthermore, soluble sugars are crucial stabilizers of cellular membranes and proteins (Hassan et al., 2023). Through hydrogen bonding, sugars protect the phospholipid bilayers of cell membranes, ensuring integrity and fluidity during osmotic stress, while also stabilizing protein structures (Wilhelm et al., 2020, Oubohssaine et al., 2024). These sugars interact with proteins, including enzymes involved in photosynthesis and respiration, preventing their denaturation or misfolding. Specifically, sucrose and trehalose can form glass-like structures in the cytoplasm, which protect vital proteins from denaturation by effectively mimicking the natural hydrated state, thereby allowing critical enzymatic functions to continue under extreme stress conditions(Zulfiqar et al., 2020, Khan et al., 2020a).

These combined roles make soluble sugars some of the most effective osmolytes for protecting plants during adverse environmental conditions. Their ability to act as osmoprotectants, stabilizers, signaling molecules, and ROS scavengers highlights their multifunctionality and underscores their central role in plant stress tolerance.

III. Strategies to Optimize Plant Performance at Potentially Harmful Conditions:

Most of the Mediterranean area includes arid and semi-arid regions, the climatic conditions of this area are not optimal for crop production (Mbarki et al., 2020). However, perennial legumes such as *Medicago* have demonstrated the ability to thrive in saline, marginal soils (Slama et al., 2022). Optimizing plant performance in these environments requires improving the complex interactions within the plant–soil continuum, a system influenced by both biotic and abiotic factors (Koyro and

Huchzermeyer, 2018, Abideen et al., 2020). A combination of halophil or drought resistant species with an optimized plant–soil continuum may be very beneficial for the future of agriculture in the challenging conditions of arid and semiarid regions.

1. Improving Soil Quality by the Addition of Biochar:

Farmers are facing the big challenge of producing crop yield with water supply and soil quality below optimum. In this context, optimization of environmental conditions by improving soil properties with the application of organic amendments such biochar may be a good strategy to overcome these adverse cues in many ways (Zaheer et al., 2021). First of all, biochar is easy to prepare, as it is a pyrolysis carbon that is commonly derived from the combustion of organic materials (Sattar et al., 2020). This component has gained considerable interest in the last few years as a way to increase productivity and mitigate the detrimental impacts of environmental stresses such as heavy metals, drought, and salinity. Its application is getting widely used in the purpose to enhance plant growth and development, thus, crop production (Yildirim et al., 2021). Biochar as a soil conditioner, have the potential to improve soil physio-chemical characteristics, soil fertility by nutrient retention, influence microbial abundance and activity in soil by removing contaminants through polyvalent cation exchange and high retention, electrostatic interaction, precipitation as well as complexation and increasing the soil organic carbon (Yousaf et al., 2022).

More importantly, it has been reported that soil amendment with biochar is a promising approach to address desertification (Zainul et al., 2017), as biochar has the capacity to increase the soil water holding capacity (WHC), moisture contents, stability of soil texture and aggregates, and porosity (Zaheer et al., 2021). Indeed it is a good strategy to improve water infiltration, organic matter status, nutrient and soil aeration and respiration particularly under low soil water content (Zainul et al., 2017, Sattar et al., 2020).

These beneficial effects jointly lead to increasing water supply, or nutrients and mitigate the negative impacts of low soil moisture content on plant performance, increase its physiological and biochemical performance, promote plant biomass production, and food security (Wang et al., 2021).

However, the physio-chemical changes of soil and its water-retaining capacity due to biochar application may vary depending on biochar nature, where it is applied, its porosity, pyrolysis conditions, the source and rate of biochar application, and the preparation methods (Zaheer et al., 2021).

It should be noted that, biochar supply has a threshold (Koyro and Huchzermeyer, 2022). An improper use of biochar may be harmful to plant performance (Zainul et al., 2017). Plant growth can be reduce if the biochar applied is too much, as high amount of biochar in the soil can increase the ion binding capacity in the soil fraction which will create a competition between plant roots and biochar resulting in nutrient reduction caused by the adsorption of mineral nitrogen and dissolved organic carbon (Dahal and Vanlerberghe, 2017). Which is why, it's very important to add an appropriate amount of biochar in the soil (Zainul et al., 2017).

In addition to the optimization of water and nutrient supply to plant roots and the increased of biomass production among other things, the positive effect of biochar extends to the improvement of leaf photosynthetic rate as well, as it was reported in many published reviews (Haider et al., 2020). This beneficial effect occurred thanks to the optimization of soil physicochemical properties and structure that ultimately increased nitrogen accumulation, thereby increasing the rate of photosynthesis (He et al., 2020). Actually, this effect is very expected as biomass production and photosynthesis are strongly correlated (Gharred et al., 2022).

Moreover, soil amendment with biochar provide better conditions for synthesis of organic matter in soil and improve growth regulating substances and prevent desiccation with improved turgidity which ultimately increase the activity of anti-oxidant enzymes resulting in the mitigation of oxidative stress through high water use efficiency (H2O loss per net CO2 uptake) (Abideen et al., 2022, Haider et al., 2020).

As a matter of fact, in previous studies, soil amendment with biochar in drought and salinity proved to be very efficient in increasing stress resistance and improve productivity and water use efficiency of many plants such as cabbage (*Brassica olerecae*) (Yildirim et al., 2021) *solanaceae* (tobacco), *fabaceae* (beans) , *poaceae* (phragmites), *Triticum aestivum L* (wheat) and tomato (Haider et al., 2020, Koyro and Huchzermeyer, 2022).

2. Improvement of plant performance by priming:

There is a widespread consensus that sustainability and improvement of crop production is an urgent necessity to meet the ever-growing population demands as it was repeatedly mentioned by researchers all over the world (Kanjevac et al., 2022). In accordance with what was written before that soil amendment with biochar may be a good strategy to optimize soil in the complex interacting system of the plant–soil continuum, other strategies should be established to optimize environmental conditions and plant performance and increase crop production especially in extreme conditions. Previous findings showed that the widespread use of chemical fertilizers, pesticides, and other chemicals in crop production can have adverse effects on soil fertility and damage beneficial soil enzymes, microbial activity as well as chemical and physical properties of the soil in long term (Koyro and Huchzermeyer, 2022, Yaghoubian et al., 2022). Therefore the integration of priming may help in providing high-quality seeds, hence, overcoming losses in the yield and protect soil structure in the same time (Yaghoubian et al., 2022)

Indeed, Seed germination is a critical points for the growth of any crop (Kanjevac et al., 2022). Seed priming is an effective technique related to the progression of seed germination. It is a pre-planting treatment that is often used to improve seed performance and enhance synchronized uniform germination, seedling growth and ultimately increase crop yield (Shah et al., 2019).

The basics of seed priming consist on the invigoration of seeds with different reagents for certain amount of time and initiate the germination process in the metabolic machinery (Kanjevac et al., 2022). Since it’s a pre-sowing step, the process should be interrupted before radicle protrusion by placing the seeds on desiccation until they are used again for growing a culture (Seleiman et al., 2021). Most of the times, chemical agents and nutrients are used in this process (Kanjevac et al., 2022).. This mechanism can be applied in different ways depending on the morphology physiology and type of seeds and plants. It includes hydro-priming, osmo-priming, hormone-priming, vitamin priming and biotic treatments (Shah et al., 2019). These different approaches and techniques, enable the seeds to a brief exposure to an environmental stimulus and cut back the sensitivity of exotic factors which would lead to the development of vigorous seedlings in extensive agro-climatic environments and eventually enable the plant to be more prepared to cope more successfully and respond more quickly to an imminent environmental stimulation and hopefully be able to tolerate different types of stresses, thereby enhance crop production (Shah et al., 2019, Sherin et al., 2022).

The benefit of priming lies in its affordability and minimal risk, making it an effective approach to promote stress resistance (Sherin et al., 2022). Even though, the stress stimuli used for priming are short and weak, they are very capable to increase stress resistance. When primed seeds are rehydrated for planting, water uptake initiates essential biochemical and molecular processes such as nucleic acid and protein synthesis, de novo ATP production, and the synthesis of sterols and phospholipids, which are crucial for maintaining membrane integrity. Antioxidant mechanisms are activated, reducing oxidative stress, while DNA repair systems address any damage accumulated during previous dehydration or stress. These metabolic preparations allow primed seeds to germinate more quickly and efficiently, while also enhancing their resilience to abiotic stresses (Kanjevac et al., 2022).

Moreover, when encountering a second stress stimulus (drought, salinity..), primed plants initiate the formation of metabolic marks that affect signal transduction and gene expression which would activate defense mechanisms and anti-oxidant machinery much faster and more efficiently than unprimed plants (Sherin et al., 2022). In this context previous studies reported that primed seeds of crops like wheat, maize and chickpea grown in drought conditions expressed more resistance to the stress by producing more crop yields than non-primed ones (Seleiman et al., 2021).

2.1 Vitamin priming with ascorbic acid:

Priming seeds with vitamins was suggested as an effective strategy to alleviate stress effects on plants (Sherin et al., 2022). Many reviews reported the advantages of seed priming with ascorbic acid (vitamin C) simply because this vitamin is very important for plant metabolism (Ghobadi et al., 2022). It's one of the most powerful non-enzymatic antioxidant that can be accumulated in different parts of the plant (Hasanuzzaman et al., 2019). It can efficiently absorbs water-soluble vitamins and modulates a number of fundamental functions in plants under optimum and non-optimum conditions (Sherin et al., 2022). Indeed, Ascorbate roles in plant metabolism are very numerous and diversified, it's involved in cell division, expansion, and elongation in plants, it can regulate biochemical reactions like tocopherol cycle, GSH-ASA cycle and GPX cycle which helps plants defend themselves against ROS (as it was described above) and enhance the synthesis of stress- responsive proteins (Shah et al., 2019). Moreover, as a co-factor for certain enzymes, ascorbic acid is involved in the process of hormone biosynthesis, phytohormone mediating signaling as well as several other physiological plant processes like photosynthesis (Alves et al., 2021). It's involvement in phytohormone-mediated signaling is mainly to regulate plant growth and development, for example, when it's shifting from the vegetative to the reproductive stage and in leaves senescence (Shah et al., 2019).

As a matter of fact, many research on crop seeds has shown that it is a very good strategy to improve mature seeds by exogenous application of some antioxidants like ascorbate (Ghobadi et al., 2022). In accordance, Khan (2019) reported that seed priming with ascorbic acid optimized the emergence index and the final emergence percentage in in *Triticum aestivum L* (wheat) (Khan et al., 2020b), in addition to the reduction of the emergence time to 50% as well. Zoya Baig (2021) also confirmed it's positive effect on growth and physiological activities of the same variety in saline conditions (Baig et al., 2021). In the same way, George Sherin (2022) reported the positive effect of seed priming with ascorbic acid on growth and photosynthesis of salt treated *Vicia faba*. It did also increase its chlorophyll and carotenoid contents resulting in a stable photosynthetic efficiency under stress conditions (Sherin et al., 2022).

Chapter 2

Materials and methods

1. Plant Material

In the present thesis two species of *Medicago* were used: *M. cilliaris* and *M. polymorpha.* The genus *Medicago* is one of the most important genera of forage plants (Reid et al., 1989). It's classified as glycophyte and comprises approximately 85 species (Abdelly et al., 2006). *M. ciliaris* and *M. polymorpha* share a basic chromosome number of x = 7. While both species are autogamous (Cherifi, 2016), *M. ciliaris* is a diploid with (2n = 16) and can readily intercross with *M. intertexta* and *M. muricoleptis* (Lesins and Lesins, 1979). On the other hand, *M polymorpha* is a diploid with (2n = 14) (Lesins and Lesins, 1979).

M. ciliaris (TNC) *and M. polymorpha* (TNP) seeds used in this work were provided by the Laboratory of Extremophile Plants in the Center of Biotechnology at the Technopole of Borj Cedria in Tunisia. These lines were originated from a local Tunisian population of Enfitha (TNC 1.11) and Mateur (TNP 11.10).

M. Polymorpha seeds collected from natural populations in Mateur were also utilised in this study (second experiment)

Healthy and uniform seeds were used for our experiments

Photo 2.1 Morphology of *M. Cilliaris* plants

Photo 2.2 Morphology of *M. polymorpha* plants.

1.1. First experiment: Impact of biochar on drought resistance of *M. ciliaris*

A First experiment was conducted to assess the influence of biochar application during water scarcity on biochemical and physiological processes in *M. ciliaris.*

1.1.1. Growth Conditions and treatments

M. ciliaris line used in this experiment is originated from a local Tunisian population of Enfitha (TNC1.11) (Badri et al., 2016). In *M. ciliaris*, germination is strongly limited by the presence of a hard seed coat; thus, to obtain a maximal rate of germinated seeds, scarification with liquid nitrogen was necessary. Scarified seeds were germinated in Petri dishes in the dark at 25 °C for 3 days then transferred into black pots with a 1.55 L capacity (pot 13 × 13 cm; dimensions at top: 13 × 13 cm; dimensions at the bottom: 9.5 × 9.5 cm, height: 12.5 cm) filled with a mixture of 70% soil, and 30% sand mixed or not with 2% Bc.

Coniferous wood and hardwood chips (1:4 ratio by weight) were mixed to produce Biochar through pyrolysis in a 36-h cycle at 750 °C using a Schottdorf-type reactor (Carbon Terra, Augsburg, Germany). The experiment was carried out at Justus Liebig university of Giessen, Germany, in a controlled environment greenhouse equipped with an automated greenhouse climate control system (including air conditioner) at a temperature of 24 °C/15 °C (day/night), a relative humidity of 55–60% and a photoperiod of 16/8 h. The emerged seedlings of uniform size were subjected to pretreatment phase (72 days).

Two irrigation modes were retained in this study: 60 and 36% of water holding capacity (WHC). The selection of these two percentages of water-holding capacity was based on a preliminary experiments carried out on *M. ciliaris* watered with 100, 75, 60 and 36% WHC, showing that for our line and soil type, 60% corresponded to the optimal conditions for growth and 36% WHC led to a significant decrease of growth parameters. Thus, plants irrigated with 60% WHC corresponded to control plants and plants irrigated with 36% of WHC corresponded to the stressed ones. Pots were weighed every day to control and maintain a stable WHC. For all treatments, water was enriched with diluted nutrient solution (Hewitt, 1952) containing: 3,5 mM $Ca(NO_3)_2$, 3,0 mM KNO_3, 1,5 mM $MgSO_4$, 1,6 mM KH_2PO_4, 0,6 mM K_2HPO_4, 3 µM Fe–K-EDTA, 0.05 µM H_3BO_3, 0,5 µM $MnSO_4$, 0,04 µM $CuSO_4$, 0,05 µM $ZnSO_4$, and 0,02 µM $(NH_4)6Mo_7O_{24}$. Whether they were watered with 60% or 36% of WHC, plants received the same quantity of nutrients. The WHC measured according to the technique of Bouyoucos (1983) was estimated at around 13,33%.

After 42 days of treatment, a final harvest was carried out on 114-day-old plants separated into shoots and roots for analysis.

Photo 2.3 Constant weighing of *M. cilliaris* pots to control WHC

Photo 2.4 Culture of *M. cilliaris* Enfitha in greenhouse under control conditions

1.2 Second experiment: Impact of priming on drought and salinity resistance of *M. polymorpha*

1.2.1 Growth conditions and treatments

A second experiment was conducted to assess the effects of ascorbic acid seed priming on *M. polymorpha L.* seedlings and their response to both salinity and PEG-induced osmotic stress. The aim was to demonstrate seed priming as a cost-effective short-term solution to enhance the performance of *M. polymorpha L.* under constantly changing environmental conditions for farmers.

Healthy uniform seeds of *M. polymorpha* L. (Mateur origin, North Tunisia) were disinfected with 10% (w/v) sodium hypochlorite for five minutes, followed by three washes with distilled water.

In this experiment, seeds were divided into two groups: one was primed with 0,2 mM (35 mg/L) ascorbic acid solution, and the other was soaked in distilled water. Both treatments were carried out at room temperature in the dark for 12 hours, with a seed/solution ratio of 1:5. After soaking, the seeds were thoroughly rinsed with distilled water, spread out on filter paper, and dried under controlled conditions until they returned to their original weight.

Subsequently, the primed (P) and non-primed (NP) control seeds were germinated in Petri dishes under controlled conditions. After three days, with a germination rate of approximately 85%, the germinated seeds were transferred to 3.5 L plastic pots for hydroponic culture.

To simulate osmotic stress, the plants were exposed to solutions with an osmotic potential of -0,3 MPa, induced by either 100 g/L PEG 6000 or 50 mM NaCl (3g/L) (electrical conductivity: 5 dS/m)

Two approaches were carried out (see Fig 2.1): primed and non-primed seedlings were cultivated in three treatment groups: water, water mixed with salt, and water mixed with PEG. A total of 300 plants were used, with each treatment group consisting of 50 plants, distributed across 5 pots (10 plants per pot). The rectangular pots measured 24 cm in length, 18 cm in width, and 8.1 cm in height. From each pot, eight uniform plants were selected for physiological measurements. To maintain optimal growing conditions, the culture solutions were aerated continuously to prevent root anoxia, and were replaced every 3 to 4 days. After 30 days of treatment, the final harvest was conducted.

Figure 2.1 Experimental design for primed and non-primed seedlings under water, salinity and PEG induced osmotic stress conditions.

The culture experiment was carried out in the greenhouse of the Center of Biotechnology in Borj Cedria at 30 ± 5°C/16 ± 2°C (day/night) temperature, 60 ±

5%/90 ± 5% (day/night) relative humidity, and 14 h light/10 h dark regime.

1.3 Third Experiment: Impact of salinity on drought resistance of *M. ciliaris* and *M. polymorpha*

1.3.1 Plant material and growth conditions

Local Tunisian lines of Enfitha, TNC1.11 of *M. ciliaris* and TNP1.11 of *M. polymorpha,* were utilized in this study. Healthy uniform seeds from both lines were disinfected with 10% (w/v) sodium hypochlorite for 5 minutes and then rinsed three times with distilled water. Subsequently, the seeds were germinated in 3,5 L pots filled with limono-sandy soil under greenhouse conditions.

One-week post-sowing, seedlings were thinned to ensure one plant per pot for uniformity. For the first three weeks, plants were watered at 80% FC. Afterward, the plants were divided into four treatment groups:

(i) Control plants (C), irrigated with tap water at 80% FC.
(ii) Water-deficit stressed plants (D), irrigated with tap water at 33% FC.
(iii) Plants irrigated to 80% FC with an additional 50 mM NaCl (S).
(iv) Plants irrigated to 33% FC with an additional 50 mM NaCl (D+S).

Each treatment included eight replicates per line, and plants were harvested one month after treatment began.

2 Physiological and biochemical measured parameters

2.1 Growth Parameter

Upon harvest, we measured root, shoot fresh weight (FW) separately. Dry weight (DW) was determined after drying the samples at 60 °C in the oven until a constant weight was reached.

The number of leaves, root and shoot lengths as well as, the number of ramification and internodes were determined. Simultaneously, five repetitions of fresh leaf, stem and root samples from each treatment were stored at -80°C for further analysis.

2.2 Chlorophyll Fluorescence

Chlorophyll fluorescence was measured in the third fully expanded leaf starting in the morning from 08:30 am to 15:00 am, using a portable chlorophyll meter (JUNIOR

PAM, WALZ GmbH, Effeltrich, Germany). Before measuring, leaves needed to be adapted to the darkness for 40 min to evaluate maximum quantum efficiency of PSII photochemistry (Maxwell and Johnson, 2000). Fluorescence parameters characterizing either the dark-adapted state or light-adapted state were measured at four plants from each of the four applied treatments. The following chlorophyll fluorescence parameters were calculated using WINCONTROL software (2.133/03.00) with standard settings for rapid light curves (Heinz Walz Gmbh, Effeltrich, Germany; (Genty et al., 1989, Schreiber et al., 1986)), which are the potential maximal efficiency of PSII (Fv/Fm), electron transport rate (ETR), photochemical quantum yield of photosystem II (Y(II)); the quantum yield of regulated non-photochemical energy loss in PS II (Y(NPQ)), quantum yield of non-regulated non-photochemical energy loss in PS II, equivalent to Y(NO); and photosynthetic photon flux density (PPFD) (µE m^{-2} s^{-1}).

2.3 CO_2/H_2O Gas Exchange

CO_2/H_2O gas exchange was determined using a Li-Cor LI-6400XT portable photosynthesis system (Li-Cor Biosciences; Lincoln, NE, USA) with a 6400-02(B) LED light source attached to the leaf chamber. Temperature in the leaf was set at 22.0 °C. Carbon dioxide levels in the leaf chamber were controlled by using CO_2 cartridge and a fixed flow rate of 300 µmol s^{-1}. CO_2 concentration within the leaf chamber (Ca) was fixed at 400 µmol mol^{-1}. Intercellular CO_2 concentration (Ci) [µmol CO_2 m^{-2} s^{-1}], net CO_2 assimilation rate (A_{net}) [µmol CO_2 m^{-2} s^{-1}], dark respiration (RD) [µmol CO_2 m^{-2} s^{-1}], transpiration rate (E) [mol H_2O m^{-2} s^{-1}] and stomatal conductance (SC) [mmol H_2O m^{-2} s^{-1}] were determined on the third fully expanded leaf from 08:30 to 15:00 am. Water use efficiency (WUE) was calculated as a A_{net}/E ratio. Photorespiration (RL) [µmol(CO_2) m^{-2} s^{-1}] was estimated as $1/12\,(ETR - 4\,(A_{net} + RD))$ (Valentini et al., 1995). Gross CO_2 assimilation (A_{gross}) [µmol (CO_2) m^{-2} s^{-1}] was calculated as the sum of A_{net}, RD and RL. The slope in the linear range of the light response curve represents the photosynthetic efficiency

Photosynthetic efficiency (Vc) was calculated as described in (Schulte et al., 2003). All measurements were carried out in the greenhouse at light saturation conditions with 750 or 1500 µE m^{-2} s^{-1} photosynthetic photon flux density (PPFD) (high water potential: 1500 µmol m^{-2} s^{-1} PPFD and low water potential: 750 µmol m^{-2} s^{-1} PPFD) with 25 ± 15 °C air temperature and 60 ± 10% relative air humidity.

2.4 Pigment analysis

2.4.1 Chlorophyll Content (first experiment)

Leaf SPAD readings (SPAD 502; Minolta Co., Osaka, Japan) provide a nondestructive surrogate method for determining leaf chlorophyll (Chl) concentration (Jifon et al., 2005). Leaf chlorophyll (Chl) concentrations were measured in the third fully expanded leaf in the morning. The mean of three SPAD readings for each leaf was recorded.

2.4.2 Chlorophyll and carotenoid content (second experiment)

Leaf chlorophyll and carotenoid concentrations were determined spectrophotometrically according to the method described by Lichtenthaler and Welburn (1983). Briefly, 5mL of 80% acetone was added to 120 mg of each fresh leaf sample. The extraction time was 72h at 4°C in the dark at 4°C. The extract was centrifuged for 5 min at 14,000 g. The absorbance of supernatant was recorded at 663, 646, and 470 nm for chlorophyll a, chlorophyll b, and total carotenoids. respectively.

2.5 Water relations

The leaf water content (LWC) was determined as:

LWC (ml g^{-1} DW) = (FW − DW)/DW (Scholander et al., 1965).

The osmotic potential (Ψs) was measured of the third leaf from the top was measured for eight plants per treatment. Fresh leaf segments were placed in perforated Eppendorf tubes and immediately frozen in liquid nitrogen. The samples were then sealed in a second intact Eppendorf tube, thawed for 30 minutes, and centrifuged at 15,000 g for 15 minutes at 4°C (Martínez-Ballesta et al., 2004). The supernatant was collected to ensure complete sap extraction. Osmolarity (Cs) was measured using a vapor pressure osmometer (Wescor 5500) and converted from mosmoles kg^{-1} to MPa using the Van't Hoff equation: $\Psi s = -RTCs$, where R is the universal gas constant and T is the temperature (K).

2.6 Proline Content

Free proline was qualified spectrophotometrically according to Bates (Bates et al., 1973). An amount of 0,2 g of plant fresh material was homogenized in 4 mL of sulphosalicylic acid (3% *w*/*v*), then mixed with 2 mL of acid ninhydrin solution and 2 mL of glacial acetic acid. The mixture was heated at +100 °C for 1 h in a water bath. The reaction was stopped by transferring the mixture to an ice bath. Proline was extracted by adding 4 mL of toluene to each tube, and the absorbance of toluene fraction (aspired from the liquid phase) was measured at λ j 520 nm using a UV/VIS spectrophotometer CAMSPEC M550 double beam (Spectronic CamSpec, Leeds, UK). Proline concentration was determined using calibration curve as µmol proline g^{-1} FW.

2.7 Lipid Peroxidation

The extent of lipid peroxidation was estimated by determining the concentration of malondialdehyde (MDA) according to Rao and Sresty (Rao and Sresty, 2000). Leaf material (50 mg FW) was homogenized with a prechilled mortar and pestle in 2 mL of ice-cold trichloroacetic acid TCA (0,1%, *w*/*v*) and centrifuged at 15,000× *g* for 15 min and at 4 °C. Assay mixture containing 2 mL aliquot of supernatant and 2 mL of 0,67% (*w*/*v*) thiobarbituric acid (TBA), was heated at 95 °C for 20 min and then rapidly cooled in an ice bath. The samples were centrifuged (10,000× *g* for 10 min at 4 °C) and the supernatant absorbance was measured at λ 532 and λ 600 nm using UV/VIS spectrophotometer CAMSPEC M550 double beam (Spectronic CamSpec, Leeds, UK). The concentration of MDA was calculated from the extinction coefficient 155 mM^{-1} cm^{-1}.

2.8 Hydrogen Peroxide Content

The hydrogen peroxide (H_2O_2) concentration was measured according to the method previously described by Loreto and Velikova (Loreto and Velikova, 2001). Frozen leaf samples (500 mg) were homogenized in 5 mL of 1% (*w*/*v*) ice-cold trichloroacetic acid (TCA) and centrifuged at 14,000× *g*, for 20 min at 4 °C. Subsequently, 0,5 mL of supernatant was mixed with 0.5 mL of potassium phosphate buffer (10 mM, pH 7.0) and 1.5 mL of potassium iodide (1 M) in a ratio 2.1 (*v*/*v*). The absorbance was measured at λ 390 nm using a UV/VIS spectrophotometer CAMSPEC M550 double beam (Spectronic CamSpec, Leeds, UK). The hydrogen peroxide content was calculated using a standard curve using different concentrations of H_2O_2.

2.9 Protein Quantification and Antioxidant Enzyme Assay

Fresh leaves (100 mg) were homogenized with ice-cold sodium phosphate buffer (50 mM, pH 7,2) containing 1 mM ascorbic acid, 1mM dithiothreitol (DTT), 0,1% of triton, 10 mM ethylene diamine tetra acetic acid (EDTA, disodium salt) and 10% (*w*/*v*) Polyvinylpolypyrrolidone (PVPP). The homogenate was centrifuged at 12,000× *g* for 20 min at 4 °C. The supernatant was collected and stored in small Eppendorf at −80 °C. Protein content was determined after mixing the supernatant with an acid solution of Coomassie–Brillant–Blau G-250 and subsequent incubation in the dark for 10 min (see Bradford, 1976). The absorbance was measured at λ 595 nm using a UV/VIS spectrophotometer CAMSPEC M550 double beam (Spectronic CamSpec, Leeds, UK). Soluble protein concentration in the enzyme extracts were estimated using a standard curve of different concentrations of bovine serum albumin (BSA). Superoxide dismutase activity was assayed by its ability to inhibit photochemical reduction of nitroblue tetrazolium chloride (NBT) at 560 nm. According to Beyer and Fridovich (Beyer et al., 1987), we prepared a reagent containing 10 mM of L-methionine, 0,1 mM of nitroblue-tetrazolium chloride (NBT) and 0,75% of Triton X-100 in 50 mM potassium phosphate pH 7,8 in a dark bottle. Of this reagent, 1 µL was added to the reaction mixture (3 mL) containing 40 µL of enzyme extract followed by 10 µL of 0,12 mM riboflavin. The mixture was prepared twice, one of them was incubated under fluorescent lamps (40 W) for 7 min and the second was kept in the dark to be used as blank for the measurements. The absorbance of the mixture was measured at λ 560 nm. The enzyme activity was calculated as the percentage inhibition per minute.

Ascorbate peroxidase (APX, EC 1.11.1.11) activity was assayed according to Nakano and Asada (Nakano and Asada, 1981). The reaction mixture (3 mL) consisted of 50 mM of potassium phosphate buffer (pH 7.0), 0,2 µM of EDTA, 0,5 mM of ascorbate, 2 mM H_2O_2 and 50 µL of enzyme extract. The reaction was initiated by the addition of H_2O_2. Ascorbate peroxidase was assayed by monitoring the decrease in absorbance at λ 290 nm. The molar extinction coefficient was 2,8 $mM^{-1}cm^{-1}$.

Guaiacol peroxidase (GPOX) activity was measured by recording the increase of the absorbance at λ 470 nm due to a tetra-guaiacol formation (ε = 26,6 L mol^{-1} cm^{-1}) according to Zaharieva et al. (1999). The reaction mixture (3 mL) contained 50 mM of potassium phosphate buffer (pH 7,0), 2 mM H_2O_2, Guaiacol 2,7 mM and 50 µL of enzyme extract. The enzyme activity was calculated as the percentage of inhibition per min. The molar extinction coefficient was 26,6 L mol^{-1} cm^{-1}.

According to Foyer and Halliwell (Foyer and Halliwell, 1976), Glutathione reductase (GR, EC 1.6.4.2) activity was determined by the oxidation of β-NADPH at λ 340 nm (ε = 6,2 L mol^{-1} cm^{-1}). The reaction mixture (3 mL) contained 100 mM Tris-HCl (pH 7.8), 0,5 mM GSSG, 0.03 mM β- NADPH, 5mM EDTA and 100 µL of enzyme extract. The molar extinction coefficient was 6,2 L mol^{-1} cm^{-1}.

2.10 Extraction and Determination of Non-Enzymatic Antioxidant Ascorbate (AsA) and Dehydro-Ascorbate (DHAsA)

Frozen leaf samples (400 mg) were ground in liquid nitrogen and homogenized in 2 mL of ice cold 6% TCA. The mixture was centrifuged at 16,000× *g* for 20 min at 4 °C and supernatant was collected. Ascorbate (AsA) and dehydro-ascorbate (DHAsA) were determined with a dipyridyl assay based on the reduction of Fe^{+3} by reduced ascorbate, followed by complex formation between Fe^{+2} and bipyridil, which absorbs at λ 525 nm. Total ascorbate was determined after the reduction of DHAsA to AsA by reacting with dithiothreitol. A standard curve was prepared for the estimation of total ascorbate (with pretreatment DTT) and DHAsA (subtracting AsA from total ascorbate). This method was described by Kampfenkel (Kampfenkel et al., 1995).

3 Statistical analysis

3.1 Statistics for the first experiment

Between four and five replicates were used for data analyses. Statistical analyses were carried out by two-way analysis of variances using SigmaPlot software. A two-way analysis of variance (ANOVA) was performed to test the independence of variation among conditions (equal variance test) and normal distribution of data of each variable

(Shapiro–Wilk). The Holm–Sidak method (all pairwise multiple comparison procedures) was used to check whether the means of the posterior homogeneous subgroups differed significantly at $p < 0{,}05$.

3.2 Statistics for the second experiment

The program AV1W MSUSTAT was used for statistical analysis in the second experiment with orthogonal contrasts and mean comparison procedures to detect differences between treatments. Mean separation procedures were carried out using the multiple range tests with Fisher's least significant difference (L.S.D.) ($P < 0{,}05$).

3.3 Statistics for the third experiment

Data were analyzed using the statistical software STATISTICA 5.0 (StatSoft France 1998). Pearson coefficients were calculated to assess correlation between different variables. Interaction were determined with two-way analysis of variance. Significant differences between means were separated using the Tukey test ($P = 0{,}05$).

Chapter 3

IMPACT OF BIOCHAR APPLICATION AT WATER SHORTAGE ON GROWTH, WATER RELATIONS AND PHOTOSYNTHESIS IN *MEDICAGO CILIARIS*

Abstract

Soil amendment with biochar is mostly used to improve soil fertility, water retention capacity, and nutrient uptake. The present study was conducted in order to study the impact of biochar at water deficiency conditions on growth, water relations, and photosynthesis in *M. ciliaris* seedlings.

Seedlings were cultivated under greenhouse conditions in pots filled with a mixture of soil and sand mixed in the presence or absence of 2% biochar. Plants of uniform size were subjected after a pretreatment phase (72 days) either to low (36% water holding capacity, water potential low) or high soil water potential (60% water holding capacity, water potential high). Pots were weighed every day to control and maintain a stable water holding capacity. In *M. ciliaris*, drought led to a significant reduction in plant growth and an increase in the root/shoot ratio. The growth response was accompanied by a decreased stomatal conductance and a reduction of the net CO_2 assimilation rate and water use efficiency.

Soil amendment with biochar enhanced the growth significantly and supported the photosynthetic apparatus of *M. ciliaris* species by boosting chlorophyll content and A_{net} both under well and insufficient watered plants and water use efficiency in case of water shortage. These results confirmed the hypothesis that biochar application significantly reduces the degree of stress on *M. ciliaris* plants. These results implied that this species could be suitable as a cash pasture plant in the development of agriculture on dry wasteland in a future world of water shortages.

Keywords: drought; *M. ciliaris*; biochar; growth; photosynthesis; chlorophyll.

1. Introduction

In the next decades, experts are estimating a rise in global temperature around 1.5 to 2°C (IBCC, 2021). The cold seasons will get shorter and the warm seasons will get longer and heatwaves will be more often according to the report released by the Intergovernmental Panel on Climate Change (IBCC, 2021).

Drought impacts are not confined to arid and semi-arid regions but increasingly spreading to more temperate and humid areas (Holman et al., 2021). Although droughts affect a range of economically important sectors, their impacts are usually more evident within agriculture, including crop failure and reduced yields, abandoned farmland, increased soil degradation and reduced mortality (Holman et al., 2021, Zhu et al., 2021). This situation creates an urgent need for attaining agricultural sustainability toward building resilience and adaptive capacity.

Preferably, endemic plants should be chosen to ensure sustainability and environmental compatibility on dry wasteland. Some representatives of the family Fabaceae and genus *Medicago* are suited candidates. They are high-quality protein-rich food sources with low demand on nutrients, a distinct resistance to saline conditions, and the ability to grow on low-quality sandy soils (Elouafi et al., 2020, Alandia et al., 2020). *Medicago* is already in use as an alternative crop, because of its ecological adaptability, morphological diversity, resistance to pests, high forage quality, high biomass production and ability to reduce soil erosion and to improve soil fertility and pasture in arid Mediterranean regions (Reid et al., 1989, Lodge, 1996, De Haan et al., 1997, Sheaffer et al., 2002). However, there is a high variation of drought resistance in this genus (Echeverria et al., 2021). Badri et al. (2016) studied the variation for tolerance to water deficit in 47 lines of *M. truncatula Gaertn., M. polymorpha L.* and *M. ciliaris* (L.) All. Latter one was the latest-flowering and produced highest biomass at low soil water availability. Therefore, we selected *M. ciliaris* as test species.

The suitability of *M. ciliaris* depends on the efficiency of its strategies during adaptation to water shortage. Volaire (Volaire, 2018) proposed the existence of three primary plant eco-physiological strategies: avoidance, tolerance and escape.

Desiccation tolerance is relatively seldom in dicot plants and *M. ciliaris* does not have this ability. In dicots, only resurrection plants are a small polyphyletic group of plant species capable of desiccation tolerance in their vegetative tissues while being able to recover full metabolic competence within hours to days following rehydration (du Toit et al., 2021). Therefore, *M. ciliaris* seems to use the escape or avoidance strategy or a mixture of both (Matos et al., 2021). Both remaining strategies have also in common a large energy demand, the necessity to optimize water uptake and to minimize water loss by a high water use efficiency of photosynthesis and during osmotic adjustment, the osmo-protection of macromolecules, the limitation of temperature rise in the leaf, the control of the respiration rate, and the protection against reactive oxygen species (ROS) (Upadhyay, 2019).

In fact, decreased diffusion of CO_2 and metabolic constraints are affecting photosynthesis as one of the key phenomena of water deficiency (Pinheiro and Chaves, 2011). Photosynthesis is a decisive point of attack and at the same time, the key process controlling plant growth and development and thus crop yield. Water shortage for example can reduce photosynthesis in plants through stomatal and non-stomatal limitations (Salmon et al., 2020) but coordinated regulation of photosynthesis in plants can increase biomass production and resistance to environmental stress (Koyro and Huchzermeyer, 2018).

Water shortage can harm photosynthesis directly by the restriction of CO_2 uptake, which leads to damage in photosynthetic machinery and as a final consequence, to the development of reactive oxygen species (ROS) (Koyro and Huchzermeyer, 2018, Siddique et al., 2016).

In order to carry out a successful re-cultivation at low soil water availability it is necessary to improve soil quality and so to the ability of plants species to resist this harsh environment.

A mean to improve soil quality is the amendment of biochar (Bc). Bc can significantly increase the organic matter content, the water holding capacity and the plant-available water of poor-quality sandy soil, latter one due to its porous nature (Kammann et al., 2011, Koyro and Huchzermeyer, 2018). The addition of Bc to the soil proved to be beneficial to plants in many ways such as enhancing water retention capacity, nutrient uptake (Paneque et al., 2016b, Sharma et al., 2019, Haider et al., 2020) water infiltration (Asai et al., 2009, Ippolito et al., 2012), soil aeration and respiration (Case et al., 2012, Haider et al., 2020). Moreover, Bc stimulated root growth and thus water uptake from fine Bc pores. It also provided at low water supply better conditions for the synthesis of organic solutes, prevented desiccation with improved turgidity, and reduced oxidative stress through high water use efficiency (Paneque et al., 2016a, Tayyab et al., 2018).

The soil improvements with Bc jointly contribute to the increase of physiological and biochemical performances of plants and consequently promote plant biomass production. These beneficial effects of Bc were reported in several species such as maize and rice (Sattar et al., 2019).

Currently, no data in the literature are available regarding the effect of Bc on drought resistance of *M. ciliaris* seedlings. We hypothesize that Bc application significantly reduces the degree of stress on *M. ciliaris* plants. This study aims to invistigate the impact of drought and Bc on the photosynthesis and water use efficiency of this promising species. By exploring the physiological and biochemical mechanisms underlying *M. ciliaris'* response to water shortage, we aim to enhance the response of this species to water deficit through the addition of Bc to the culture medium and boost the productivity of alternative crops. This approach could pave the way for transforming dry wastelands into productive agricultural

2. Methodology

The present chapter investigates the effects of biochar at water deficiency conditions on *M. ciliaris* line originated from a local Tunisian population of Enfitha (TNC1.11). Scarified seeds were germinated in Petri dishes in the dark at 25 °C for 3 days then transferred into pots filled with a mixture of 70% soil, and 30% sand mixed or not with 2% Bc. The emerged seedlings of uniform size were subjected to pretreatment phase of 72 days and treatment phase of 42 days.

Two irrigation modes were retained in this study: 60 and 36% of WHC. Plants irrigated with 60% WHC corresponded to control plants and plants irrigated with 36% of WHC corresponded to the stressed ones. The WHC measured according to the technique of Bouyoucos was estimated at around 13,33%. The parameters measured were growth parameters , water status and photosynthetic parameters.

Photo 3.1 Comparison of morphological parameters of *M. ciliaris* plants in high and low water potential (Overall plant height and biomass). The comparison highlights the potential of biochar in mitigating the adverse effects of water scarcity on plant morphology, with notable differences in the vigor and structure of the plants in each treatment

3. Results

3.1. Growth

The dry weight of control plants (0% Bc WPh) was about 10 g at the time of harvest. Biochar amendment significantly increased the biomass (~24%), (Figure 3.1 A). Instead, water deficit led to a significant reduction. However, this reduction was more pronounced in 0% Bc (~45%) than in 2% Bc. Shoot and root dry weight variations were in the mean similar to those of the whole plant dry weight (Figure 3.1 B and C).

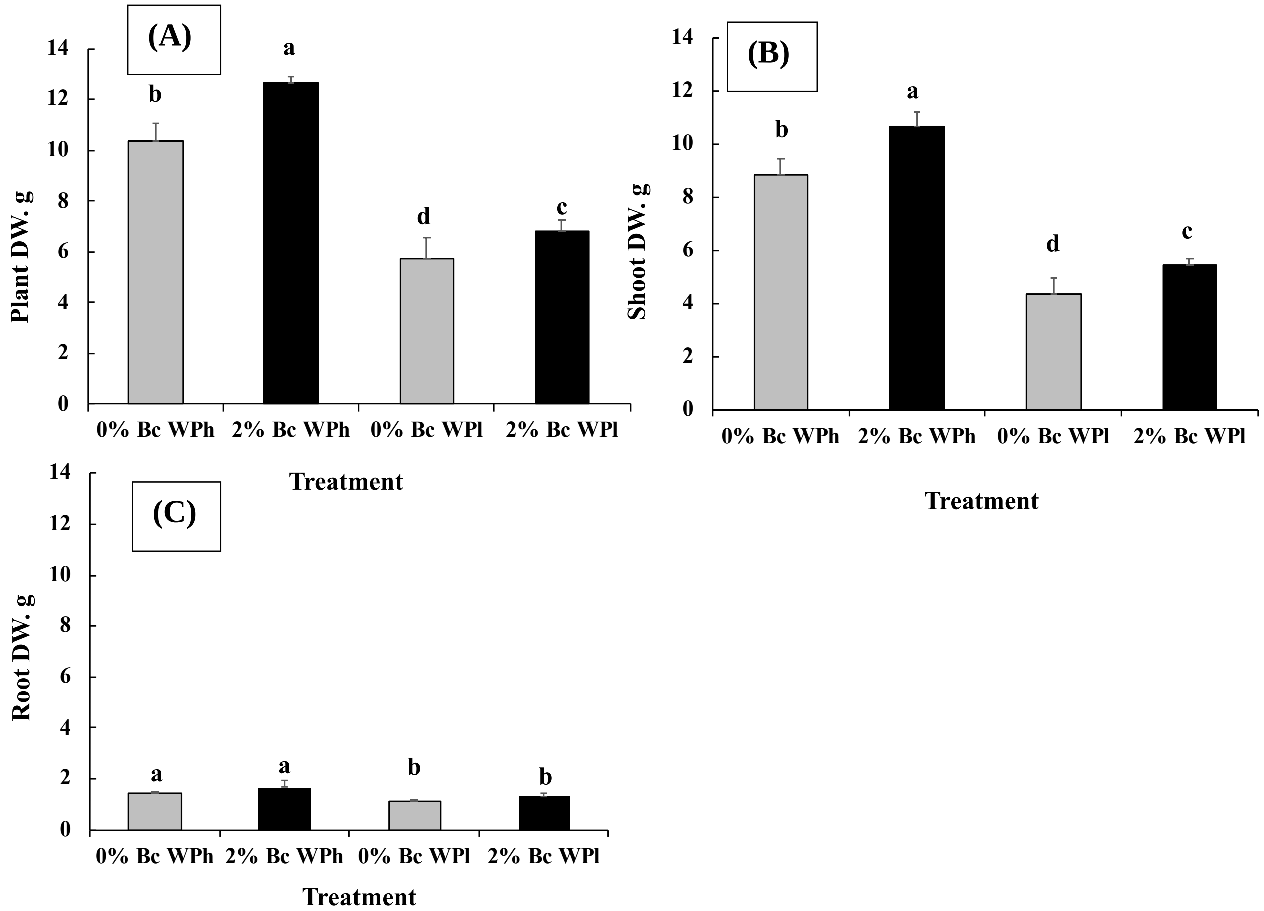

Figure 3.1. Varietal differences in plant growth parameters; dry weight of the whole plant (**A**), shoot (**B**), and the root (**C**), in *M. ciliaris* after 3 weeks of drought treatment. Values represent mean SE (*n* = 5) and the different letters a to d indicate significant differences between the treatments. Low soil water potential (WPl), high soil water potential (WPh), Biochar (Bc).

As shown in Figure 3.2, the root/shoot ratio was significantly higher in plants subjected to water deficit stress than in controls. The biochar amendment caused a significant increase of the root/shoot ratio with sufficient water supply but not under drought conditions.

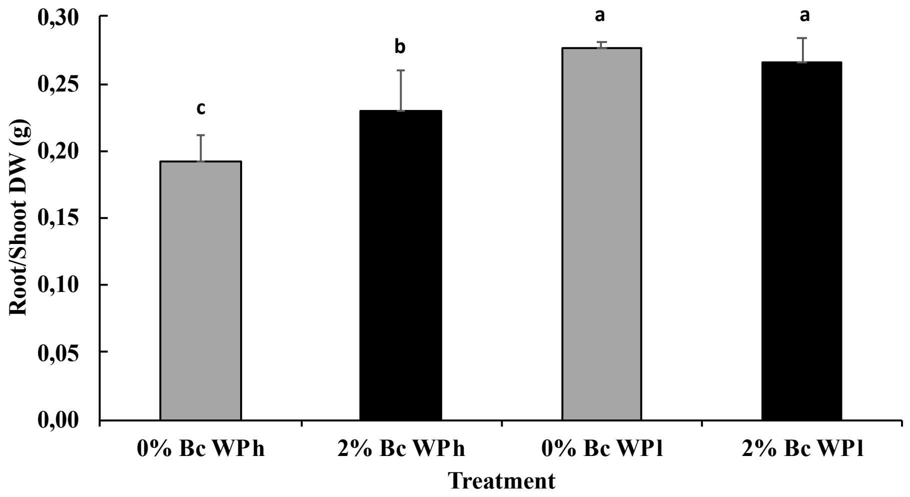

Figure 3.2. Root–shoot ratio in *M. ciliaris* after 3 weeks of drought treatment. Values represent mean SE (*n* = 5) and the different letters a to c indicate significant differences between the treatments. Low soil water potential (WPl), high soil water potential (WPh), Biochar (Bc).

3.2. Tissue Water Status

As shown in (Figure 3.3), shoot and root water content were significantly reduced by water deficit by 55% and 27%, respectively, indicating the depressive effects of drought on water status in *M. ciliaris*. Biochar had hardly any effect on the water status with the exception of a further reduction in water content in the roots (Figure 3.3 B).

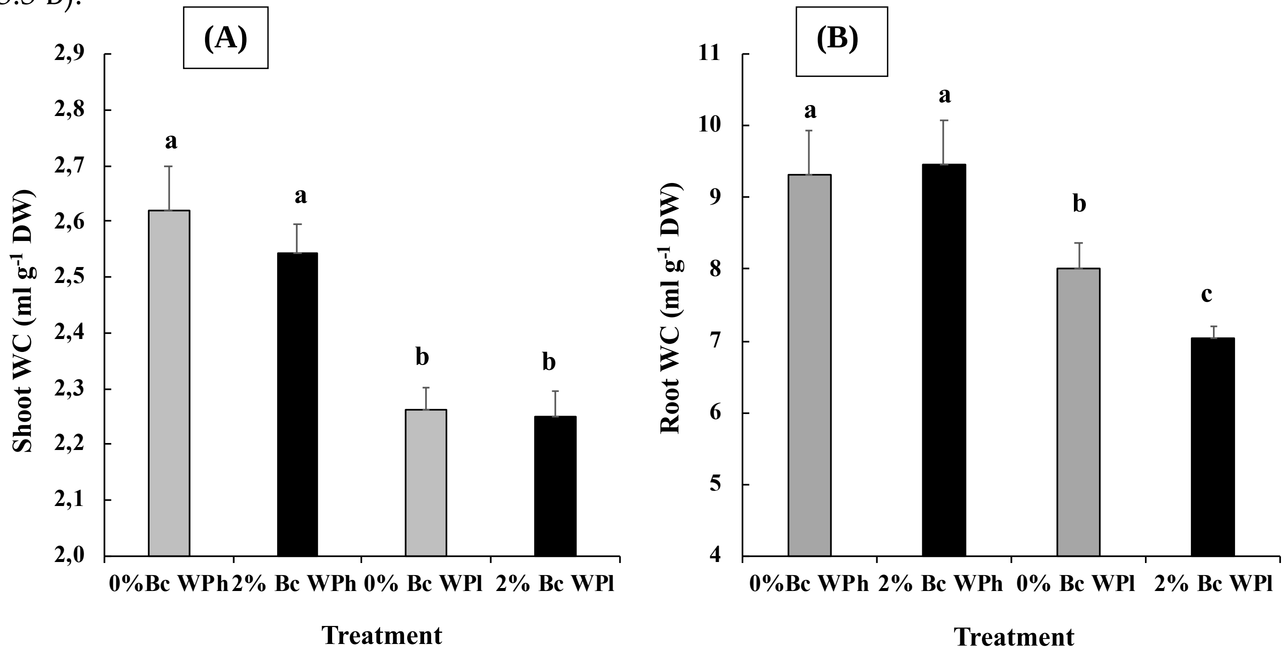

Figure 3.3 Shoot water content (**A**) and root water content (**B**) in *M. ciliaris* after 3 weeks of drought treatment. Values represent mean ± SE (*n* = 5) and the different letters a to c indicate significant differences between the treatments. Low soil water potential (WPl), high soil water potential (WPh), Biochar (Bc).

3.3. Chlorophyll and Protein Content

Water deficit stress significantly reduced chlorophyll content (Figure 3.4). The biochar amendment caused a significant increase in chlorophyll content at low and high soil water potential.

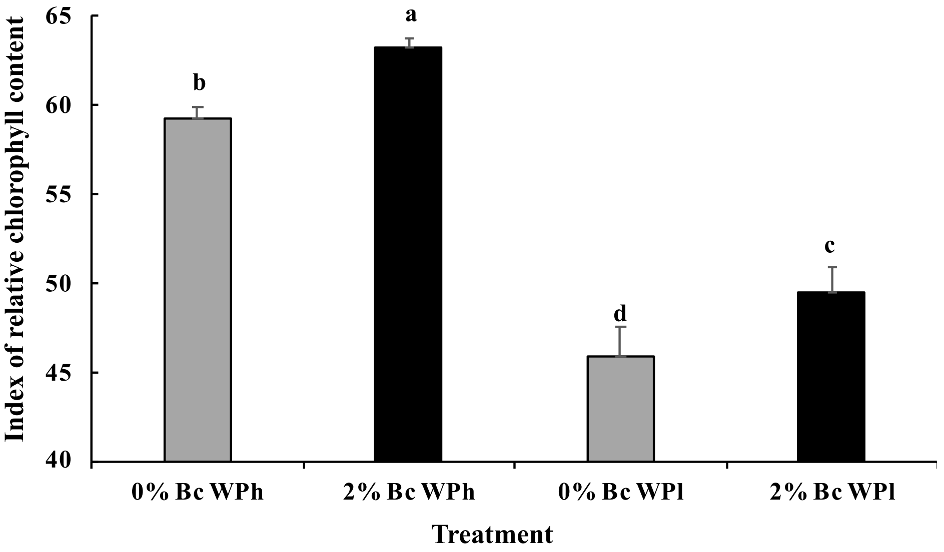

Figure 3.4. Chlorophyll concentration in *M. ciliaris* after 3 weeks of drought treatment. Values represent mean SE (n = 5) and the different letters a to d indicate significant differences between the treatments. Low soil water potential (WPl), high soil water potential (WPh), Biochar (Bc).

The development of the protein content was nearly reciprocal to the chlorophyll content (Figures 3.4 and 3.5). Drought led to a significant increase of the protein content and biochar to a non-significant decrease (in the mean).

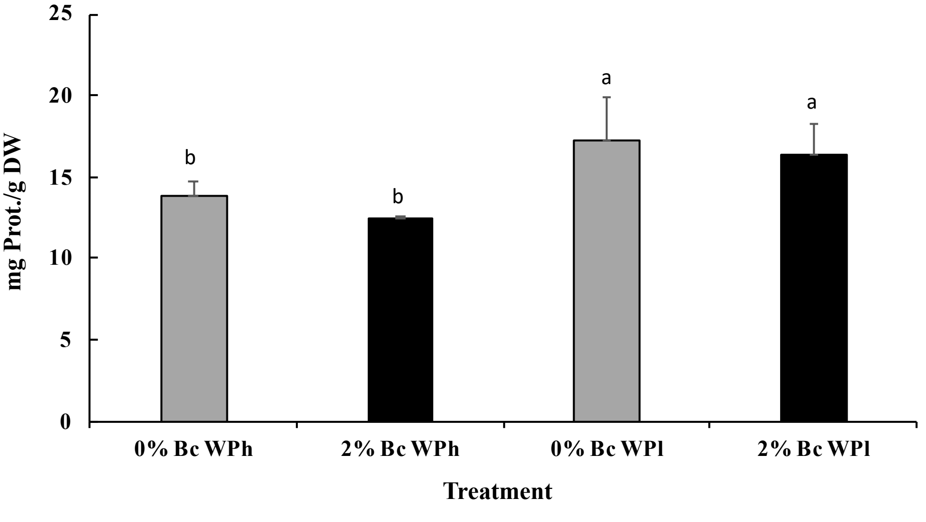

Figure3.5. Leave protein content in *M. ciliaris* after 3 weeks of drought treatment. Values represent mean SE (n = 5) and the different letters a and b indicate significant differences between the treatments. Low soil water potential (WPl), high soil water potential (WPh), Biochar (Bc).

3.4. Leaf CO_2/H_2O Gas Exchange

The highest A_{net} was reached at high water potential without biochar amendment (10,66 µmol m^{-2} s^{-1}) (Table 3.1). Drought led to a significant decrease of A_{net} (2,513 µmol m^{-2} s^{-1}). The Biochar amendment buffered significantly the drought induced reduction of A_{net}. The differences in A_{net} correlated nicely with the photosynthetic efficiency (V_c).

There was a clear correlation between drought induced reduction of A_{net} and ETR leading finally, and under incorporation of dark and light respiration rates (latter one not shown), to homeostatic and stable ETR/A_{gross} ratio in all four treatments.

There was a clear direct correlation between the drought induced reduction of A_{net} and the stomatal conductance, with the logic consequence of low C_i/C_a ratios in both generously watered treatments and high C_i/C_a ratios in both water deficient treatments).

The application of biochar didn't have any effect on C_i/C_a and Sc. However, biochar had a significant positive impact on the water use efficiency at low water potential. This effect was reached mainly by the maintenance of high A_{net} rates.

Table 3.1. CO_2/H_2O gas exchange and chlorophyll fluorescence parameters (A_{net}, V_c, SC, C_i/C_a ratio, WUE, R_L, R_D, ETR, ETR/A_{gross}, and Y(NPQ)) of *M. ciliaris* leaves at saturating light intensity after 3 weeks of drought treatment. Values represent mean ± SE (n = 5) and different letters indicate significant differences between treatments. Net CO_2 assimilation rate (A_{net}) [µmol(CO_2) m^{-2} s^{-1}], photosynthetic efficiency (V_c), stomatal conductance (S_c) [mol(H_2O) m^{-2} s^{-1}], ratio of intercellular and atmospheric CO_2 concentration (Ci/Ca ratio) [µmol(CO_2) m^{-2} s^{-1}], ratio of net CO_2 assimilation rate and transpiration (A/E) (µmol/mmol), Photorespiration (R_L) (µmol(CO_2)*m^{-2}*s^{-1}), Dark respiration (R_D) (µmol CO_2 $m^{-2}s^{-1}$), electron transport rate (ETR) (µmol electrons m^{-2} s^{-1}), Gross CO_2 assimilation (A_{gross}) [µmol(CO_2) m^{-2} s^{-1}], Quantum yield of regulated non-photochemical energy loss in PS II (Y(NPQ)), electron (e^-).

Treatment **Parameter**	**WPh** (at 1500 µE m^{-2} s^{-1} PPFD)		**WPl** (at 750 µE m^{-2} s^{-1} PPFD)	
	0 % Bc	**2 %Bc**	**0 % Bc**	**2 % Bc**
A_{net} (µmol CO_2 m^{-2} s^{-1})	10,667 a ±0,566	8,029 b ±0,803	2,513 d ±0,294	4,495 c ±0,801
V_c (µmol CO_2*m^{-2}*s^{-1})	0,057 a ±0,008	0,048 ac ±0,012	0,030 b ±0,001	0,045 bc ±0,012
Sc (µmol CO_2 m^{-2} s^{-1})	0,07 a ±0,008	0,05 b ±0,004	0,021 c ±0,008	0,03 c ±0,007
C_i/C_a ratio	0,367 a ±0,05	0,323 a ±0,06	0,510 b ±0,09	0,422 b ±0,01
WUE (A/E)	9,476 b ±1,27	6,595 c ±0,21	6,4 d ±1,85	11,04 a ±0,61
R_L (µmol(CO_2)*m^{-2}*s^{-1})	11,88 b ±0,73	13,59 a ±0,95	9,05 c ±0,56	8,01 c ±0,72
R_D (µmol CO_2 m^{-2} s^{-1})	1,189 ab ±0,141	0,640 b ±0,157	1,675a ±0,600	0,900 b ±0,452
ETR (µmol e^- m^{-2} s^{-1})	182,23 b ±7,71	195,73 a ±8,15	112,38 c ±3,9	109,6 c ±6,42
ETR/A_{gross} ratio (e^- / CO_2)	7,75 a ±0,2	8,65 a ±0,26	8,43 a ±0,63	8,4 a ±0,68
Y(NPQ)	0,382 a ±0,04	0,301b ±0,06	0,329 a ±0,03	0,371 a ±0,029

4. Discussion

In agreement with the present study, scientists worldwide are exploring possibilities in order to create the best possible growing conditions for drought-resistant crops that are able to maintain high productivity even in dry wastelands (Mancosu et al., 2015; Ngumbi and Kloepper, 2016). We decided to select *M. ciliaris* because it is a relatively salt-resistant medic species.

4.1. Adjustment of Growth and Water Relations

It was obvious that *M. ciliaris* reduced biomass production by 45% in cases of water deficit (Figure 3.1). This result matches with results from Ines et al. (2022), showing that the reduction of plant biomass production of medic plants ranged between 12 and 73%. However, it could be shown that the addition of Bc (biochar) to the soil substrate led to a significant increase in the biomass production of *M. ciliaris* in both water regimes. Similar Bc effects were reported for rice and maize grain yields (increase of 12.1% and 28% respectively) (Ahmed et al., 2019, Wang et al., 2014, Wu et al., 2014). Independently of the presence of Bc in the culture medium and in line with our findings, drought-stressed plants exhibited a higher root/shoot DW ratio than plants growing under adequate water supply (Figure 3.2). This may be related to the preferential allocation of dry matter to roots (Ines et al., 2022) and may facilitate adaptation to drought by limiting the transpiring leaf area and extracting water residuals (López-Galiano et al., 2019).

The drought-induced increase of the root/shoot ratio may be also a consequence of decreasing RWC in both organs (Figure 3.3). The decrease of the RWC in case of water shortage seems to be a typical response of *M. ciliaris*. For instance, the exposure of several annual *Medicago spp. (M. rugosa, M. scutellata, M. littoralis, M. truncatula, M. murex, M. polymorpha, M. intertexta, M sativa*) to five days of drought led to a reduction of RWC up to 40% (Naidu et al., 1992). In cases of *M. ciliaris* varieties, the decrease amounted to around 60% in severely dehydrated plants (Jungklang et al., 2017). From this perspective, it is surprising that Bc amendment led not only to a further reduction of the root RWC during water shortage but also to higher growth rate. This apparent contradiction can be explained by the enhanced osmotic adjustment with organic osmoprotectants as a strategy to tolerate the adverse effects of drought conditions (López-Galiano et al., 2019). Furthermore, Bc improved soil quality by producing higher organic matter that enhanced growth-regulating substances and plant functioning (Manolikaki and Diamadopoulos, 2019).

Similar results were also found for soybean (Mannan et al., 2021). The authors reported that Bc application significantly enhanced crop growth rate, increased total biomass production approximately twice and improved WUE but did not improve RWC, water retention or uptake capacity in leaves. The assumption seems obvious that the enhanced biomass production after addition of Bc to the soil substrate may be caused by an improvement in plant nutrition rather than by increasing water uptake. Indeed, biochar application increased soil-available potassium (K) and enhanced its uptake and the stress resistance in soybean.

4.2. Regulation of Photosynthesis

The establishment of a new suited equilibrium and a high efficient use of the resources is the main strategy during adaptation to water shortage in *M. ciliaris*. The following adaptation of photosynthesis to a new optimum is a good example for a coordinated regulation in *M. ciliaris*: The suppressive impact of drought on photosynthesis (A_{net}, table 3.1) contributed together with a reduction of the chlorophyll content (figure 3.4), leaf area and photosynthetic electron transport rate (ETR) to the maintenance of a constant ETR/A_{net} ratio (table 3.1). This mechanism reduced the generation of ROS and counteracted the otherwise possible destruction of chloroplasts (Raza et al., 2021, Gill and Tuteja, 2010).

Usually, a reduced chlorophyll concentration, implies a reduced ability for light harvesting and thus reduced photosynthesis (Fathi and Tari, 2016). *M. ciliaris* seems to use this correlation in case of drought actively or forced for backwards regulation and for the protection of the plants against oxidative stress as confirmed in the current study. Latter one could happen because of the limited stomatal conductance and CO_2/H_2O gas-exchange at water shortage (Geissler et al., 2015). Indeed, our studies showed that A_{net} and S_C but not C_i (or the C_i/C_a ratio, Table 3.1) increased and decreased simultaneously. Stomatal conductance was higher in plants receiving normal irrigation than drought-treated plants (Raza et al., 2021). The increase of the C_i/C_a ratio in the leaf intercellular is a very common response at limited water supply because of reduction of the flow through the closing stomates (Turner et al., 1985). However, the opposite happened in *M. ciliaris* (table 3.1). This can be explained by the fact that the photosynthetic carbon assimilation capacity decreased under water shortage to a higher degree (76%) than the leaf conductance (70%) and illustrates nicely the resultant photosynthetic shifts from stomatal to non-stomatal limitations. These results are similar to some earlier findings published by He et al., (He et al., 2020) where drought stress was also accompanied by increasing intercellular CO_2 concentrations of bamboo leaves (*D. minor var. amoenus).* In this context it was shown that photosynthesis is primary affected under mild and moderate stress conditions by stomatal limitation but under severe water deficiency by non-stomatal limitation in chloroplast CO_2 fixation ability, rather than CO_2 diffusion resistance (Bota et al., 2004, Grassi and Magnani, 2005, Chaves et al., 2009, Lawlor and Tezara, 2009).

It is well known that Bc improves plant performance in the form of higher organic matter production, an increased synthesis of growth regulating substances and an improved plant functioning (Manolikaki and Diamadopoulos, 2019). Bc amendment also supports at water shortage increasing activity of anti-oxidant enzymes and the maintenance of high leaf chlorophyll contents (Keshavarz Afshar et al., 2016, Iqbal, 2017). A number of previously published reports (Paneque et al., 2016a, Lyu et al., 2016, Xiao et al., 2016) showed that Bc application can improve soil water availability in general and buffer the effect of reduced water supply on plant photosynthetic carbon assimilation capacity. This is in line with our finding that soil amendments with Bc boost chlorophyll content both under well and insufficient watered plants.

In comparison to the results of the water shortage treatment (0% Bc WPl, see above) and in agreement with above cited literature was the Bc-induced higher maintenance (2% Bc) of net photosynthesis (A_{net} only 40% less as WPh, table 3.1) accompanied by a proportional higher chlorophyll content (figure 3.4, (Wang et al., 2021, Raza et al., 2021, Zainul et al., 2017)) and less pronounced decrease of stomatal conductance (S_c, only 44% less as WPh). In order to survive in dry arid zones, optimizing photosynthesis as well as stomatal conductance is essential for plant species with the aim to preserve net CO_2 assimilation and reduce evaporation (Fetjah et al., 2021). Under these unfavorable conditions it seems to be beneficial that Bc application enhanced WUE in case of water shortage (table 3.1). The high WUE correlated with the maintenance of a high A_{net} and the reduction of non-stomatal limitation. By increasing WUE, oxidative stress is most likely reduced and consequently the resistance against drought stress is increased (H_2O loss per net CO_2 uptake) (Paneque et al., 2016a). This interpretation is in line with some previous studies in which soil amendment with Bc alleviated drought stress symptoms by significantly enhancing the water use efficiency, stomatal conductance, chlorophyll contents, and photosynthesis of tomato, cowpea and okra leaves during water shortage (Farooq et al., 2021, Singh et al., 2019).

A remarkable feature of the photosynthetic apparatus is its ability to adapt to changes in environmental conditions by sensing light quality and quantity, CO_2 levels, temperature, and nutrient availability (Rochaix, 2011). The water shortage and the resulting low A_{net} and low WUE led in *M. ciliaris* (0%Bc) to a reduced demand and the necessity of regulation of light energy coming through the chloroplast electron transport chain. While light is essential for photosynthesis, it can also lead to light-induced damage when the absorbed light energy exceeds the capacity of the photosynthetic machinery. To avoid that, the excess photons and electrons need to be dissipated. This occurs through photoinhibition or a rapidly inducible non-photochemical quenching process Y(NPQ) in which the absorbed excess light energy is dissipated as heat (Niyogi, 1999, Massacci et al., 2008). Chlorophyll fluorescence is an important photosynthetic parameter that reflects the absorption and utilization of light energy from Photosystem II (PSII). However, *M. ciliaris* responds to water shortage not as expected with higher Y(NPQ) (table 3.1) but a significant decrease of ETR ($P < 0.05$) together with an increase of dark-respiration (R_D) and decrease of light-respiration (R_L), leading finally to no significant change of the ETR/A_{gross} ratio. The reduction of ETR correlated in *M. ciliaris* with a reduction of the chlorophyll content, indicating that PSII had been damaged to varying degrees, photosynthetic organs had been altered, and the effects of excessive light energy could not be disposed through heat dissipation (Y(NPQ)) instead, through photochemistry by producing large amounts of reactive molecules causing oxidative damage to photosynthetic organs (Dietz and Pfannschmidt, 2011) and the resultant photosynthetic shifts from stomatal to non-stomatal limitation.

In the current study, we applied Bc treatment in order to increase productivity and escape ROS damage. Indeed, biochar application during water shortage led to significant higher chlorophyll concentrations, higher A_{net} and also lower R_D and R_L (Table 3.1), giving the overall picture of a moderate stress response.

5. Conclusion

The results presented in this study support our assumption that Bc application enhances the performance of *M. ciliaris* during times of limited water supply. Indeed, biochar application during water shortage led to a more moderate exposure to water deficiency and in this way supported an improved and interactive plant adjustment. It led to a reduced impact of limited water supply on growth and water relations and included a balanced regulation of photosynthesis and the buffering of reactive oxygen species with photoprotective mechanisms indirectly by the response of the CO_2/H_2O gas exchange parameters (Table 3.1). We conclude that amendment through Bc may be a helpful approach to improve the performance of *M. ciliaris* during water shortage and to increase plant productivity in the arid land regions. This species seems to be suited to use a cash pasture plant in the development of agriculture on dry wasteland in a future world of water shortage. However, further field trials should be carried out under corresponding conditions as an intermediate step before agricultural use to verify the presented results on an agro-ecosystem level.

Chapter 4

IMPACT OF BIOCHAR APPLICATION AT WATER SHORTAGE ON PROLINE CONTENT AND ANTIOXYDANT CAPACITY IN *Medicago ciliaris*

Abstract

The impact of biochar at water deficiency conditions on biochemical processes of *M. ciliaris* seedlings was investigated. Proline, malondialdehyde (MDA) and Hydrogen Peroxide Content, the activity of enzymatic antioxidants (SOD, APX, GPOX and GR) and non-enzymatic antioxidants were determined. Plants of uniform size were subjected after a pretreatment phase (72 days) either at low (36% water holding capacity, water potential low) or high soil water potential (60% water holding capacity, water potential high).

Under drought conditions *M. ciliaris* showed a higher risk of ROS production that was indicated by a high level of lipid peroxidation, high antioxidant activities and high proline accumulation. Soil amendment with biochar enhanced the growth significantly and water use efficiency in case of water shortage. This increase of water use efficiency was correlated with the biochar-mediated decrease of the MDA and proline contents in the leaves buffering the impact of drought on photosynthetic apparatus by increasing the activity of enzymatic antioxidants SOD, APX, GPOX and GR and non-enzymatic antioxidants, such as AsA and DHAsA, giving the overall picture of a moderate stress response. These results confirmed the hypothesis that biochar application significantly reduces both the degree of stress and the negative impact of oxidative stress on *M. ciliaris* plants.

Key words: *Medicago ciliaris,* drought, biochar, proline, antioxidant capacity.

1. Introduction

Water shortage can harm photosynthesis directly by the restriction of CO_2 uptake, which leads to damage in photosynthetic machinery and as a final consequence, to the development of reactive oxygen species (ROS) (Koyro and Huchzermeyer, 2018; Siddique et al., 2016). As oxygen is produced by the water-splitting system located adjacent to PSII, ROS formation is a real risk. Therefore, the degree of ROS damage and the efficiency of the ROS detoxification system in the chloroplasts are of crucial importance for survival. In fact, ROS has a high affinity to react with macromolecules such as lipids, proteins and nucleic acids and cause the malfunctioning of these macromolecules (Kocsy et al., 2013). For this reason it is very helpful that the extent of ROS damage can be estimated by measuring the peroxidation rate of membrane lipids with the malondialdehyde method (Koyro et al., 2013; Sharma et al., 2012).

However, in order to cope with an increased ROS production, plant cells display a complex and high-energy consuming array of both enzymatic and non-enzymatic detoxification mechanisms (Seminario et al., 2017). The latter group includes the production of low-molecular weight compounds such as AsA (ascorbate, vitamin C), glutathione (GSH), carotenoids or flavonoids (Das and Roychoudhury, 2014; Jithesh et al., 2006). AsA is one of the most abundant water-soluble reducing compounds present in plant tissues, serving also as an electron donor in numerous reactions (Foyer and Noctor, 2011) such as the effective quenching of H_2O_2 (Dewhirst and Fry, 2018).

The addition of Bc to the soil also provided at low water supply better conditions for the synthesis of organic solutes, prevented desiccation with improved turgidity and reduced oxidative stress through high water use efficiency (Paneque et al., 2016a; Tayyab et al., 2018). The soil improvements with Bc jointly contribute to the increase on the physiological and biochemical performances of plants and consequently promote plant biomass production (Sattar et al., 2019).

Lyu reported that the plant defense mechanism is strengthen by Bc application through the increase of protective enzymatic activities and the electron transfer chain, thereby, minimizing the deleterious effects of drought on photosynthetic apparatus (Lyu et al., 2016a).

Currently, no data in the literature is available regarding the effect of Bc on drought resistance of *M. ciliaris* seedlings. We hypothesize that Bc application significantly reduces both the degree of stress and the negative impact of oxidative stress on *M. ciliaris* plants. We intended to study the impact of drought and Bc on photosynthesis, water use efficiency, oxidative stress damage, ROS defense, and growth performance of this promising species. Regarding the study of biochemical mechanisms implied in the response of *M. ciliaris* to water shortage, we aim to improve the response of this species to water deficit through the addition of Bc to the culture medium.

2. Methodology

The present chapter deals investigates the effects of biochar at water deficiency conditions on proline, malondialdehyde (MDA), hydrogen peroxide content, and the variation of the enzymatic (SOD, APX, GPOX, and GR) and non-enzymatic antioxidant activities. The plants used in the present study were those obtained according to the protocol described in the previous chapter and whose growth, water relations and photosynthetic parameters were previously analyzed.

3. Results

3. 1. Proline and MDA Accumulation in leaves

Drought led to a significant increase of proline content (Figure 4.1A) and MDA content (Figure 4.1B). However, biochar caused a significant decrease of proline and MDA content at low and high soil water potential.

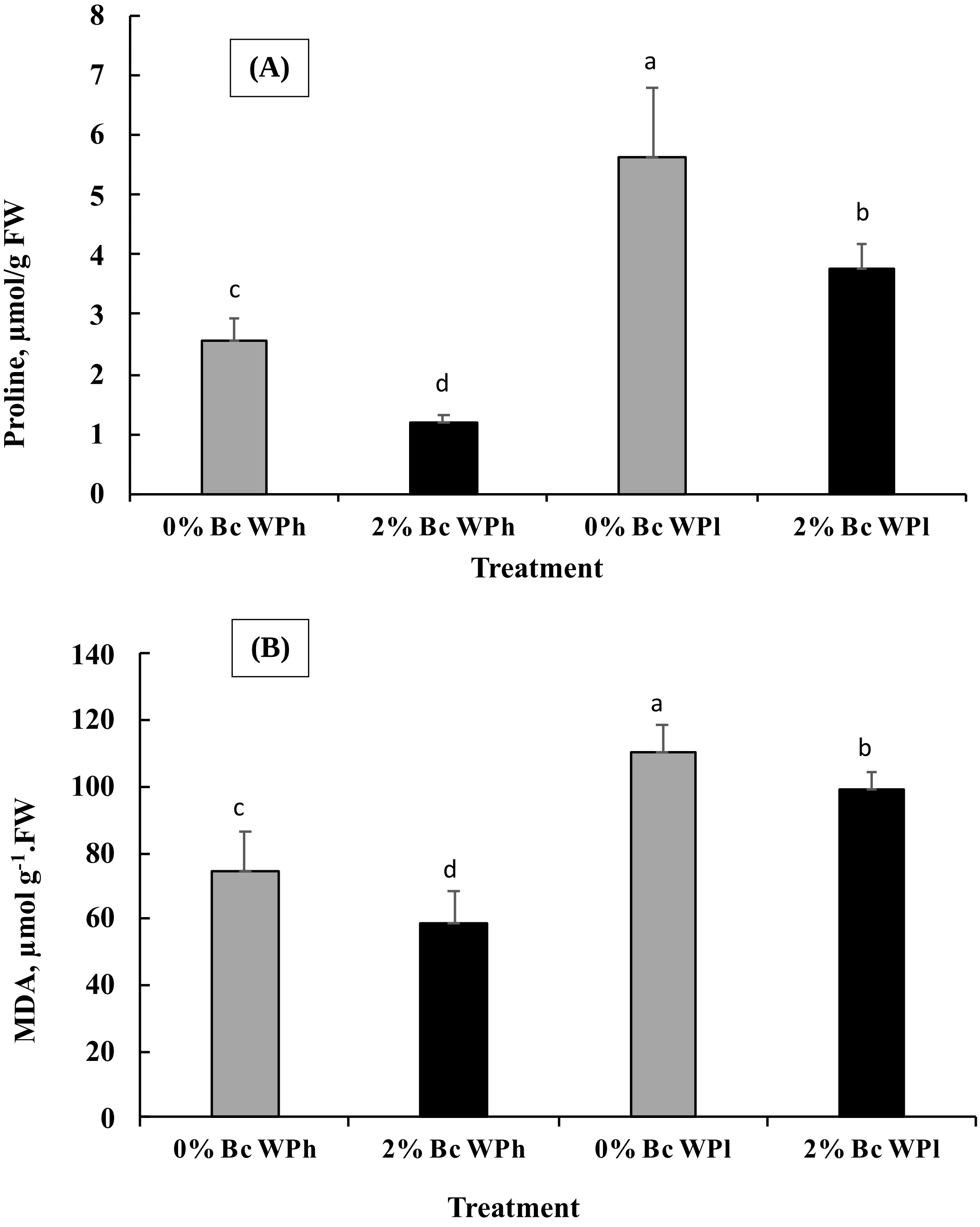

Figure 4.1. Changes in Proline (**A**) and MDA (**B**) content in *M. ciliaris* leaves after 3 weeks of drought treatment. Values represent mean ± SE (*n* = 5) and the different letters a to d indicate significant differences between the treatments. Low soil water potential (WPl), high soil water potential (WPh), Biochar (Bc).

3. 2. Enzymatic Antioxidant Assays

Drought stress caused an increased accumulation of H_2O_2 in the leaves of *M. ciliaris* treated with and without biochar (Figure 4.2 A). It is noticeable that higher H_2O_2 values correlate with lower chlorophyll content (see Figure 3.4) and lower photosynthetic activity (see Table 3.1). The limited water supply induced an increase in reactive oxygen species (ROS), such as H_2O_2, . It was the logical consequence to measure the antioxidant enzyme activities. The drought-induced adjustment of *M. ciliaris* to an enhanced attack by reactive oxygen species (ROS) is reflected by increased activities of SOD, APX, GPOX and GR (Figure 4.2). However, biochar reduced the APX and SOD activities in plants. Both enzymes constitute first line of defense against oxidative stress. Their reduced activities might be an indicator of a reduced demand for an adaptive response to ROS. The nonsignificant reduction of H_2O_2 content and GR activity points in the same direction.

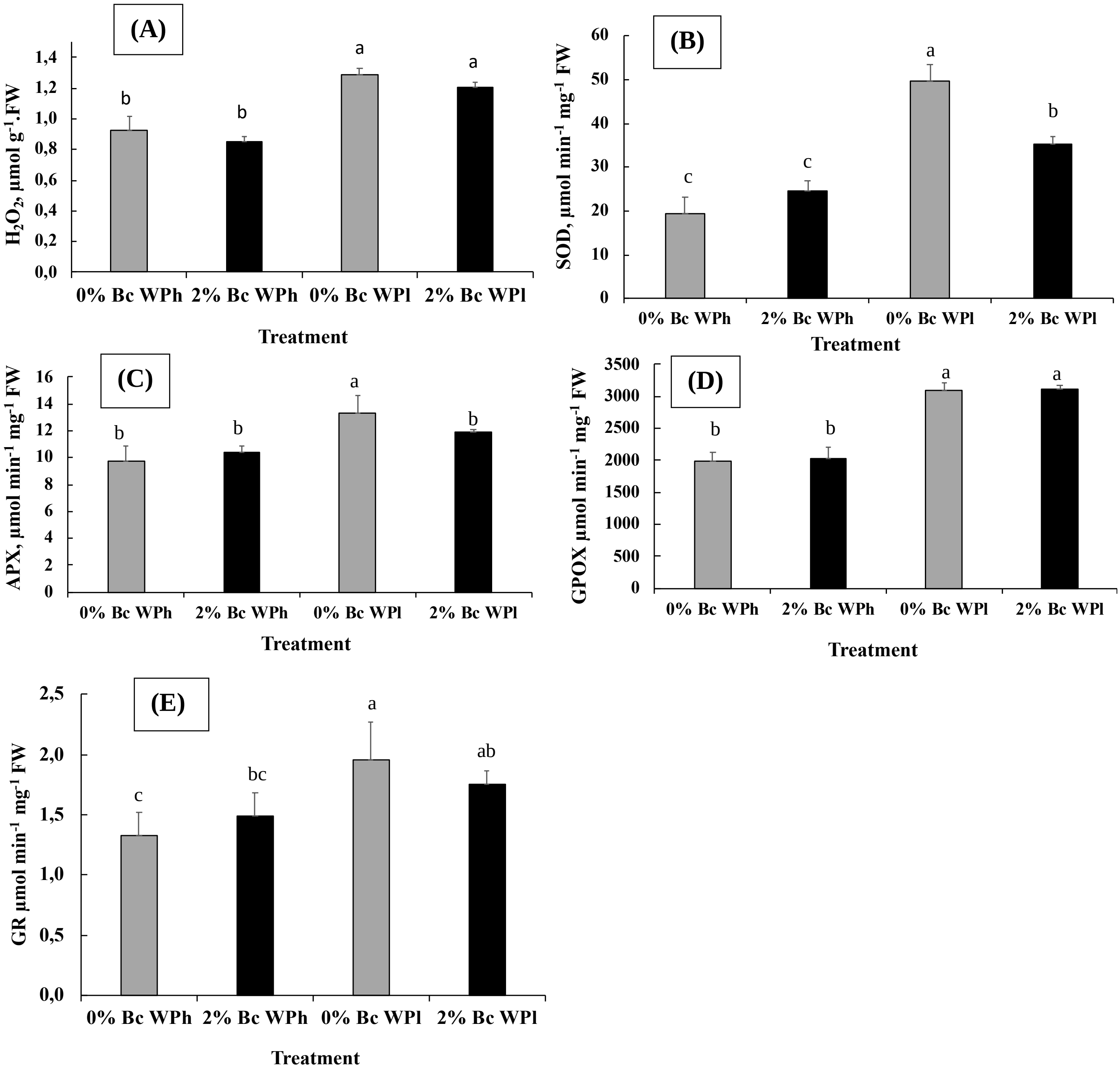

Figure 4.2. Varietal differences in the content of H_2O_2 (**A**) and the enzymatic activities of SOD (**B**), , APX (**C**), GPOX (**D**) and GR (**E**) in *M. ciliaris* leaves after 3 weeks of drought treatment. Values represent mean ± SE ($n = 5$) and the different letters a to c indicate significant differences between the treatments. Low soil water potential (WPl), high soil water potential (WPh), Biochar (Bc).

3. 3. Non Enzymatic Antioxidant Assays: Ascorbate Determination

Both factors water deficiency and biochar induced a significant increase in the total ascorbate concentration in the leaves of *M. ciliaris* (Figure 4.3 A). Drought reached this effect by a joint increase of the reduced ascorbate (AsA, Figure 4.3 B) and oxidized ascorbate (DHAsA, Figure 4.3 C) concentration. However, biochar had a significantly higher impact on the concentration of DHAsA in drought, leading to an overall significant decrease in the AsA/DHAsA ratio in both biochar treatments (Figure 4.3 D)

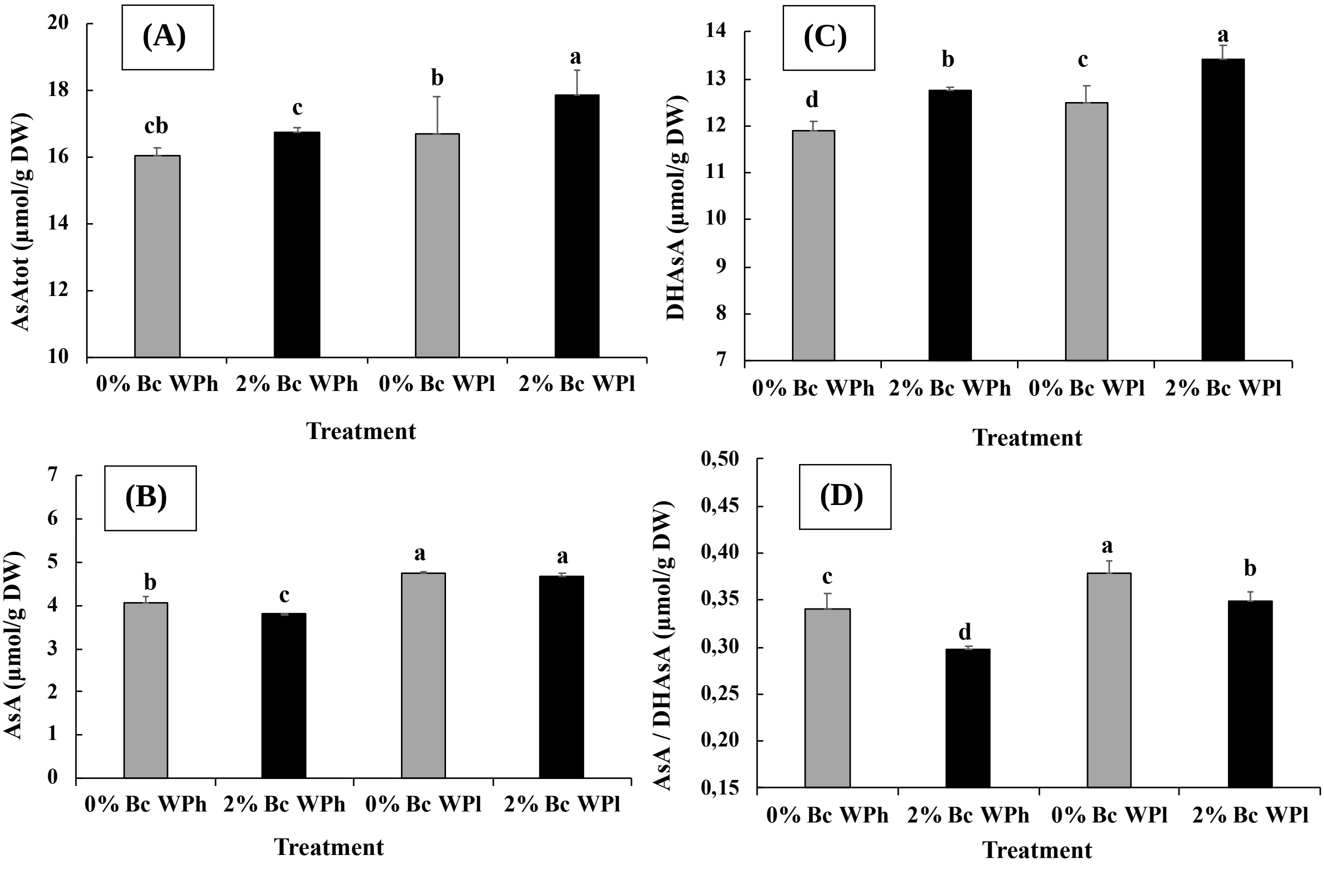

Figure 4.3. The content of total ascorbate (AsA + DHAsA) (**A**), AsA, (**B**), DHAsA (**C**), and AsA/DHAsA ratio (**D**) in *M. ciliaris* leaves after 3 weeks of drought treatment. Values represent mean ± SE (*n* = 5) and the different letters a to d indicate significant differences between the treatments. Low soil water potential (WPl), high soil water potential (WPh), Biochar (Bc).

4. Discussion

4. 1. Indicators of Oxidative Stress

The results of CO_2/H_2O gas exchange and PSII chemistry confirms the acceptance of a possible increasing oxidative stress at water deficiency in soils without Bc amendment resulting in malfunctioning and eventually death of the affected cells (Yadav and Sharma, 2016). In general, several photoprotective mechanisms exist such as plastid antioxidant enzymes and molecules (Niyogi, 1999), repair processes, for damaged PSII (Nixon et al., 2010) and lipid peroxidation (Rochaix, 2011). In this study we used MDA, proline and H_2O_2 as biomarkers for oxidative stress (MDA figure 4.1 B), (Khaleghi et al., 2019a), non-enzymatic photo-protection (proline figure 4.1 A, (Anwar Hossain et al., 2014) and redox regulation (H_2O_2, figure 4.2 A, (Marinho et al., 2014).

In agreement with our expectations water shortage led to a significant increase of the MDA content in *M. ciliaris*, indicating a substantial stress by reactive oxygen species leading to lipid peroxidation, fatty acid saturation, and consequently damage to the membranes (Ramachandra Reddy et al., 2004; Shafiq et al., 2015). The formation of MDA is actually the consequence of enzymatic breakdown in cells. . *M. ciliaris* plants grown with Bc-amendment had lower MDA content than the non-treated ones. This effect can be explained by the coordinated activation of protective enzymes (see figure 4.2) which leads to attenuate ROS production hence oxidative stress (Lyu et al., 2016b; Rondon et al., 2007). Our results are similar to some earlier findings in which soil amendment with Bc decreased MDA content of *Phragmites karka* and *Brassica olerecae* (Cabbage seedling) under drought stress conditions (Abideen et al., 2020; Yildirim et al., 2021).

We got similar responses of both, proline and MDA to water stress as to Bc amendment. Proline, produced under harmful conditions, can act as a free radical scavenger for photo-protection but is also a compatible solute in osmotic adjustment (Anjum et al., 2011a), a metal chelator, an activator of ROS detoxification pathways, a cell redox balancer, a cytosolic pH buffer, a source of energy, a source of nitrogen and carbon, a stabilizer of subcellular structures and membranes including photosystem II (Nadeem et al., 2019), and can act as a signaling molecule (Hossain et al., 2014). Water shortage led in *M. ciliaris* leaves (0% Bc) to a considerable increase of leaf proline accumulation which shows the importance of proline as photoprotectant and osmoprotectant (see also (Khatun et al., 2021; Kosar et al., 2021). In agreement with our findings, Yildirim (Yildirim et al., 2021) reported that Bc treatment lowered proline content in the plants. Our results suggest reduced osmotic and oxidative stress in Bc-treated plants.

The last used biomarker H_2O_2 regulates plant growth, development, acclimatory and defense responses (Slesak et al., 2007). Moreover, among oxidative species, H_2O_2 is very vigorous metabolite that deteriorate the structure of biological membranes during abiotic stresses (Jajic et al., 2015). The increased production of ROS (such as H_2O_2) in drought-stressed plants of *M. ciliaris* (figure 4.2) is a common phenomenon taking place under stress conditions (Farooq et al., 2009). Plants use antioxidant defense (enzymatic or non-enzymatic) to deal with oxidative stress (Lyu et al., 2016c). Plants employ diverse defensive adaptive mechanisms to survive under such adverse cues such as the activation of a signaling pathway, expression of genes, and accumulation of stress-related proteins (Chen et al., 2013) and enzymes. Latter effect may also be used to explain the increase of protein content in *M. ciliaris* plants subjected to water shortage (figure 3.5).

4. 2. Photoprotective Mechanisms: Enzymatic Oxidants

In the present investigation, water shortage boosted enzymatic and non-enzymatic antioxidant activity in *M. ciliaris* plants. Enzymes like superoxide dismutase (SOD), ascorbate peroxides (APX), guaiacol peroxidase (GPOX) and glutathione reductase (GR) are directly engaged in catalyzing ROS degradation reactions by directly scavenge ROS (Farooq et al., 2013; Khaleghi et al., 2019b), and reduce indirectly membrane lipid peroxidation and alleviate the damage in PSII structure and function.

The first enzyme in the antioxidant pathway is SOD which removes superoxide radical by catalyzing its dismutation to H_2O_2 and another oxidized to O_2 (Halliwell, 2006) The increase of SOD activity observed in the leaves of *M. ciliaris* (figure 4.2 B) as a function of the applied water stress levels might be correlated to enhanced protection from damages, among them lipid peroxidation, associated with oxidative stress. In *M. ciliaris* water shortage led to an increased concentration of APX, GPOX and GR (see figure 4.2) suggesting the involvement of the Halliwell–Asada pathway, where APX reduces H_2O_2 to water and MDHA using ascorbic acid as substrate (Koyro et al., 2012) at the expense of NADPH (Ahanger et al., 2017; Duarte et al., 2013; Koyro and Huchzermeyer, 2018). Khaleghi (Khaleghi et al., 2019b) reported that APX activity increased in drought-stressed *Maclura pomifera*, *Picea aspertata* and *Nicotiana tabacum*, respectively. The stimulation of APX activity might be correlated to the increased H_2O_2 generation by the observed enhanced SOD activity (both figure 4.2). Wang (Wang et al., 2014b) reported that peroxidase activity is closely related to PSII electron transport properties and PSI but the activity of latter one could be inhibited with the increase in SOD activity.

SOD and APX showed maximum activity in the leaves of untreated plants and minimum activity in the leaves of Bc-treated plants (figure 4.2). These results correlate with the Bc-mediated decrease of the MDA and proline contents in the leaves and an improvement of photosynthetic parameters. It was shown before, that Bc application can buffer the impact of drought on photosynthetic apparatus by regulating the activity of protective enzymes and affecting electron transfer (Chaves et al., 2009; Lyu et al., 2016b). Our results are similar to some earlier findings at water shortage conditions where Bc application lowered enzymatic activity and lipid peroxidation and enhanced photosynthesis in *Pyrus ussuriensis* Maxim (Lyu et al., 2016b), *Brassica olerecae* (Yildirim et al., 2021), and *Phragmites karka* (Abideen et al., 2020).

4. 3. Photoprotective Mechanisms: Non-Enzymatic Antioxidants

Besides enzymatic antioxidants there are non-enzymatic antioxidants such as reduced (AsA) and oxidized (DHAsA) ascorbate, glutathione (GSH), carotenoid which can play a role in the antioxidant system in two ways either directly interact with ROS or function as substrates in enzyme-catalyzed ROS-degrading reactions (Anjum et al., 2011b; Farooq et al., 2013; Yadav and Sharma, 2016). Ascorbate (AsA, Vitamin C) is one of the universal non-enzymatic antioxidants having the ability to donate a hydrogen atom and form a relatively stable ascorbyl-free radical. It protects plants against oxidative damage by environmental stresses, such as drought (Foyer and Noctor, 2011; Pehlivan, 2017). It participates in diverse redox and ROS neutralization reactions in the chloroplast and can be facultative electron donor for photosynthetic electron transport chain (Akram et al., 2017). DHAR is responsible for regenerating AsA from the oxidized state and regulates the cellular AsA redox state, which is crucial in the response to abiotic stresses. Ascorbic acid (AsA) and its oxidized form dehydroascorbate (DHAsA) play a key role in redox state-based signaling mechanisms by detoxification of ROS and its products, as well as transmission of redox signals (Miret and Müller, 2017). To obstruct levels exceeding the anti-oxidative capacity of cells ROS formation has to be closely regulated.

The increased activity of APX at water shortage correlates with the increase of AsA and DHAsA and can be explained by the high demand and capacity to eliminate H_2O_2 in the leaves of *M. ciliaris* (s.a.). Increased AsA-GSH cycle enable chloroplast to prevent photoinhibition by maintaining the $NADP^+/NADPH$ ratio so that ETR gets least affected (Ahmad et al., 2018).

Water shortage caused a significant increase of the DHAsA content (figure 4.3). Several plant studies revealed that the upregulation of AsA-GSH pathway enzymes and the enhancement of the DHAsA and GSSG levels conferred plants better tolerance to abiotic stresses by reducing the ROS (Hasanuzzaman et al., 2019). DHAsA is supposed to be concerned in zeaxanthin biosynthesis dissipating excess light energy in the thylakoid membranes of chloroplast and prevents oxidative stress by maintaining the activity of antioxidant enzymes. In accordance to our expectations water shortage also led to a significant increase of AsA (reduced form of ascorbate) content and AsA/DHAsA ratio. Alteration in AsA/DHA ratio is involved in stress sensing and redox homeostasis is one of the important factors to protect cells from ROS toxicity (Foyer and Noctor, 2009). The leaf apoplast redox status specifically modulate plant growth and response to hormones, antioxidant enzyme activities, expression patterns of catalase, glycolate oxidase and some other genes, MAPK activity and regulation of transcripts associated with calcium channels (Pignocchi et al., 2006). For instance, AsA/DHAsA ratio is a prominent modulator of enzymes responsible for carbon assimilation under stress conditions (Kocsy et al., 2013) and usually calculated to evaluate AsA availability and, thus, used as an indicator of oxidative stress and initiators of plant defense system (Kocsy et al., 2013). Several authors mentioned that the increase of the AsA/DHAsA ratio above a distinct limit is leading to an enhanced production of abscisic acid in plants (Jubany-Marí et al., 2010) and can cause, in accordance with the available data shown in this paper, the closing of stomata, reduction of CO_2 fixation, cell expansion and plant growth (see (Chen and Gallie, 2004)). Moreover, the increase of the AsA/DHAsA ratio at 0% Bc and water shortage up to the highest level of all four treatments deepens the impression that the increase of SOD, APX, GPOX and GR activities were not high enough to hinder extensive ROS damage in *M. ciliaris* leaves.

However, soil amendment with biochar (2% Bc) led at water shortage to a significant reduction of the AsA/DHAsA ratio down to the level of well-watered control plants (0% Bc). This Bc-mediated effect was mainly reached by the significant higher increase of both, the total ascorbate AsA_{tot} (oxidized and reduced) and DHAsA content, which optimize *M. ciliaris* chances in buffering oxidative stress by directly scavenging ROS (Foyer and Halliwell, 1976; Foyer and Noctor, 2009).

Chapter 5

SEED PRIMING WITH ASCORBIC ACID IMPROVES RESPONSE OF MEDICAGO POLYMORPHA L. SEEDLINGS TO SALINITY AND PEG-MEDIATED OSMOTIC STRESS

Abstract

This study assessed the effect of ascorbic acid (0,2) seed priming on the performance of *Medicago polymorpha* L. under water shortage induced by irrigation with either 50 mM NaCl or 100g/L PEG. For this purpose, parameters related to plant morphology, CO_2/H_2O leaf gas exchanges, osmotic adjustment, pigment content and proline accumulation were determined.

Both NaCl-salinity and PEG-mediated osmotic stress, reduced plant biomass (-30% and -40% respectively), leaf and ramification number, stem length, net CO_2 assimilation (-31% and 63% respectively) and leaf water content. However, both treatments and especially PEG led to an increase in root/shoot ratios and leaf proline content.

Interestingly, ascorbic acid seed priming significantly enhanced CO_2/H_2O gas exchange and biomass production, with increases of 66%, 100%, and 92% in control, NaCl-treated, and PEG-treated plants, respectively. Ascorbic acid seed priming also improved water relations, as reflected by the decrease in leaf osmotic potential and the increase in leaf proline accumulation, with gains of 67% and 120% in PEG- and NaCl-treated plants, respectively. Additionally, it enhanced leaf water content, particularly under PEG treatment.

Besides, ascorbic acid seed priming supported an increase of the leaf carotenoid and chlorophyll contents (+65 and +45% respectively for chl a and chl b), thereby contributing to a better photosynthetic activity, and hence plant performance under salinity.

We concluded that ascorbic acid seed priming is an easy, cost-effective and promising approach to mitigate the impact of osmotic stresses like drought and salinity, by especially improving plant water relations and photosynthetic activity.

Keywords

Seed priming, ascorbic acid, *Medicago polymorpha* L, growth improvement, proline, salinity osmotic stress, drought.

1. Introduction

Drought and salinity are among the major factors limiting crop production in the arid and semi-arid areas not only by strongly reducing crop productivity of vegetable crops but also by affecting food security (Jiao et al., 2021). Plants responses to salinity and drought are often similar and multifactorial and complex. Therefore, the physiological and biochemical backgrounds of plant resistance to both stresses is poorly understood.

Seed priming before germination can induce drought and salinity resistance and offset the damages associated with biotic and/or abiotic stresses. It is one of the most promising authentic and affordable approaches to mitigate the deleterious effects of osmotic stress and is considered as an effective technique for achieving successful germination and for the improvement of the nutrient acquisition from nutrient-poor soils (Arun et al., 2022). During the early developmental stages, seed priming improves the germination rate and uniformity as well as the later behavior of seedlings in terms of plant productivity and stress resistance to drought, salinity, low temperature and chilling (Arun et al., 2022, Ben Youssef et al., 2021, Shah et al., 2019, Peyvast et al., 2009). Balmer reported that plants grown from primed seeds react more efficiently and rapidly to a stress (Balmer et al., 2015). It is adopted that exposure of plants to a primary constraint triggers a set of temporary metabolic adaptation inducing a stress memory that allows plants to adapt more efficiently to subsequent episodes of stress (Devika et al., 2021). However, the underlying physiological and biochemical background of this process is still poorly understood.

Ascorbic acid (AsA) is one of the most important metabolites involved in cell division, and osmotic adjustment (De Gara et al., 2003). AsA Application via seed priming may thus be helpful in improving the stand establishment and resistance to water shortage and salinity in plants (Farooq et al., 2013, Baig et al., 2021). Priming with this agent lowers the impact of salt by changing protein abundance, destination and storage (Ali et al., 2019). The over-production of reactive oxygen species (ROS) and their dousing is one of the key responses of plants to environmental stresses (Farooq et al., 2013). AsA is one of the most important antioxidant at cellular processes like cell division and expansion. It plays an important role in the metabolic activity during the germination process, cell detoxification, protection from reactive oxygen species as well as protecting against cell death (Arrigoni et al., 1992, Conklin and Barth, 2004).

Using different seed priming agents, including hydropriming, low temperature, scarification, urea, KNO_3, KCl, NaCl and ascorbic acid, Sadeghi and Robati (2015) found that ascorbic acid priming is the most effective tool to trigger water stress tolerance. Similar published results revealed that seed priming with AsA and salicylic acid proved to be effective in enhancing growth characteristics and yield attributes of *Praecitrullus fistulosus* (stocks) (Qamar et al., 2021).

M. polymorpha L., due to its fodder value, is an important local legume. It is considered as a potential species for pasture enhancement in the Mediterranean regions (Sheaffer et al., 2002). It's used in traditional Chinese medicine for treating indigestion, urinary tract calculi, jaundice and intestinal inflammation (Guo et al., 2020). However, *M. polymoroha* L. is particularly salt-sensitive species. According to Ibrar et al. (2003), the germination process in this plant is completely inhibited at 15dS/m (175 mM NaCl), whereas, at the vegetative stage, parameters including plant height, leaf and branching number, as well as shoot dry weight are severely inhibited at 5dS/m (50 mM NaCl) (Ibrar and Hussain, 2003). Besides, Cherifi et al. (2016) showed reduction of growth in four populations of *M. polymorpha* L. in a range between 40% and 60% under 50mM NaCl in plants cultivated in sand filled pots (Cherifi et al., 2016). Based on these data, we decided to use 50 mM NaCl as salt concentration for our study.

The present study was conducted to evaluate the potential of AsA (0,2 mM) to improve the drought (PEG-induced) and NaCl resistance of *M. polymorpha* L.. It was hypothesized that seed priming with AsA may improve key traits (osmotic adjustment, morphological parameters, water relations, pigment content, photosynthetic activity, and antioxidant potential) responsible for plant productivity. The intension behind this study was to prove that seed priming technique is an easy and affordable approach that can improve *M. polymorpha* L. performance under changing environments.

2. Methodology

The present chapter will be devoted to assess the analysis of the effect of ascorbic acid seed priming on *M. polymorpha L.* seedlings and their response to both salinity and PEG-induced osmotic stress. the plants were exposed to solutions with an osmotic potential of -0.3 MPa, induced by either 100 g/L PEG 6000 or 50 mM NaCl and the parameters measured were: growth and morphological parameters, photosynthetic parameters, water relations, Pigments concentrations, and proline accumulation.

3. Results

3.1 Growth and morphological parameters

3.1.1 Biomass production and leaf number

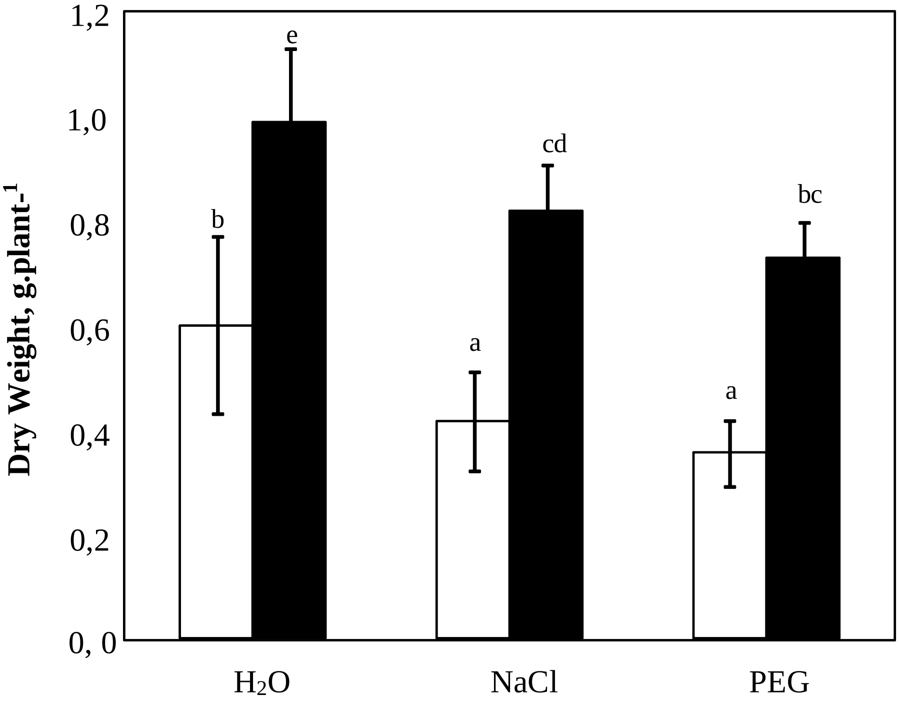

Figure 5.1: Whole plant biomass production of *M. polymorpha* seedlings derived from non-primed (*open columns*) or 0,2 mM Ascorbic Acid primed (closed columns) seeds subjected to three water treatments during one month: Control, NaCl 3g/L PEG 100g/L. Values are the means of eight replicates, vertical bars are SEs. Values sharing a common letter are not significantly different at $P < 0.05$.

The total plant dry weight as well as leaf and stem biomass were significantly reduced by PEG or NaCl salinity (-30 and -40 % respectively) (Figs.5.1 and 5.2). No significant differences were observed regarding root development between plants subjected to salt or PEG. Seed priming with ascorbic acid improved whole plant biomass production by 66%, 100% and 98% in the control, NaCl- and PEG-treated plants respectively. A similar trend was observed for growth of the different plant organs (leaves, stems and roots) (Fig.5.2). However, the increase in root DW under control salt or PEG in seed primed plants was less pronounced when compared to leaves. Indeed, Leaves of control plants produced 3-folds more biomass than roots (Fig. 5.2). Root/shoot ratio was significantly higher in plants challenged with osmotic stress, as compared to the control, but the highest values were observed in plants cultivated in the presence of PEG. Independently of the osmotic stress treatment, seed pretreatment with ascorbic acid led to a significant decrease in root shoot ratio (Fig.5. 3a).

Water deficiency conditions led to a significant reduction of leaf number (Fig.5.3b) (PEG: -40%, NaCl: -26%). Seed priming with ascorbic acid led to a significant increase of the leaf number in plants either by salt or PEG treatment and reached similar values as control plants.

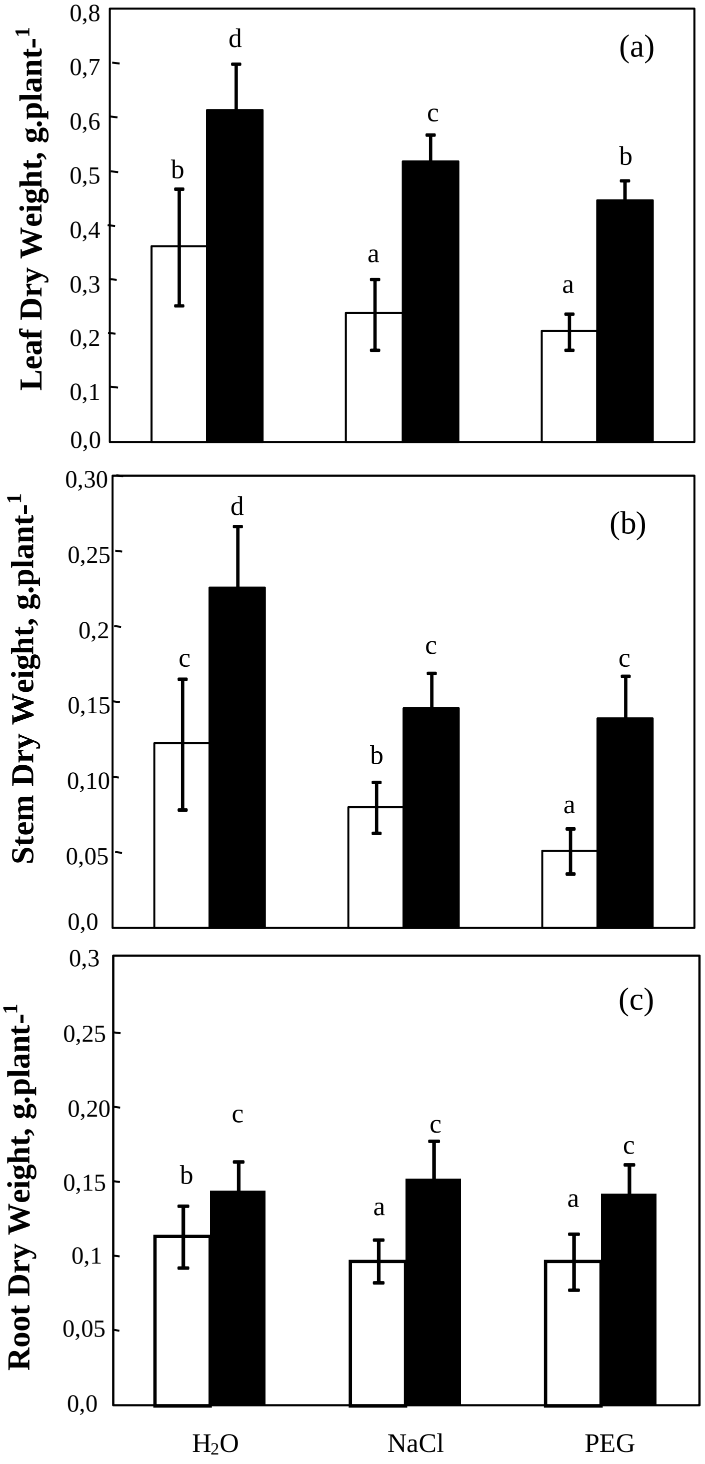

Figure 5.2: Leaf (a) , shoot (b) and root (c) dry weights of *M. polymorpha* seedlings derived from non-primed (*open columns*) or 0,2 mM Ascorbic Acid primed (closed columns) seeds subjected to three water treatments during one month: Control, NaCl 3g/L PEG 100g/L. Values are the means of eight replicates, vertical bars are SEs. Values sharing a common letter are not significantly different at P <0.05.

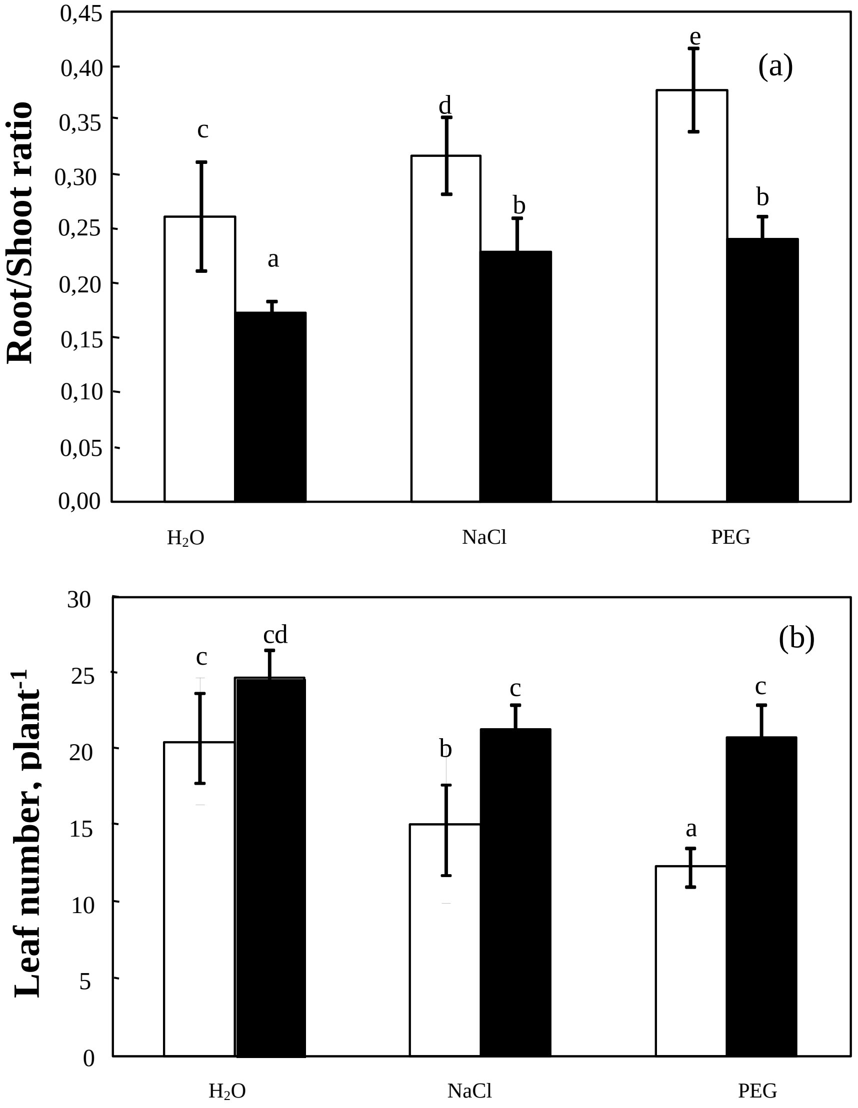

Figure 5.3 (a) Root/Shoot dry weight ratio **(b)** Leaf number of *M. polymorpha* seedlings derived from non-primed (*open columns*) or 0,2 mM Ascorbic Acid primed (closed columns)seeds subjected to three water treatments during one month: Control, NaCl 3g/L PEG 100g/L. Values are the means of eight replicates, vertical bars are SEs. Values sharing a common letter are not significantly different at P <0.05.

3.1.2 Ramification and internode number, root and stem length

Salinity led to a marked reduction of ramifications and internodes number (-28% and -26% respectively) (Figs.5.4 a and b), whereas ascorbic acid priming significantly mitigated this negative effect in both parameters under saline conditions. Control and PEG treatments showed no significant impact on both parameters. However, stem length was significantly reduced by both water deficiency conditions (Figs.5.4 c and d) (-52% and -44% respectively for NaCl and PEG). Ascorbic acid seed pretreatment caused an increase in stem length only under NaCl salinity. Root length was neither affected by water deficiency conditions nor by pretreatment with ascorbic acid (Figs.5.4 c and d).

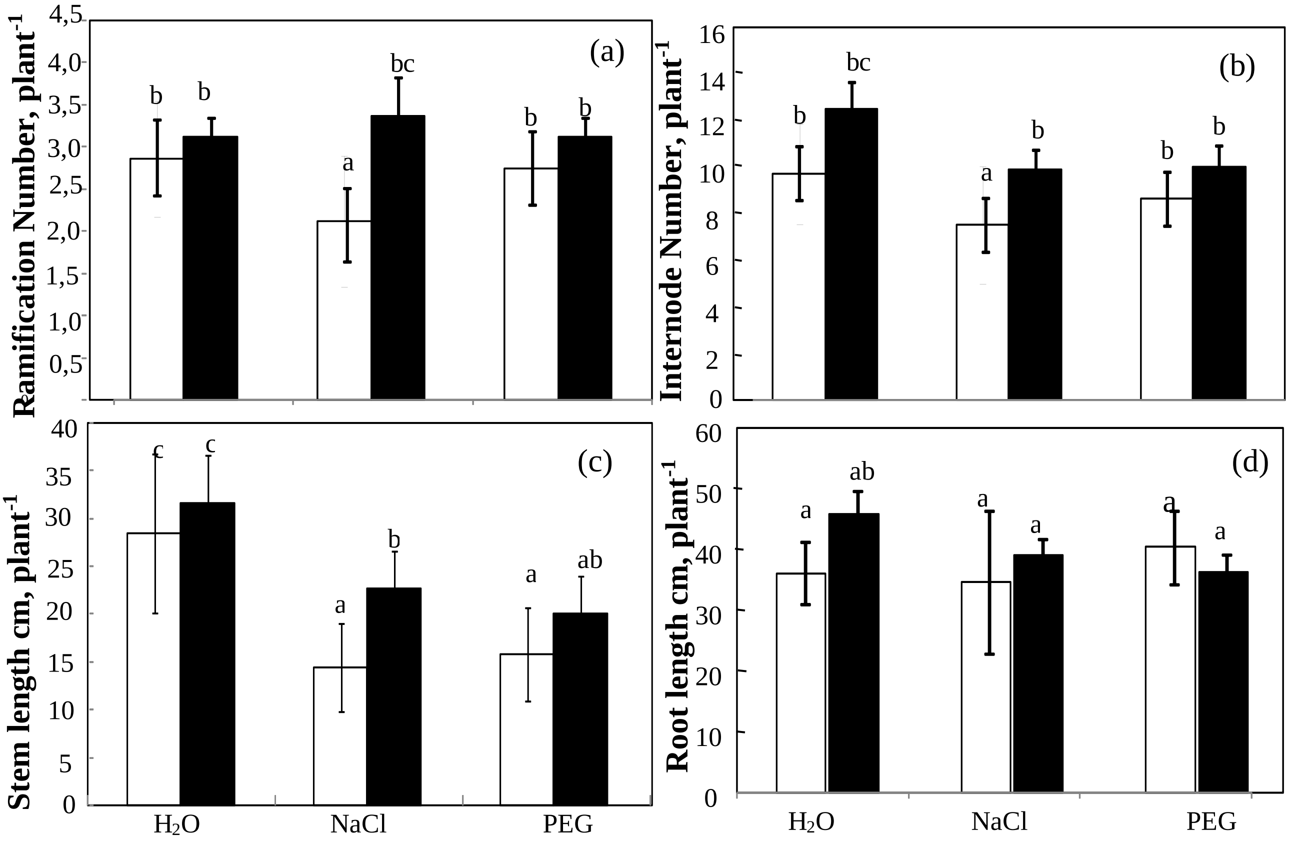

Figure 5.4: Ramification (a), internodes (b) number, shoot (c) and root (d) length of *M. polymorpha* seedlings derived from non-primed (*open columns*) or 0,2 mM Ascorbic Acid primed (closed columns)seeds subjected to three water treatments during one month: Control, NaCl 3g/L PEG 100g/L. Values are the means of eight replicates, vertical bars are SEs. Values sharing a common letter are not significantly different at P <0.05.

3.2 Photosynthetic parameters

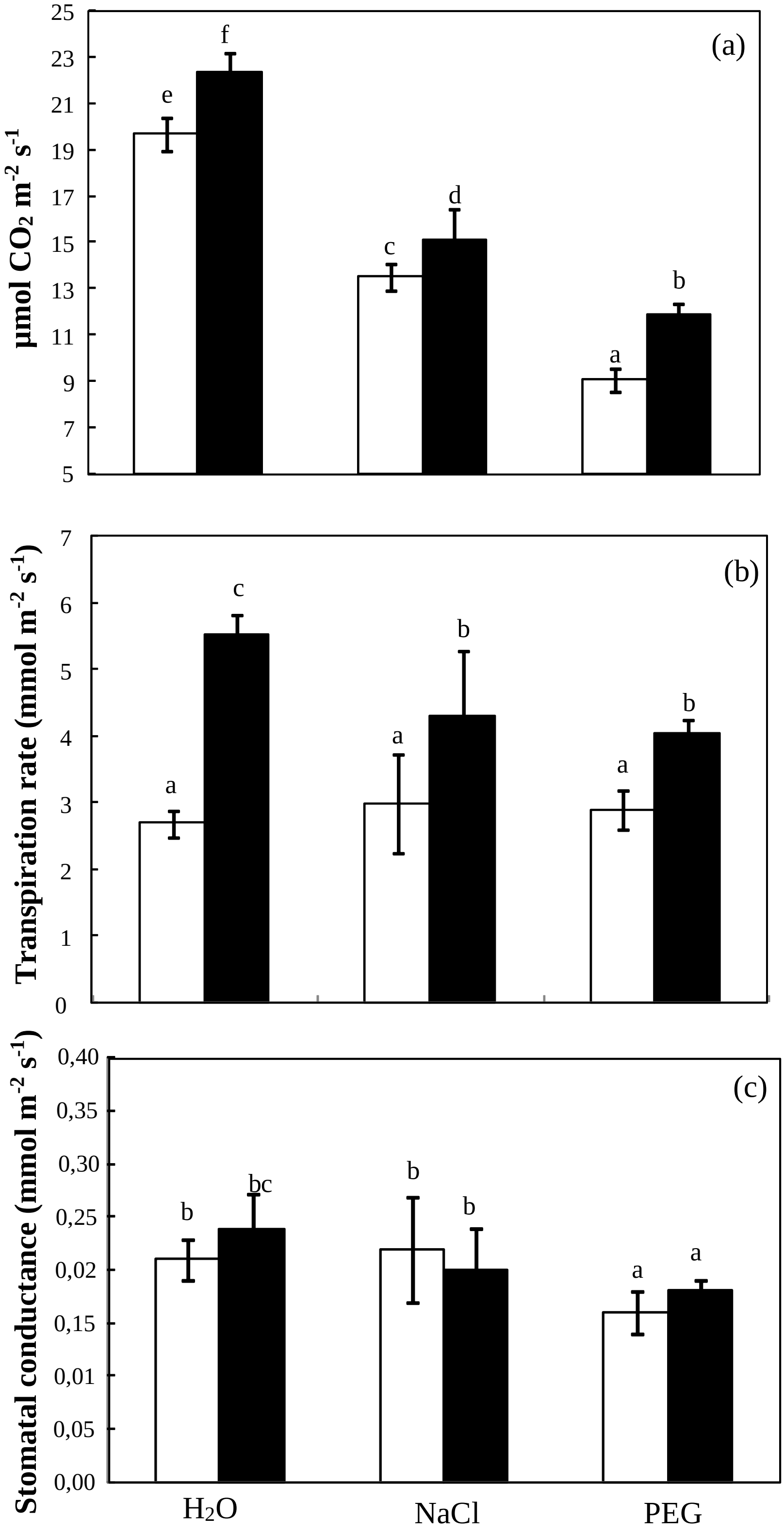

Figure 5.5: Net CO_2 assimilation (a),transpiration (b) and stomatal conductance (c) of *M. polymorpha* seedlings derived from non-primed (*open columns*) or 0,2 mM Ascorbic Acid primed (closed columns) seeds subjected to three water treatments during one month: Control, NaCl 3g/L PEG 100g/L. Values are the means of eight replicates, vertical bars are SEs. Values sharing a common letter are not significantly different at $P < 0.05$.

The leaf net CO_2 assimilation reached 19 μmolm^{-2}s^{-1} under control conditions (Fig.5. 5). Water deficiency conditions led to a significant reduction of this parameter (-31% and -63% respectively for NaCl and PEG). Interestingly, regardless of the applied stress, seed pretreatment with ascorbic acid led to a significant increase of net CO_2 assimilation. This was also true for transpiration (Fig.5.5 b) in all three treatments. The stomatal conductance was only reduced in primed or non-primed seedlings exposed to PEG treatment, whereas seed pretreated with ascorbic acid had no effect on stomatal conductance under both control and osmotic stress conditions.

3.3 Water relations

Leaf water content (LWC) was comparable in both control and NaCl-salinity treatments (about 5ml g^{-1} DW) (Fig.5.6 a). However, PEG treatment led to a significant reduction of LWC (-53%). This effect was buffered by pretreatment with ascorbic acid. Both osmotic stress conditions caused a significant decrease of the leaf osmotic potential, but this effect was more pronounced in PEG-treated plants compared to salt-treated plants. Pretreatment with ascorbic acid further reduced osmotic potential under both water deficit conditions (Fig.5.6 b).

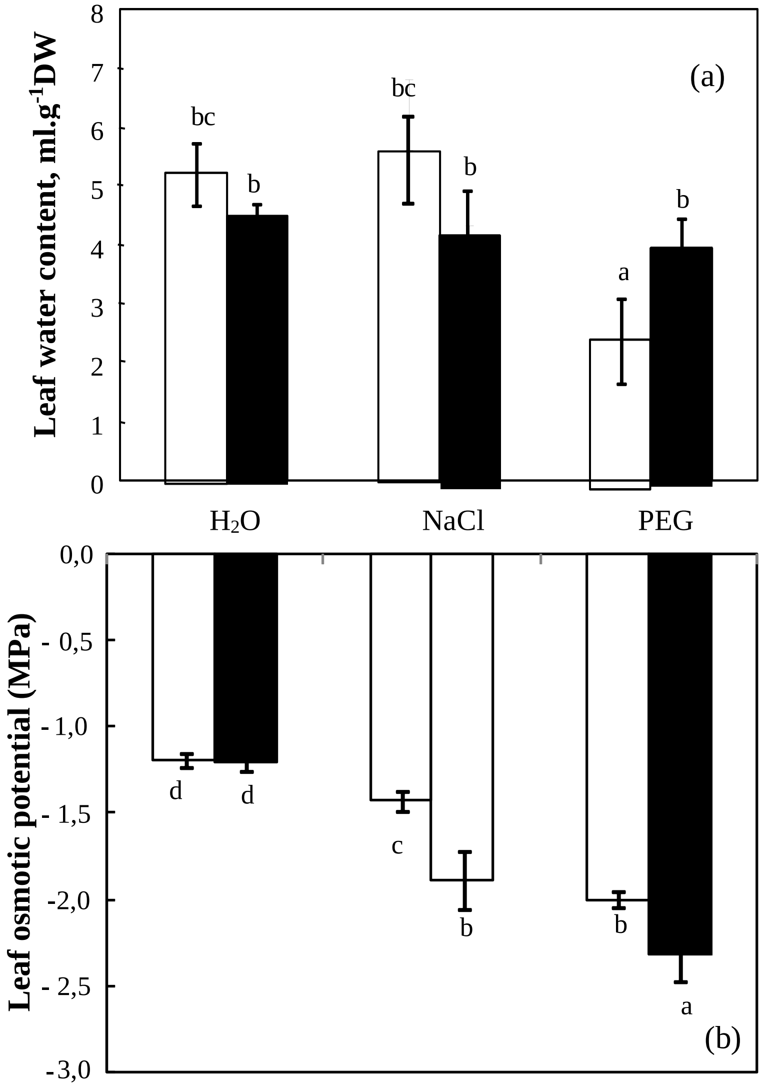

Figure 5.6: Water content (a) and osmotic potential (b) (of *M. polymorpha* seedlings derived from non-primed (*open columns*) or 0,2 mM Ascorbic Acid primed (closed columns) seeds subjected to three water treatments during one month: Control, NaCl 3g/L PEG 100g/L. Values are the means of eight replicates, vertical bars are SEs. Values sharing a common letter are not significantly different at P <0.05.

3.4 Pigment concentration and proline accumulation

NaCl salinity caused a strong reduction of the chl *a*, chl *b*, and carotenoid contents (-53%, -46% and -50% respectively) (Figs.5.7 and 8a). Priming with ascorbic acid significantly counteracted this effect for the three pigments. PEG had no effect on photosynthetic pigments in both primed and unprimed seedlings.

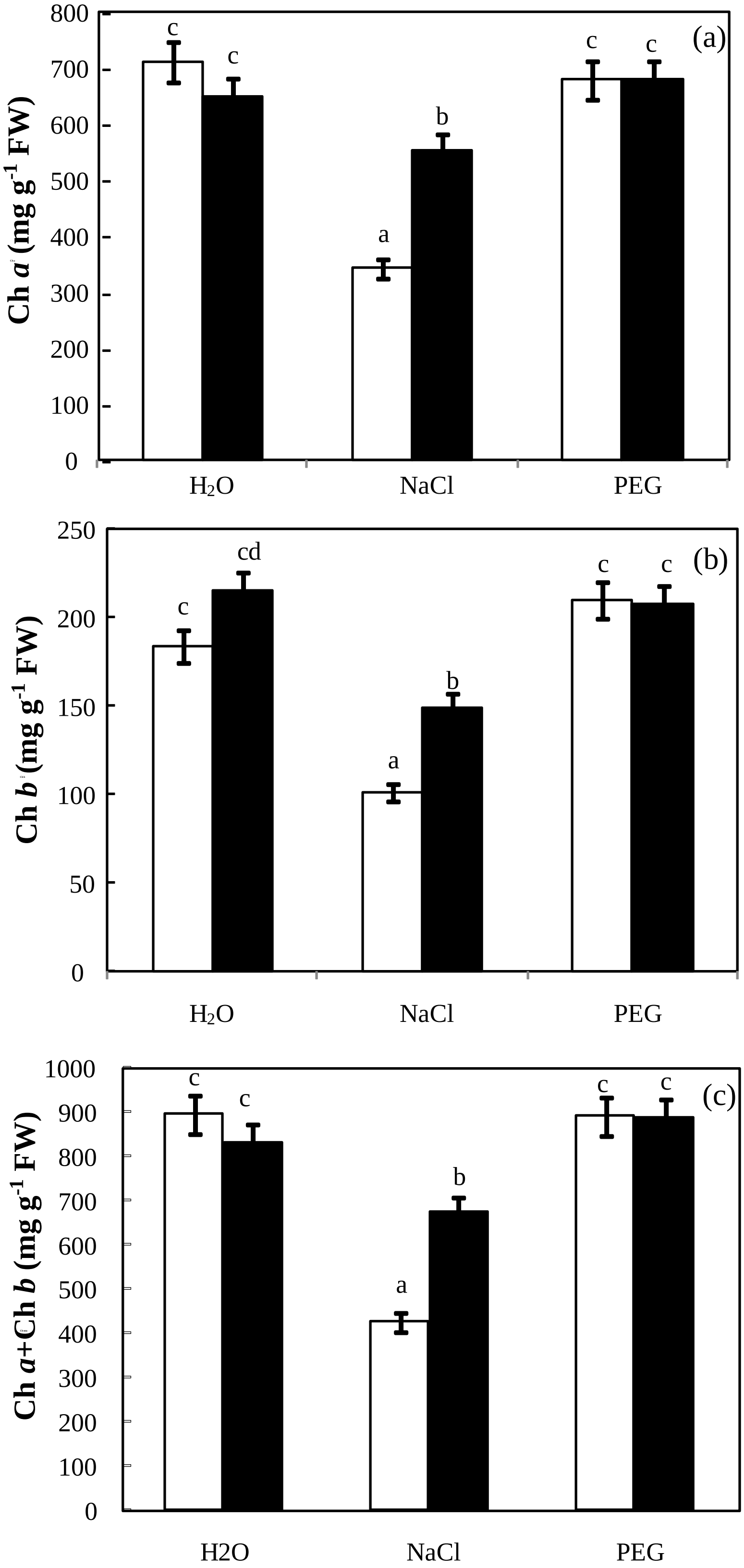

Figure 5.7: Chlorophyll a (a), chlorophyll b (b) and total chlorophyll (c) of *M. polymorpha* seedlings derived from non-primed (*open columns*) or 0,2 mM Ascorbic Acid primed (closed columns) seeds subjected to three water treatments during one month: Control, NaCl 3g/L PEG 100g/L. Values are the means of eight replicates, vertical bars are SEs. Values sharing a common letter are not significantly different at P <0.05.

Leaf proline content was about 0,7 mmol g^{-1} FW (Fig.5. 8b) under control conditions. Both water deficiency conditions led to a significant increase of the proline content. Seed pretreatment with ascorbic acid led to a further significant increase in leaf proline content.

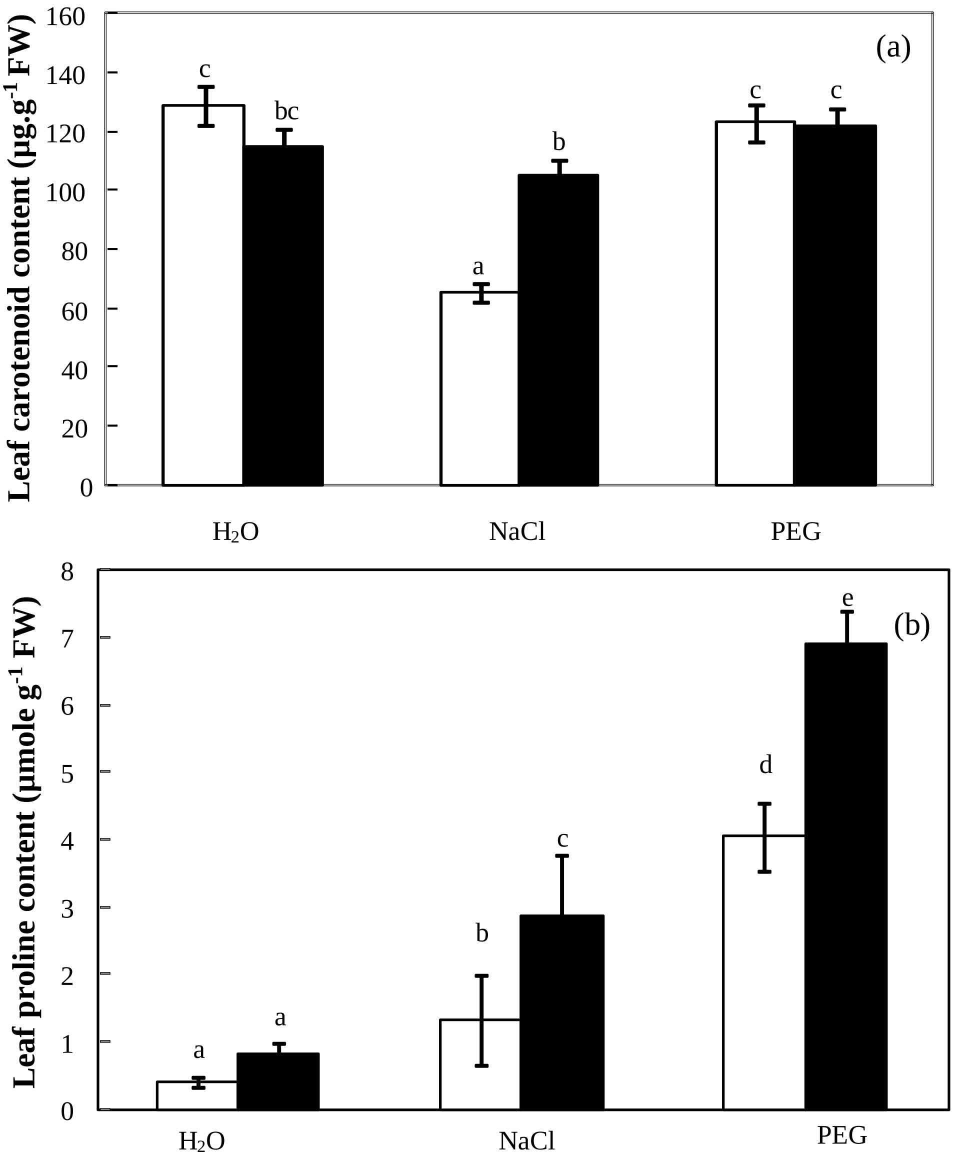

Figure 5.8: (a) Leaf carotenoid content (b) Leaf proline content of *M. polymorpha* seedlings derived from non-primed (*open columns*) or 0,2 mM Ascorbic Acid primed (closed columns) seeds subjected to three water treatments during one month: Control, NaCl 3g/L PEG 100g/L. Values are the means of eight replicates, vertical bars are SEs. Values sharing a common letter are not significantly different at P <0.05.

4. Discussion

The present study investigated the impact of AsA priming on growth, physiological and biochemical parameters *of M. polymorpha* L. plants growing in water deficiency conditions caused by 50 mM NaCl or 100g/L PEG treatments. We hypothesized that priming with ascorbic acid can induce pre-adjustment to water-deficiency resulting in improved plant productivity, enhanced morphological traits, increased photosynthetic activity, better water relations, higher pigment content and proline accumulation.

Our results showed a reduction in biomass in *M. polymorpha* by 40% and 30% when plants were subjected to osmotic stress induced by PEG and salt. In agreement with our findings, Slama et al. (2022) reported that plants biomass reduction of *Medicago* can range between 12 and 73% under water deficiency conditions (Slama et al., 2022). In the same way, Cherifi et al. (2016) showed that reduction of growth in four populations of *M. polymorpha* L. ranged between 40 and 60% at 50mM NaCl salinity (Cherifi et al., 2016).

While drought involved a pure reduction of water potential, salinity introduced an additional ionic impact (Li et al., 2022). As a result, NaCl salinity differs from drought due to the added challenges of Na^+ and Cl^- toxicity and disruption to ion homeostasis (Ma et al., 2020). In our study, osmotic stress, whether it was induced by salt or PEG, led to several similar deleterious effects on plant growth and development. However, these effects were largely mitigated by seed priming with ascorbic acid. Indeed, this latter improved plant biomass production by 66%, 100% and 98% in control, NaCl and PEG treated plants, respectively. This confirms previous studies carried out on rice, wheat, chickpea, sunflower, cotton and sesame (Nakao et al., 2018, Bhusal and Thakur, 2020, Sharma et al., 2021, Catiempo et al., 2021, Zhao et al., 2020).

Both water deficiency treatments used in this study (salinity and PEG) led to an increase in the root/shoot ratio, regardless of seed priming. Slama et al. (2022) recently highlighted that an increased root/shoot ratio is a reliable indicator of drought adjustment and resistance, practically in drought tolerant species like *M. sativa*, *M. truncatula* and *M. laciniata*. The Root/shoot ratio decreased following priming under both control and osmotic stress conditions. likely due to the higher beneficial effects of priming on shoot biomass compared to roots growth. We interpreted this as an indication of improved water uptake capacity and enhanced stress defense (Bacher et al., 2022). Maintaining optimal root quality and physiological activity is essential for healthy shoot development and in turn, for producing high quality seeds with good quality (Bláha, 2019).

It should be mentioned that root/shoot ratio is strongly linked to plant integrity throughout the entire growth and development period making it crucial parameter for the successful production of *M. polymorpha* L.

Our data indicated that both water deficiency conditions significantly affected leaf net CO_2 assimilation, which is one of the most fundamental physiological processes in plants. Water deficiency can induce photoinhibition and oxidative stress, altering the activity of photosynthetic enzymes such as Rubisco. Moreover, drought and salinity affect various pigments, photosystems, and components of the electron transport system (Scheibe and Beck, 2011). Specifically, our results showed a decrease (about -70%) in chlorophyll a and b levels in plants exposed to NaCl induced osmotic stress.

Seed priming with ascorbic acid improved CO_2 fixation in all three studied treatments. In plants subjected to salinity, this improvement was associated with an increase in photosynthetic pigments (chl a, chl b and carotenoids). Similarly, Dai et al. (2017) observed that seed priming with $CaCl_2$ and $ZnSO_4$ enhanced photosynthesis in soybean by increasing the accumulation of photosynthetic pigments (Dai et al., 2017). Ramani et al. (2006) also reported that carotenoids are involved in the adaptation of photosystem II to salinity(Ramani et al., 2006). Carotenoids function both as accessory pigments, aiding in light cupture for photosynthesis, and as photoprotective agents against oxidative stress (Slama et al., 2017).

Carotenoids are also key protective compounds against lipid membrane peroxidation (Johnson et al., 2007) . Another study conducted in Zn O priming in wheat seeds revealed that priming improved the photosynthetic efficiency through the improvement of light absorption efficiency, energy trapping by pigments and transport followed by lower production of ROS. Moreover, it has been documented that seed priming improves photosynthesis rate through the improvement of stomatal length, width and number (Khan et al., 2019). However, in our study seed priming had no effect on stomatal conductance under water deficiency condition.

In *M. polymorpha* L., leaf number was significantly impacted by water deficiency conditions, nevertheless, this effect was alleviated by seed priming. In cowpea, water deficit was shown to affect not only the production of grains but also the whole process of growth of all plant organs including the size of individual leaves and leaf number (Blum, 2017). Arun et al. (2016) attributed the beneficial effects of seed priming on leaf number and area to the advantage of rapid seedling emergence (Arun et al., 2016). The authors explained this by changes in cell number and division, increased cell wall plasticity and membrane permeability, as well as, cell enlargement and rapid cell wall elongation (Salisbury and Ross, 1992). Our results showed that, in addition to changes in the leaf number, several other morphological parameters such as leaf internodes, ramification number and stem length were affected by both water deficiency conditions. However, these adverse effects were mitigated by seed priming. These findings are in agreement with previous studies of chickpea, rice and pepper crop grown in saline conditions (Kaur et al., 2017, Zheng et al., 2016, Aloui et al., 2017).

Tissue hydration is an important process to assess osmotic stress resistance in plants. In our study, seed priming with ascorbic acid improved the water status of plants under PEG treatment by increasing leaf water content and simultaneously lowering the osmotic potential. Similarly, seed priming in wheat has been shown to enhance water status, leading to a significant yield enhancement (Meena et al., 2013). Seed priming mitigates drought effects through cytoskeletal restructuring, as well as, aquaporin formation (Ge et al., 2014). Our results also revealed that seed priming increased root biomass production in *M. polymorpha* L. which likely contributed to improved water uptake capacity.

To counteract the negative effects of unfavorable environmental conditions, plants also evolved mechanisms for osmotic adjustment and oxidative stress defense. These mechanisms involved increased synthesis of proline, secondary metabolites such as carotenoids and xanthophyll, as well as carbohydrates (Serraj and Sinclair, 2002). These compounds are involved in maintaining the structure and the integrity of cell wall (Kaur et al., 2017). Serraj and Sinclair (2002) reported that proline is one of the most common osmolytes, that helps in alleviating the negative effect of drought on plants. It has been reported that proline, when produced under stressful conditions, can act as a compatible solute for osmotic adjustment, a free radical scavenger, and a metal chelator. Additionally, proline activates ROS detoxification pathways, balances cellular redox, buffers cytosolic pH, and serves as a source of energy, nitrogen and carbon. It also stabilizes subcellular structures and membranes including photosystem II, and function as a signaling molecule (Hossain et al., 2014). Seed priming can protect plants and stimulates growth by accelerating the accumulation of osmolytes or non-enzymatic antioxidants such as proline under drought or saline conditions. In our case when cultivated in the presence of PEG, pretreatment of seeds with ascorbic acid led to a drastic increase in leaf proline content. This behavior was associated with a protection of proteins, an increased antioxidative activity and an increase in leaf water relation in plants.

Chapter 6

COMBINED EFFECTS OF SALINITY AND SOIL DRYING ON GROWTH, WATER, STATUS AND PHOTOSYNTHESIS IN *MEDICAGO CILLIARIS* AND *MEDICAGO POLYMORPHA*

Abstract

Drought and soil salinity are among the most common and frequently co-occurring abiotic stresses. They pose a significant threat to plant productivity, especially in arid and semi-arid regions of the world. This study aimed to investigate the interaction between soil drying (D) and salinity(S) in two forage species: *Medicago ciliaris* (TNC1.11 line) and *M. polymorpha* (TNP1.11 line). Plants were grown for one month in silty-sandy soil under two irrigation regimes: 80 and 33% of field capacity (FC). The amount of the evapotranspirated water was replaced by a nutrient solution containing either 0 or 50 mM NaCl. Morphological parameters such as photosynthesis, whole plant growth, water and nutrient status (Na^+, K^+, Ca^{2+}, Zn^{2+}) were investigated.

Under both control and saline conditions, biomass production in *M. ciliaris*, was significantly higher across all treatments C, S, D, and D+S compared to *M. polymorpha*, with increases of 237%, 230%, 175%, and 172%, respectively. These higher productivity in *M. ciliaris* can be attributed to: (i) Higher photosynthetic activity and improved water use efficiency. (ii) Increased leaf count per plant, higher ramifications, and longer stems. (iii) Higher root water content, particularly in treatments C, S, and D+S and (iv) a high Zn accumulation in leaves..

Both salinity and drought, when applied individually negatively affects growth, photosynthesis, water and potassium leaf content in both studied species. However, the combined effects of both constraints were not significantly additive. Salinity exerts a similar reduction in growth for both species with a 64% decrease compared to the control. The major advantage of *M. ciliaris* under dry or saline conditions appears to be its higher water use efficiency, which also mitigates the risk of oxidative stress. Additionally, *M ciliaris* showed a lower increase of Na^+ and less decrease of K^+ at salinity leading to an overall lower Na^+/K^+ ratio as *M. polymorpha*. This shows its superior ion selectivity compared to *M. polymorpha*.

Compared to *M. polymorpha*, the TNC1.11 line of *M. ciliaris* produced more biomass under drought, salinity and combined stresses, therefore we recommend its multiplication and use by farmers as a forage candidate for the rehabilitation of soils in arid and semi-arid salinized regions.

Key words: Combined stresses, growth, photosynthesis, rehabilitation, salinity, soil drying, water use efficiency.

1. Introduction

Salinity is one of the major environmental factors repressing the agricultural production worldwide, second only to drought. It threatens crop production in arid and semiarid regions, where soil salt content is naturally high, and precipitation can be insufficient for leaching (Zhao et al., 2007).

Soil salinization can arise from both natural causes and human-mediated activities (Rengasamy, 2010). Increased soil salt concentration increase osmotic pressure around the roots, reducing water uptake, leading to apparent water limitation. Additionally, excessive salt accumulation in the plant tissues particularly in leaves, would negatively affect plant and leaf growth by impairing metabolic processes and decreasing photosynthetic capacity, partly due to stomatal closure (Yan et al., 2022). The osmotic effect dominates at low salinity levels and during the initial phase of salt exposure, while the ionic effect during long term exposure and at high level of salinity (Arzani and Ashraf, 2016). Under these conditions, leaf Na^+ content can reach toxic levels, triggering the production of reactive oxygen species (ROS) that damage DNA, protein, chlorophyll and membrane function. Moreover, high contents of Na^+ and Cl^- under salinity repress nutrient-ion activities as it disturbs the nutrient ratios by producing extreme ratios of Na^+/Ca^{2+} and Na^+/K^+ (Singh et al., 2014). Adaptation to stress is a very complex process in plants and needs the elimination, compartmentalization, and optional ion uptake to preserve an appropriate Na^+, K^+ and Cl^- balance (Gil et al., 2013).

Drought alone causes more annual losses in crop yield than all pathogens combined (Gupta et al., 2020). Drought's impact on agriculture depends on the degree and duration of the reduced precipitation and soil water gradients, as well as, on plant species and developmental stages (Tardieu et al., 2018). The areas affected by drought are approximately 40% of the world's available lands. This constraint continues to be an important challenge to agricultural researchers. Drought induces various morpho-physiological and biochemical changes in plants. It often causes stomatal closure, which restricts the diffusion of CO_2 into the leaf or results in non-stomatal limitations, reducing carbon assimilation and impairing photosynthesis processes (Paul et al., 2019, Cui et al., 2022). During drought, plants can maintain physiological activities by

(i) enhancing root water uptake (higher root/shoot ratio), (ii) improving water use efficiency (via stomatal closure), (iii) adjusting osmotic processes within tissues, as well as, (iv) activating antioxidant and metabolite production (Gupta et al., 2020).

In natural environments, plants are often subjected to combination of different types of abiotic stresses, which considerably reduce their growth and productivity. While, salinity and drought have each been extensively studied individually, their combined effects present unique challenges that require further investigation (Paul et al., 2019).

Research on the combined effects of drought and salinity has shown that plant responses to combined stresses cannot simply be extrapolated from their responses to each stress applied individually (Zandalinas et al., 2018). Some salt-resistant species are unable to survive drought and *vice vers*a (Khedr et al., 2011). While salinity shares many similar features with drought stress, particularly in early responses due to osmotic effect (Munns, 2002), long-term plant responses to both stresses may behave differently; as sodium toxicity and nutrient imbalances can add to the initial stress due to its transport within plant tissues via the transpiration stream.

It has been reported that high salinity combined with soil drying may interfere with nutrient accumulation, thereby further contributing to growth inhibition. Indeed, when salt and drought are combined, the decrease in potassium phosphorus and calcium contents in shoot tissues were associated with an increase in sodium level (Brown et al., 2006).

However, the combination of both stresses may offer plants opportunities to resist above their individual resistance level. When drought and salinity occur simultaneously, plants can employ crosstalk and exhibit strategic defense responses which could be distinctive from the response to either individual stress (Ma et al., 2020). As a result, the molecular and metabolic responses to a combination of stresses are unique and cannot be extrapolated from plant response to the individual stress (Mittler and Blumwald, 2010, Mittler, 2006).

Moreover, maintaining a suited water potential and appropriate nutrient ratios under saline conditions is crucial, as low soil moisture in saline conditions can further reduce water availability, accentuate salt toxicity, and suppress root growth. This ultimately restricts water absorption from the subsoil and alters plant's ability to survive (Ahmed et al., 2013). Therefore, studying the combined effects of drought and salinity has considerable practical and ecological implications for ensuring sustainability of plants performance to abiotic stresses (Goharrizi et al., 2020).

Besides, selecting and utilizing salt and drought-resistant species is crucial for soil revegetation and preservation purposes (Morales et al., 1998). Studying morphological and physiological changes in plants under stress, such as biomass, leaf area, and plant height, alongside photosynthetic activity measurements, provides valuable insights into the mechanisms of stress induced crop yield loss. This information can be utilized in identifying and selecting resistant lines or species for sustainable agricultural practices (Badri et al., 2016).

Physiological and biochemical plant's characteristics play a crucial role in primary screening of resistant genotypes and can be determined at initial stages of plant development (Goharrizi et al., 2020). Monclus et al., 2006 suggested that producing a high level of drought tolerance requires the ability of a cultivar to increase its water use efficiency.

The specific effects of combined salinity and drought on halophytic plants have been the topic of several studies, which suggest that these stresses can interact and, in some cases, mitigate each other's effects (Chakraborty et al., 2018, Slama et al., 2007). However, in non-halophylic species, water deficit and salinity are often considered additive stress factors, with their combined effects depending on species, stress duration and its severity. There is still much to uncover regarding how various plants respond to different levels of combined drought and salinity (Munns, 2002).

Data on plant growth, biochemical and physiological responses of *Medicago ciliaris* and *Medicago polymorph* caused by the combination of salinity and drought are lacking. Considering these aspects, the aim of the study was to investigate the interacting effects of salinity and drought stress in two forage species *Medicago ciliaris* and *M. polymorpha* and to determine if these effects are additive or not on growth, photosynthesis, water and nutrient status. Additionally, we sought to select the most resistant species by the co-occurrence of salinity and drought, in order to encourage its multiplication and use by farmers for the rehabilitation of soils in arid and semi-arid salinized regions

2. Methodology

Healthy and uniform seeds of *M. ciliaris* (TNC1-11) and *M polymorpha* (TNP1-11) were disinfected with 10% (w/v) sodium hypochlorite for 5 min followed by thorough rinsing with distilled water. Subsequently, seeds were germinated in 3.5-L pots filled with limono-sandy soil in a greenhouse. After one week of sowing, seedlings were thinned to one per pot to achieve homogeneous plants. The soil field capacity (FC) of 11,5% was determined according to the technique of Bouyoucos (1983) . Following a period (three weeks) of ample watering (80% FC), plants were divided into four groups: the first group was irrigated with tap water at 80% FC (control plants) (C), the second group at 33% FC (water-deficit treated plants) (D), the third group was irrigated with tap water at 80% FC added with 50 mM NaCl (S), and the fourth group at 33% FC supplemented with 50 mM NaCl (D+S). A final harvest was carried out after one mouth of treatment.

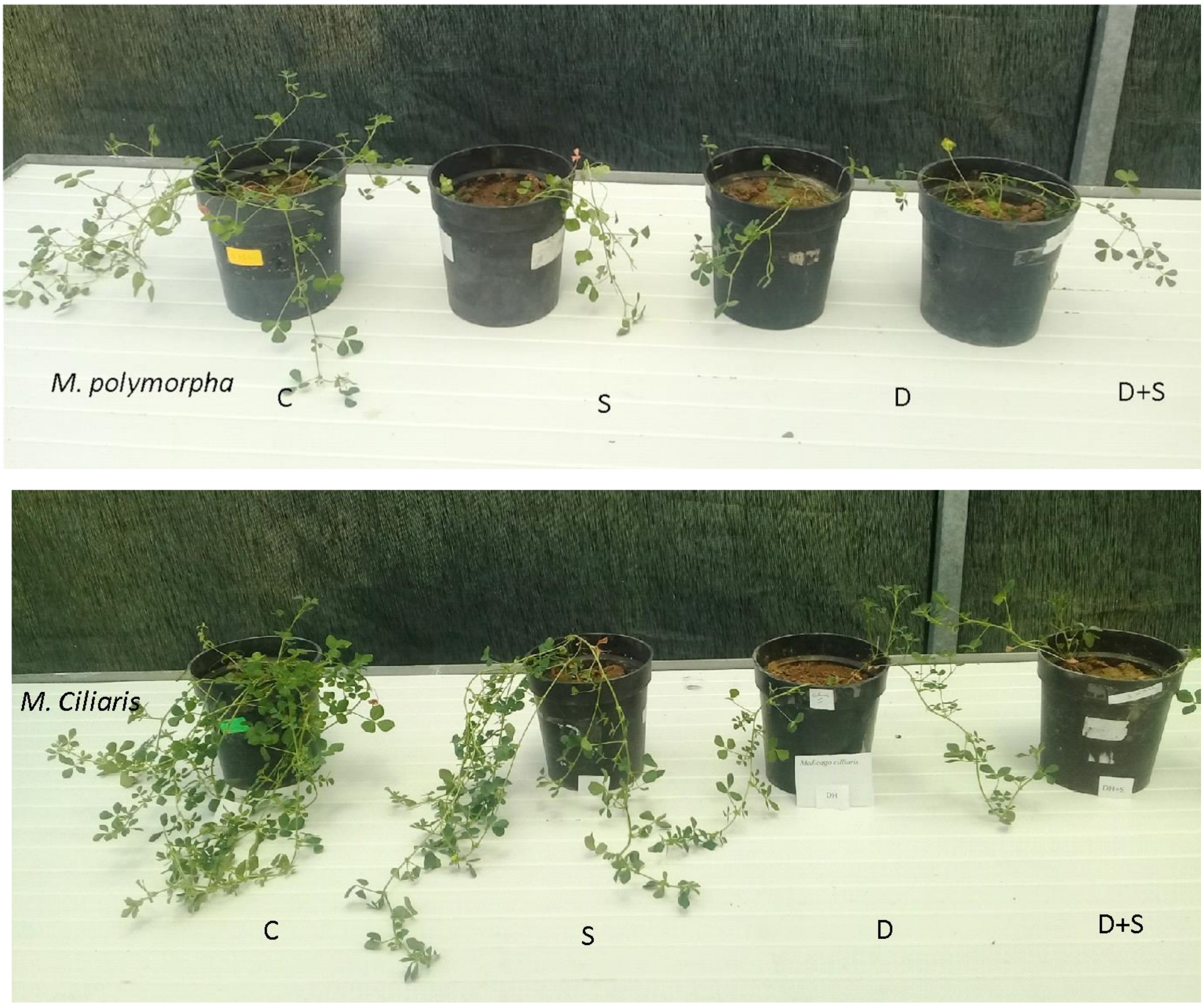

Photo 6.1 *M. polymorpha* and *M. ciliaris* aspect. Plants were cultivated under control conditions (C) (80%FC), salinity (S) 50 mM NaCl, soil drying (D) 33% FC and the combination of the latter both (D+S)

3. Results

3.1 Growth

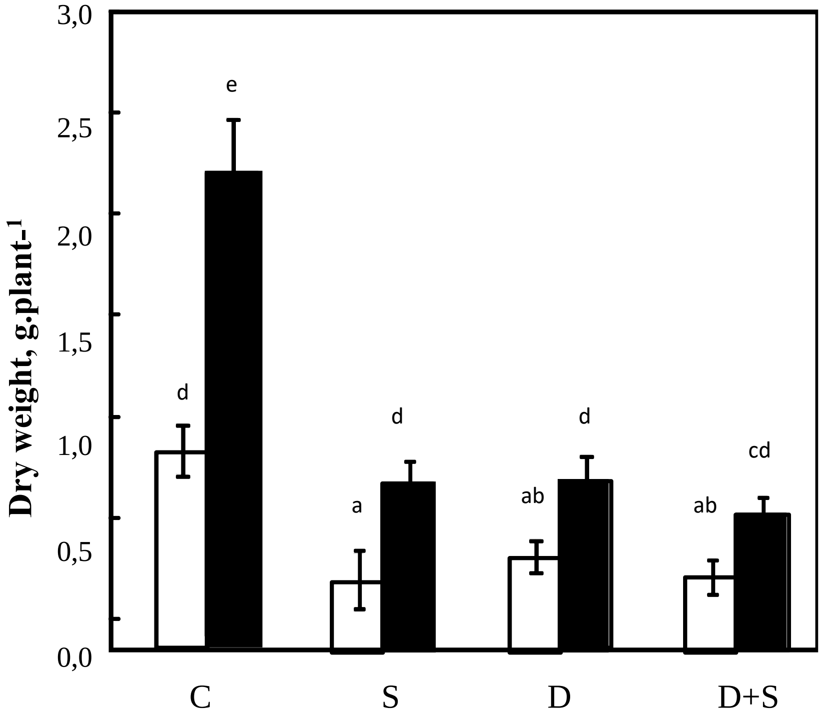

Fig. 6.1 Whole plant biomass production of *M. polymorpha and M. ciliaris* seedlings subjected to four water treatments during two month: control (C) (80%FC), salinity (S) 50 mM NaCl, soil drying (D) 33%FC and the combination of the latter both (D+S). Values are the means of eight replicates, vertical bars are SEs. Values sharing a common letter are not significantly different at $P < 0.05$.

Under both control and stress conditions, *M. ciliaris* produced more biomass than *M. polymorpha*. The whole plant biomass production in *M. ciliaris* was 237%, 230%, 175%, and 172% under C, S, D, and combined D+S treatments, respectively, compared to *M. polymorpha* (Fig. 6.1). Salinity alone significantly reduced plant growth in both species by approximately 64%. In *M. ciliaris*, biomass reductions were 63% under drought and 70% under the combined D+S treatment, while *M. polymorpha* showed reductions of 50% and 60% for the same treatments. The effects of salinity and drought were not additive in either species, as biomass production remained similar under individual or combined stress conditions.

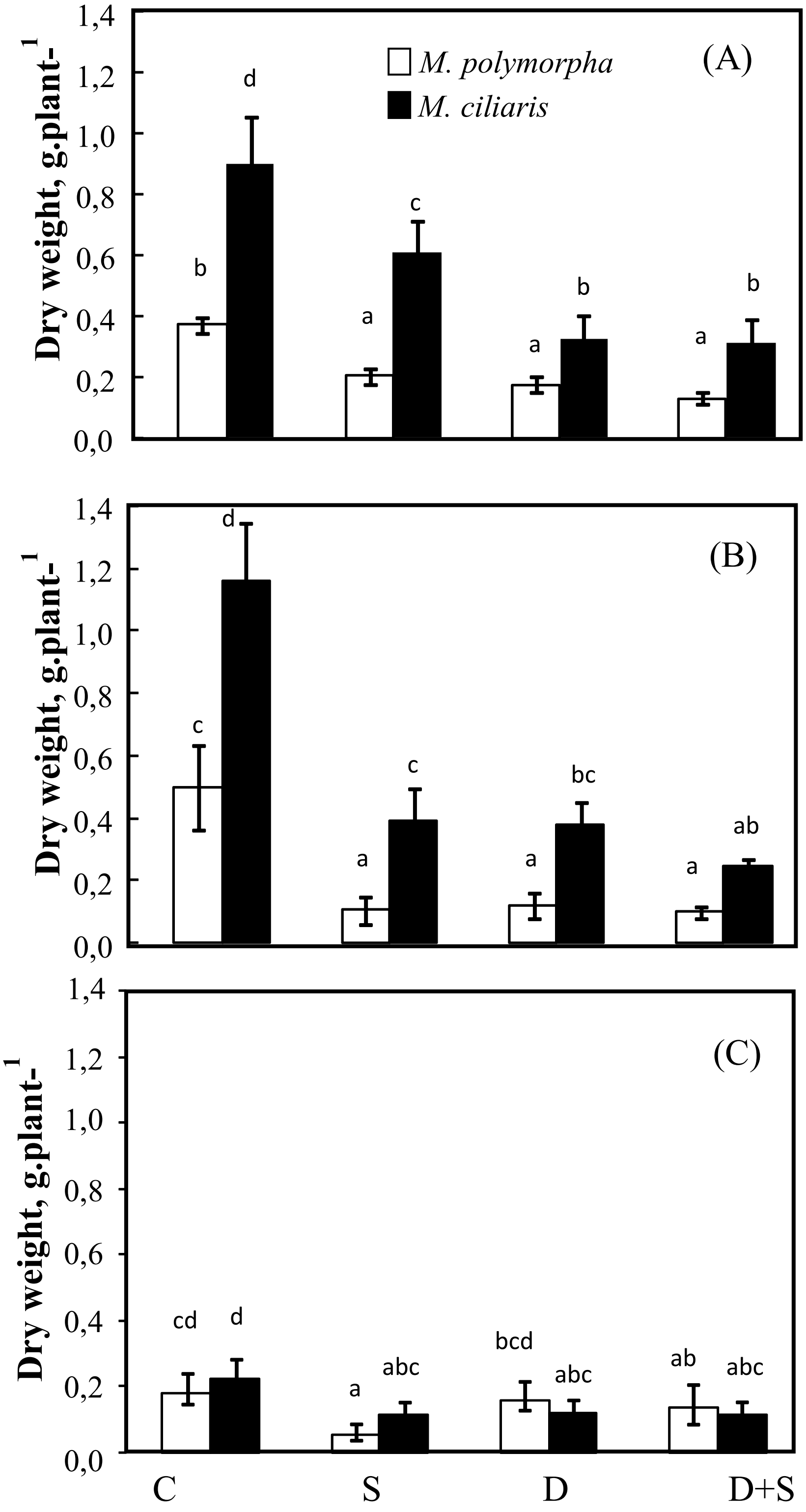

Fig. 6.2 Leaf, stem and root biomass production of *M. polymorpha and M. ciliaris* seedlings subjected to four water treatments during two month: control (C) (80%FC), salinity (S) 50 mM NaCl, soil drying (D) 33%FC and the combination of the latter both (D+S). Values are the means of eight replicates, vertical bars are SEs. Values sharing a common letter are not significantly different at P < 0.05.

In *M. polymorpha* the changes in leaf DW were similar to those observed in the whole plant (Fig.6.2). However, in *M ciliaris* the effects of D and the combination of D+S were significantly more pronounced compared to S alone. Stem biomass production variations were similar to those observed in leaves in both species. Root biomass production was lower than that of stems and leaves representing 50 %, and 25% of total biomass under control conditions for *M. ciliaris* and *M polymorpha* respectively.

In *M. polymorpha,* root growth was more sensitive under salinity applied alone or in combination with soil drying compared to soil drying alone. However, root growth in *M cilliaris* was significantly reduced under S, D, and D+S treatments .

3.2 Root / shoot ratio

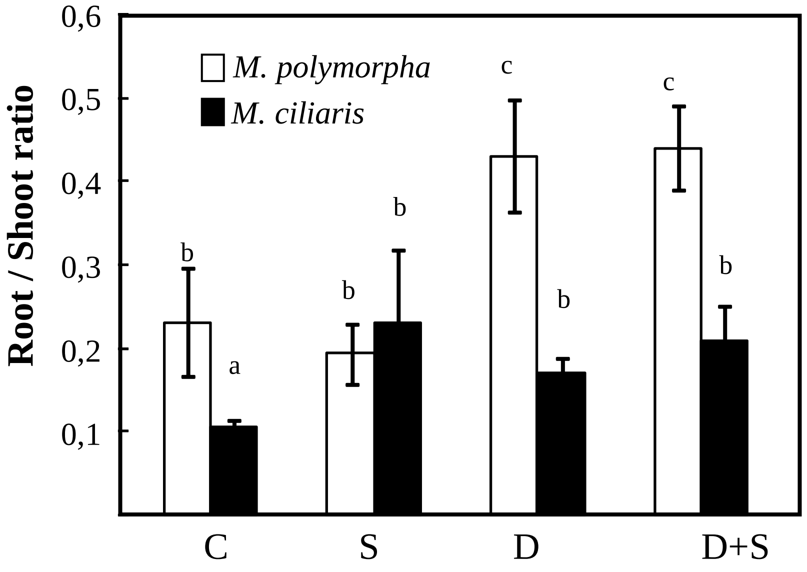

Fig.6.3 Root/shoot ratio of *M. polymorpha and M. ciliaris* seedlings subjected to four water treatments during two month: control (C) (80%FC), salinity (S) 50 mM NaCl, soil drying (D) 33%FC and the combination of the latter both (D+S). Values are the means of eight replicates, vertical bars are SEs. Values sharing a common letter are not significantly different at $P < 0.05$

In *M. polymorpha,* the root/shoot ratio (Fig.6.3) increased by 50% in plants submitted to D alone or combined to S. However in *M ciliaris* this parameter increased significantly in D, S and D+S by about 45%.

3.3 Morphological parameters

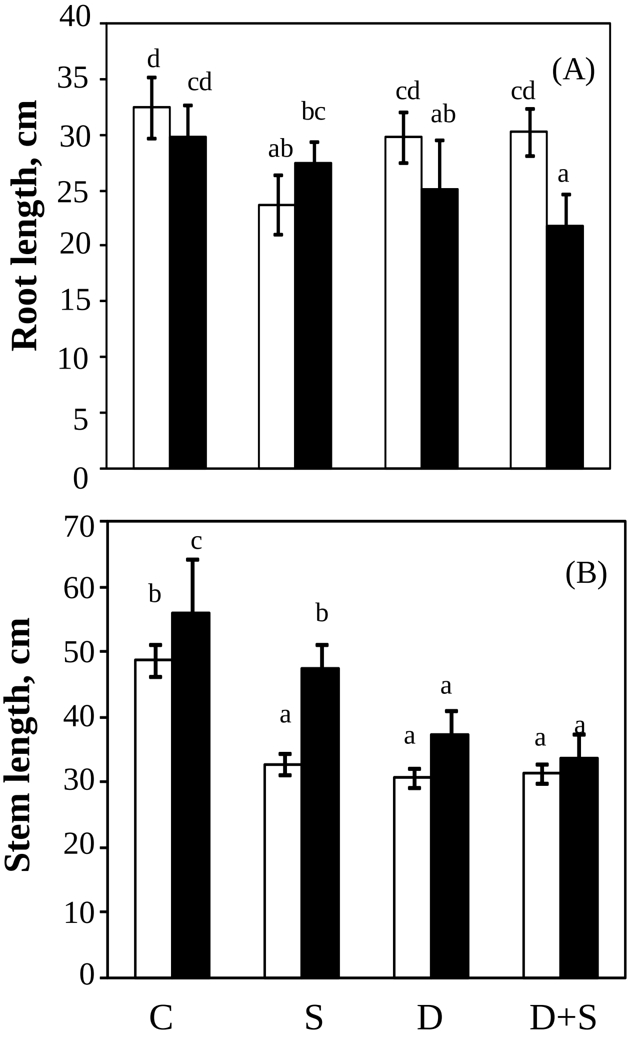

Fig. 6.4 Root (A) and stem length (B) of *M. polymorpha* and *M. ciliaris* seedlings subjected to four water treatments during two months: control (C) : 80% FC, salinity (S) : 50 mM NaCl, soil drying (D) : 33% FC and the combination of the latter both (D+S). Values are the means of eight replicates, vertical bars are SEs. Values sharing a common letter are not significantly different at $P < 0.05$.

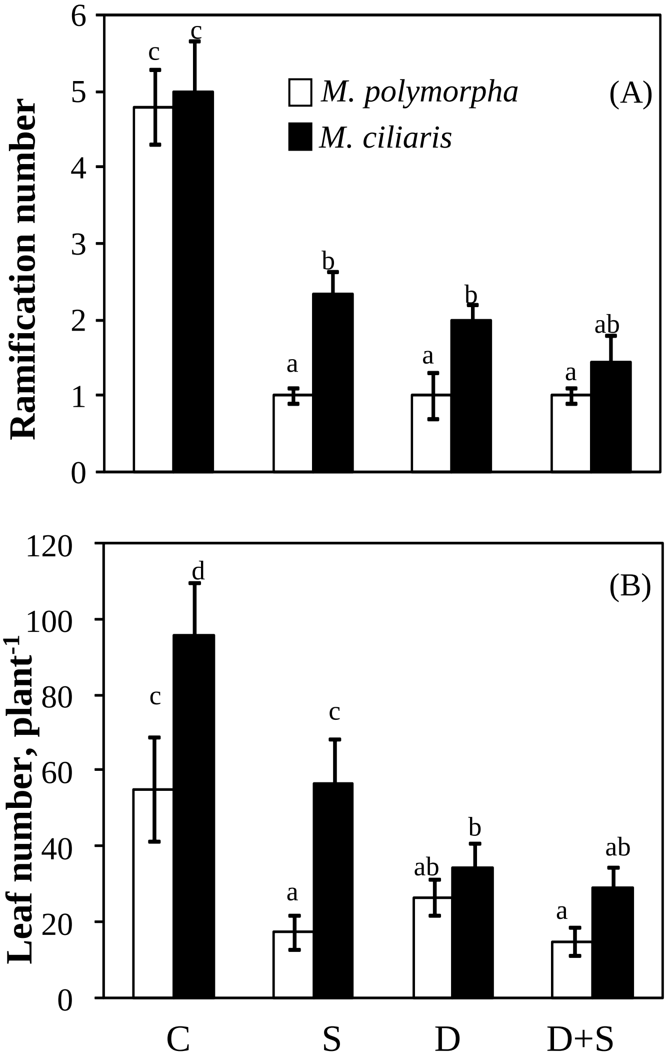

Fig. 6.5 Ramification (A) and leaf number (B) of *M. polymorpha* and *M. ciliaris* seedlings subjected to four water treatments during two months: control (C) : 80% FC, salinity (S) : 50 mM NaCl, soil drying (D) : 33% FC and the combination of the latter both (D+S). Values are the means of eight replicates, vertical bars are SEs. Values sharing a common letter are not significantly different at $P < 0.05$.

In both species the highest values of root and stem length, ramification and leaf number were observed under control conditions (Figs. 6.4 and 6.5).

Compared to *M. polymorpha*, *M. ciliaris* produced more leaves under C, S, and D+S treatments (55 vs 17 leaves under C). Moreover, *M. ciliaris* exhibited longest stems under C, and combined C+S treatments compared to *M polymorpha* .

In *M polymorpha,* leaf number was significantly reduced under S, and D+S treatments with a 72%reduction. In *M. cilliaris* the effects of D alone or in combination to S was significantly more pronounced when compared to S alone. Both species experienced significant similar reductions in root and stem length, ramification and leaf number under single or combined stress conditions. However, in *M ciliaris* the impact of D either alone or combined to S was more pronounced than that of S alone particularly on stem length and leaf number.

3.4 CO_2/ H_2O gas exchange

The highest value of CO_2 assimilation was observed in well-watered plants of *M. cilliaris* (8.8 µmol.CO_2 $m^{-2}s^{-1}$) (Fig.6.6 A). Under C and S conditions, *M. ciliaris* exhibited a higher net CO_2 assimilation compared to *M. polymorpha.* However, no significant differences were observed between the two species when subjected to D or combined D+S.

In *M. polymorpha,* S and D whether applied individually or in combination significantly reduced net CO_2 assimilation and the combined effects were not additive.

In *M. cilliaris* net CO_2 assimilation was significantly more affected under D and D+S treatments when compared to S alone.

Regarding stomatal conductance (Fig. 6.6 B), *M. polymorpha* exhibited the highest values under C, D, and combined S+D compared to *M. ciliaris*. Nevertheless, stress application significantly reduced this parameter. In *M. ciliaris*, stomatal conductance was more impacted by D and combined stress (D+S) than by S alone, compared to the C treatment.

Changes in E mirrored those observed in gs (Fig. 6.6 C).

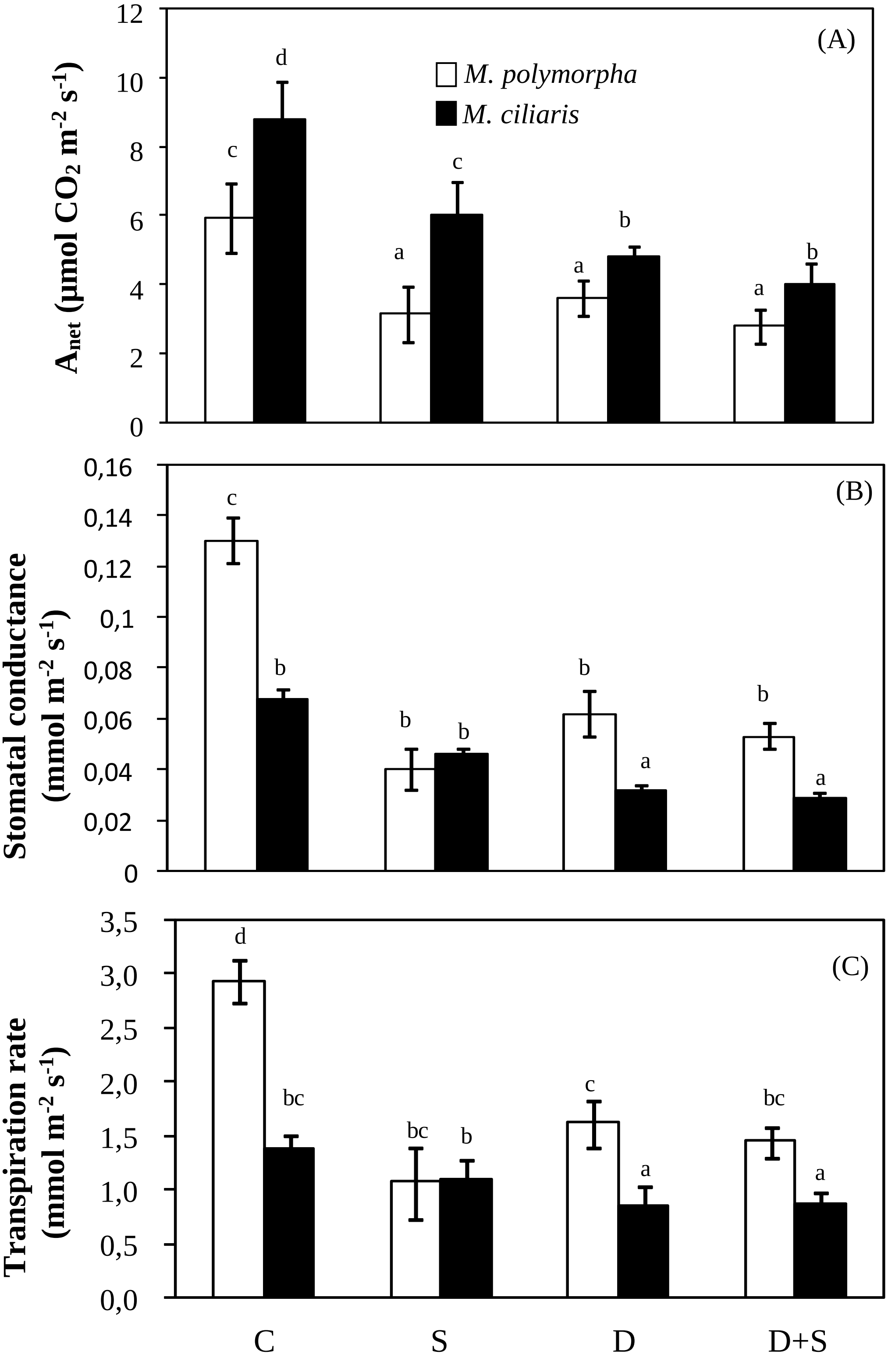

Fig. 6.6. A_{net} CO_2 (A), gs (B) and E (C) of *M. polymorpha and M. ciliaris* seedlings subjected to four water treatments during two months: control (C) : 80% FC, salinity (S) : 50 mM NaCl, soil drying (D) : 33% FC and the combination of the latter both (D+S). Values are the means of eight replicates, vertical bars are SEs. Values sharing a common letter are not significantly different at $P < 0.05$.

As shown in table 6.1 water use efficiency (WUE) was higher in *M. ciliaris* compared to *M. polymorpha* for all treatments.

Table 6.1: Water use efficiency of *M. polymorpha and M. ciliaris* seedlings subjected to four water treatments during two months: control (C) : 80% FC, salinity (S) : 50 mM NaCl, soil drying (D) : 33% FC and the combination of the latter both (D+S). Values are the means of eight replicates. Values sharing a common letter are not significantly different at P < 0.05

	C	**S**	**D**	**D+S**
M. polymorpha	3,11 c	2,61 b	2,23 b	1,93 a
M. ciliaris	6,33 f	5,49 e	5,68 e	4,63 d

3.5 Water relations

Leaf hydration was higher in *M. ciliaris* compared to *M. polymorpha* under control conditions (Fig.6.7A). However, both species exhibited similar leaf water content under S, D and combined D+S treatments. Both drought and salinity applied alone or in combination significantly reduced leaf water content in both species. The reduction was more pronounced in *M. ciliaris* compared to *M. polymorpha,* for instance, a 45% reduction vs 55% under drought conditions.

Stem hydration was affected in *M. polymorpha* under D and D+S treatments (Fig.6.7 B) but stem hydration was less sensitive to D, S, and combined D+S treatments compared to leaves and roots in both species. Root water content was affected by all applied constraints (Fig. 6.6 C). The effects of salt and drought on tissue hydration were not additive in both species.

As shown in table 6.2 water content per plant was higher in *M. ciliaris* compared to *M. polymorpha* for all treatments with *M. ciliaris* having 2,8, 3,2, 1,7 and 1,9 fold higher water content under C, S, D, and D+S treatments respectively.

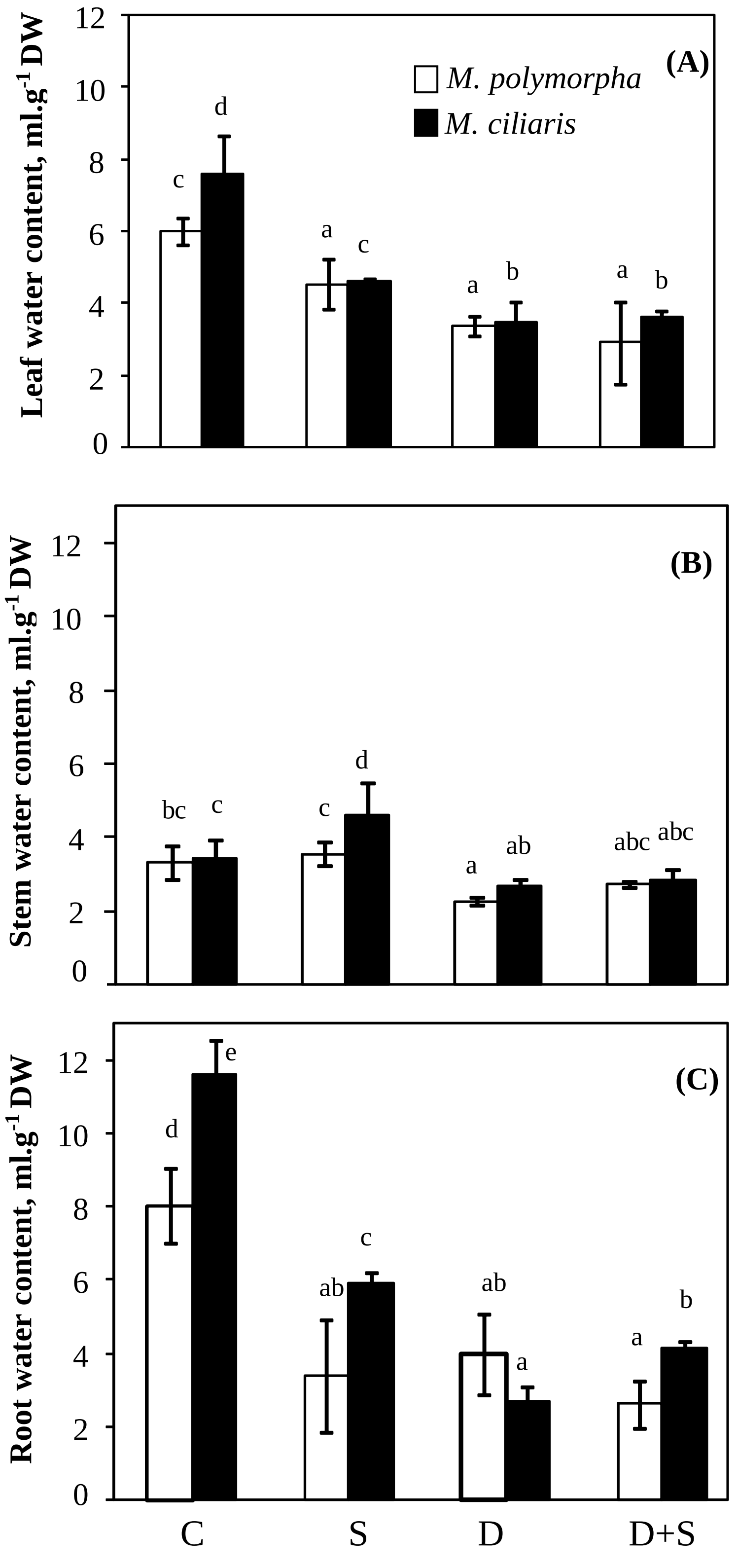

Fig. 6.7. Leaf, stem and root water content of *M. polymorpha and M. ciliaris* seedlings subjected to four water treatments during two months: control (C): 80% FC, salinity (S): 50 mM NaCl, soil drying (D): 33% FC and the combination of the latter both (D+S). Values are the means of eight replicates, vertical bars are SEs. Values sharing a common letter are not significantly different at $P < 0.05$.

Table 6.2 leaf water content ml H_2O plant^{-1} of *M. polymorpha and M. ciliaris* seedlings subjected to four water treatments during two months: control (C): 80% FC, salinity (S): 50 mM NaCl, soil drying (D): 33% FC and the combination of the latter both (D+S). Values are the means of eight replicates. Values sharing a common letter are not significantly different at P < 0.05.

	C	**S**	**D**	**D+S**
M. polymorpha	4,7 f	1,27 b	1,4 b	0,96 a
M. ciliaris	13,14 g	4,15 e	2,44 d	1,86 c

3.6. Nutrient status

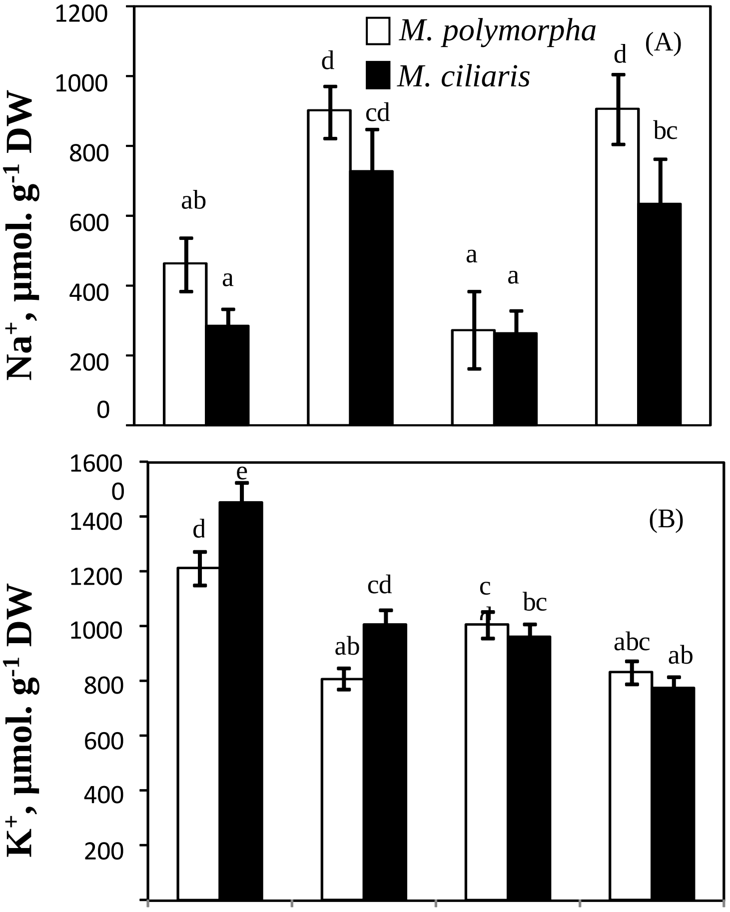

Fig. 6.8. Leaf sodium (A), potassium (B) contents of *M. polymorpha and M. ciliaris* seedlings subjected to four water treatments during two months: control (C): 80% FC, salinity (S): 50 mM NaCl, soil drying (D): 33% FC and the combination of the latter both (D+S). Values are the means of eight replicates, vertical bars are SEs. Values sharing a common letter are not significantly different at P < 0.05.

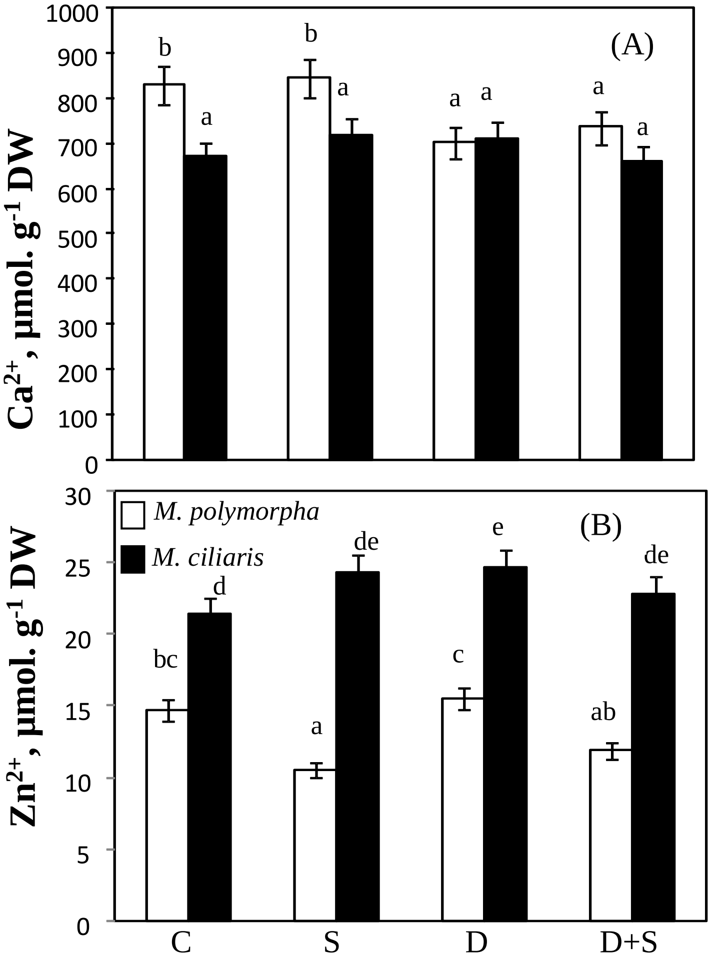

Fig. 6.9. Leaf calcium (A) and zinc (B) contents of *M. polymorpha and M. ciliaris* seedlings subjected to four water treatments during two months: control (C): 80% FC, salinity (S): 50 mM NaCl, soil drying (D): 33% FC and the combination of the latter both (D+S). Values are the means of eight replicates, vertical bars are SEs. Values sharing a common letter are not significantly different at $P < 0.05$.

In both species, high sodium (Na$^+$) concentration was observed in the leaves of salt-treated plants, and those exposed to drought combined to salinity. Under S+D treatment, *M. polymorpha* accumulated significantly more sodium compared to *M cilliaris* (Fig. 6.8 A).

Potassium(K$^+$) content was higher in *M. ciliaris* under C and S treatments compared to *M. polymorpha*. In *M. polymorpha,* this parameter was not affected by drought, however it decreased significantly under S, alone or combined to D. In *M. ciliaris*, there was no significant interaction between the two constraints, but potassium content decreased significantly by 25, 30 and 45% under S, D and D+S treatments, respectively (Fig.6.8 A).

The Na^+/K^+ ratio remained unchanged under D in both species compared to control conditions. However, this parameter increased significantly under S and D+S treatments by 150% and 200% in *M. polymorpha* versus 237 and, 275% in *M. ciliaris,* respectively. Under salinity, the Na^+/K^+ ratio was higher in *M. polymorpha* compared to *M. ciliaris* (Fig. 6.10).

Calcium (Ca^{2+}) content decreased significantly under D and D+S in *M polymorpha.* However, in *M cilliaris* leaf calcium content was not significantly affected by all treatments (Fig.6.9 A).

In all treatments, *M. polymorpha* accumulated a higher amount of Zn^{2+} in the leaves compared to *M. polymorpha* under C, S, D and D+S, respectively. In *M. cilliaris,* leaf Zn^{2+} content remained unaffected by all applied treatments. However, in *M. polymorpha* Zn^{2+} content decreased under S and remained unchanged under D and D+S treatments (Fig.6.9 B).

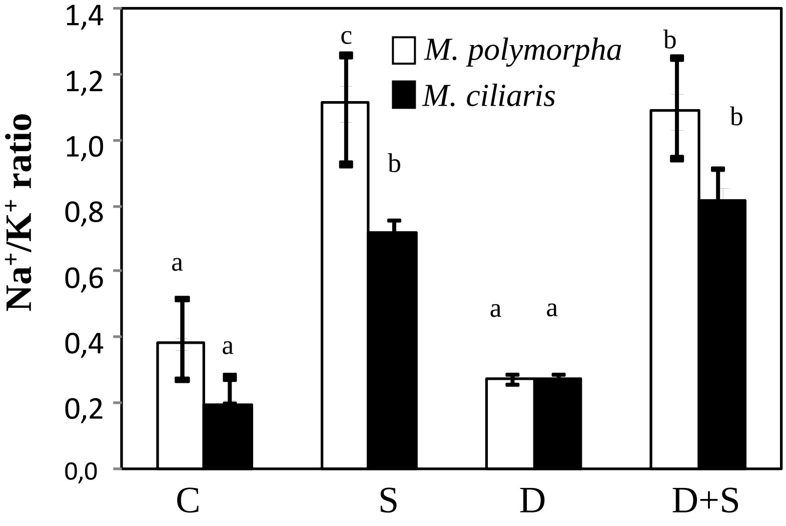

Fig. 6.10. Na^+/K^+ ratio in leaves of *M. polymorpha and M. ciliaris* seedlings subjected to four water treatments during two months: control (C): 80% FC, salinity (S): 50 mM NaCl, soil drying (D): 33% FC and the combination of the latter both (D+S). Values are the means of eight replicates, vertical bars are SEs. Values sharing a common letter are not significantly different at $P < 0.05$.

3.7 Correlations and Interaction effects of drought and salt

The impact of drought on leaf and stem dry weight, hydration, leaf number, and photosynthetic parameters (A, gs, and E) was found to be highly significant in both species, as demonstrated in Tables 6.3 and 6.4. Conversely, drought showed no significant effect on Na^+, K^+ and Zn^{2+}levels. In *M. polymorpha,* leaf calcium content was slightly affected by drought.

Salinity had a significant effect on whole plant dry weight, root hydration, leaf Na^+ and K^+ contents in both species. Moreover, S highly affected leaf Zn^{+2} content in *M. polymorpha*. In case of *M. ciliaris,* S had no significant effect on leaf calcium and Zn^{+2} contents.

The interaction effects between the two constraints observed in *M. polymorpha,* (Table 6.3) affected the whole plant, leaf and stem dry weight, in addition to E and gs. No significant interactions were observed on root dry weight, root/shoot ratio, leaf and stem hydration as well as Na^+, Ca^{2+}, and Zn^{2+} leaf contents.

In *M. ciliaris,* the two constraints (Table 6. 4) had no interaction effects on the root/shoot ratio, stem hydration, E, gs, root and stem length, or leaf Na^+, Ca^{2+} and Zn^{2+} contents.

Table 6.3 Results from a two-way analysis of variance examining the effect of drought (D), salinity (S) and their interaction (D*S) on parameters describing plant growth, gas exchanges, water relations, and nutrition status in *M. polymorpha* plants

M. polymorpha			
	D	S	D*S
	F	F	F
WP DW	13,21*	32,02***	25,38***
Leaf DW	76,51***	120,67***	42,32***
Stem DW	25,84***	30,30***	30,84***
Root DW	0,50ns	12,65*	2,28ns
Root/shoot	13,08*	0,01ns	0,23ns
H_2O leaves	30,82***	6,33*	0,82ns
H_2O Stems	30,94***	8,02*	1,68ns
H_2O Root	20,04**	31,71***	10,59*
A_{net}	16,62**	1,83ns	11,46*
E	24,01***	45,12***	29,14***
GS	30,06***	45,88***	29,83***
Leaf number	17,82**	43,67***	11,49*
Root length	2,25ns	9,69*	12,50*
Stem length	21,83**	13,74*	16,32**
Na^+	1,29ns	41,21***	1,20ns
K^+	0,09ns	25,92***	7,00*
Na^+/K^+	1,42ns	15,77**	0,73ns
Ca^{2+}	12,07*	0,55ns	0,06ns
Zn^{2+}	2,41ns	31,68***	0,13ns

Table 6.4 Results from a two-way analysis of variance examining the effect of drought (D), salinity (S) and their interaction (D*S) on parameters describing plant growth, gas exchanges, water relations, and nutrition status in *M. ciliaris* plants.

M. ciliaris			
	D	S	D*S
	F	F	F
WP DW	38,4866***	39,4192***	26,1386***
Leaf DW	99,4562***	12,2892*	10,6613*
Stem DW	65,5093***	61,7872***	30,4791***
Root DW	8,5725*	10,6804*	8,5509
Root/shoot	0,4855 ns	6,8886*	1,9943 ns
H_2O leaves	75,190***	24,083**	29,821***
H_2O Stems	19,9458**	6,2195*	3,4040 ns
H_2O Root	334,671***	52,228***	149,758***
A_{net}	121,670***	17,681**	5,591*
E	34,5137***	1,2206 ns	1,8297 ns
GS	62,0534***	7,4882*	3,6587 ns
Leaf number	92,4492***	26,1318***	7,3546*
Root length	3,5277 ns	0,3489 ns	0,6778 ns
Stem length	67,727***	10,636*	0,881 ns
Na^+	0,5055 ns	64,0385***	1,2459 ns
K^+	4,382 ns	25,912**	34,350***
Na^+/K^+	0,0057 ns	76,6843***	1,9040 ns
Ca^2	2,189 ns	0,001 ns	0,054 ns
Zn^{2+}	2,896 ns	0,153 ns	0,411 ns

4. Discussion

The aim of our work was to compare the responses of the two forage species *M. ciliaris* and *M. polymorpha* to drought and salinity applied separately or in combination to select the most resistant species and identify some traits involved in this resistance.

Our results showed that when subjected to salinity, the two species were affected by the same magnitude (64%) but *M. ciliaris* kept always under all treatments the highest biomass production (absolute value). However, when drought was applied individually or in combination with salt, *M. ciliaris* was more affected, compared to control (relative value). The reduction of plant biomass production in forage species subjected to stress conditions was reported by several studies (Ben Salah et al., 2011, Badri et al., 2016a, Badri et al., 2016b, Slama et al., 2022, Gharred et al., 2022).

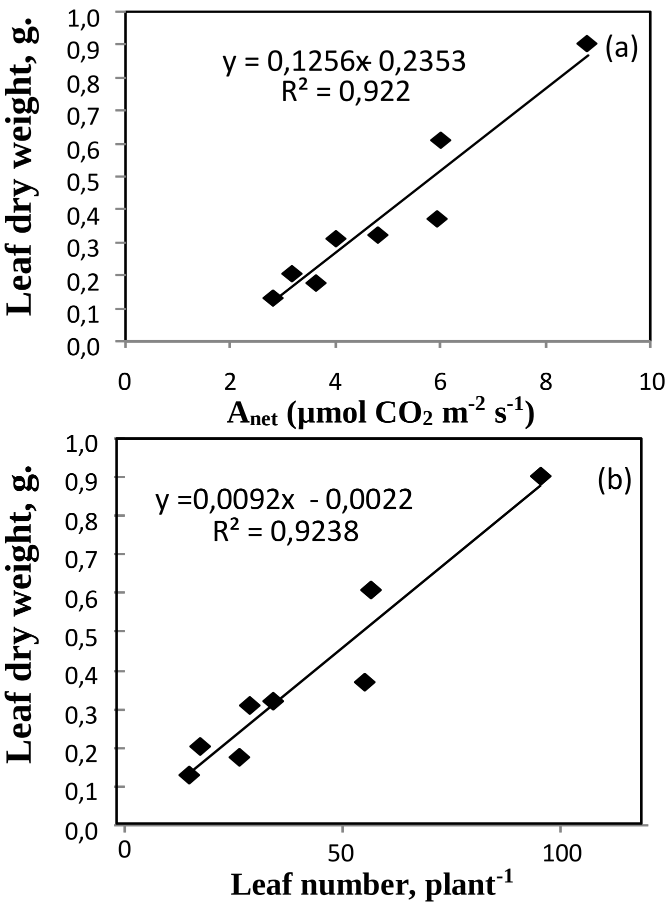

Fig. 6.11. Correlation between leaf dry weight and net CO_2 assimilation (a), leaf dry weight and leaf number (b).

Our results showed a close relationship between net CO_2 assimilation and leaf dry weight ($R^2 = 0.922$) as well as between net CO_2 assimilation and leaf number ($R^2 = 0.923$) (Fig. 6.11). This suggests that the effects of soil drying and/or salinity on growth were exerted likely through their impact on photosynthetic activity and leaf production. Moreover, a high positive correlation was observed between net CO_2 assimilation and stomatal conductance ($R^2 = 0.94$ and 0.98 for *M. polymorpha* and *M. ciliaris,* respectively) (Fig. 6.12), suggesting that the reduction in net CO_2 assimilation under stress conditions is primary due to reduced CO_2 supply resulting from stomatal closure.

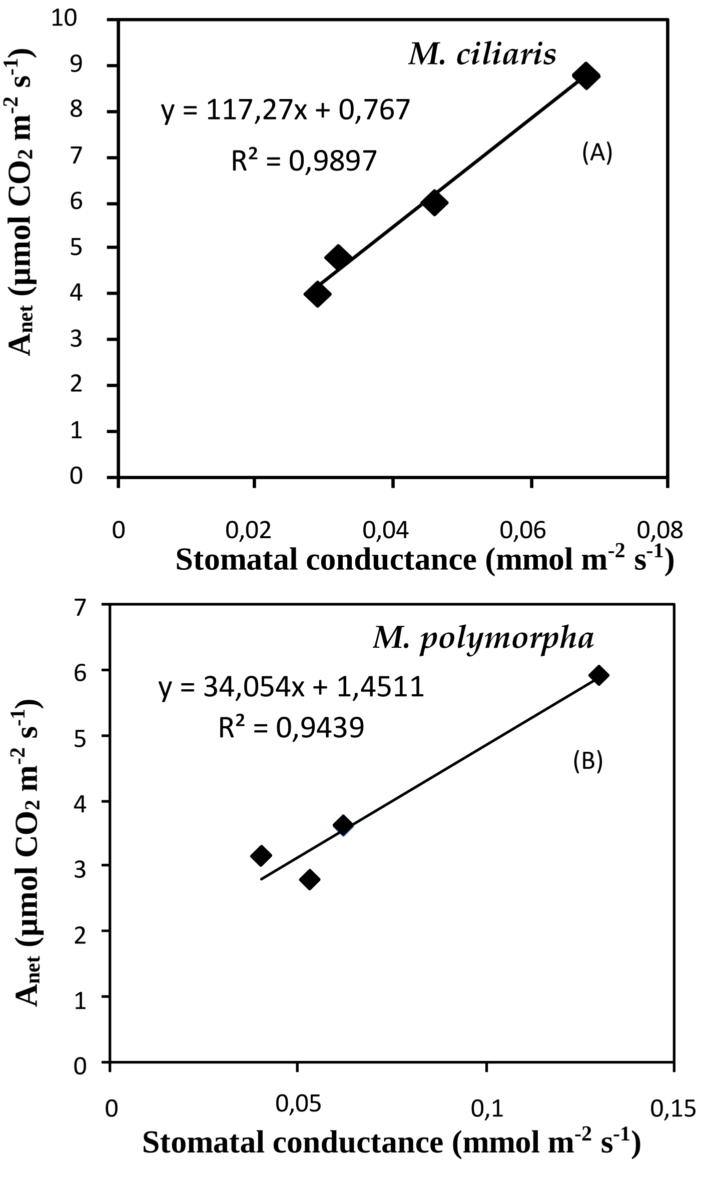

Fig. 6. 12. Correlation between net CO_2 assimilation and stomatal conductance in *M. ciliaris* (A) and *M. polymorpha* (B)

Both studied species produced more biomass in their leaves and stems compared to the roots. However, these leaves and stems seem to be more affected by S, D and the combination of S+D, compared to roots. Consistent with our findings, previous studies reported that water-deficit affects shoot biomass production more than root biomass production, resulting in an increase of the root/shoot DW ratio. This behavior is considered a criterion of adaptation to drought (Farhat et al., 2019). Indeed, our results showed that salinity and water-deficit whether applied separately or in combination significantly reduced leaf number and ramification, stem length, stomatal conductance, as well as transpiration. These responses could be considered as an important mechanisms to minimize water loss under stress conditions (Abrar et al., 2022).

Under saline conditions, *M ciliaris* exhibited a significant higher stem length, leaf and ramifications number compared to *M. polymorpha*. However, under D+S treatments no differences were observed between the two species. The combined effects of these abiotic stresses suggest that their interactions depends on both the nature of the constraint and the plant species. It has been reported by Mittler and Blumwald (2010), that some stress combinations might even benefit plants. For instance, When compared to each of the individual stresses applied separately, salinity could have a beneficial effect on the plant's ability to cope with drought. In non-halophytes, water deficit and salinity are often considered additive stress factors (Munns, 2002). However, our results pointed out, that drought and salinity did not have additive effects on plant growth parameters in the two studied species.

Plant's adaptation to water deficit involves various strategies including: (a) improving water uptake, (b) reducing water consumption, (c) as well as, improving water use efficiency (WUE) (Kang et al., 2021). WUE is an important indicator of how a particular cultivar responds to varying levels soil moisture availability. Generally, the water use efficiency of a plant decreases under water deficit and increase under optimal water availability. However, plants that maintain their physiological processes and reschedule water use in response to stress, are considered to exhibit anti-drought behavior (Kilemo, 2022).

Exhibiting a high level of drought tolerance requires cultivar's ability to increase its WUE (Hussain et al., 2022). In our study, the major advantage of *ciliaris* is indeed its higher WUE, which enabled a combination of higher A_{net} with greater biomass production. This higher WUE also helped balance the osmotic stress and hinder a negative water balance.

A high correlation (R^2 =0,86) was observed between leaf dry weight production and WUE as well as between leaf water content per plant and WUE (R^2 =0,92) (Figs 6.13 A and B). In all treatments, water content per plant was higher in *M. ciliaris* compared to *M. polymorpha* .

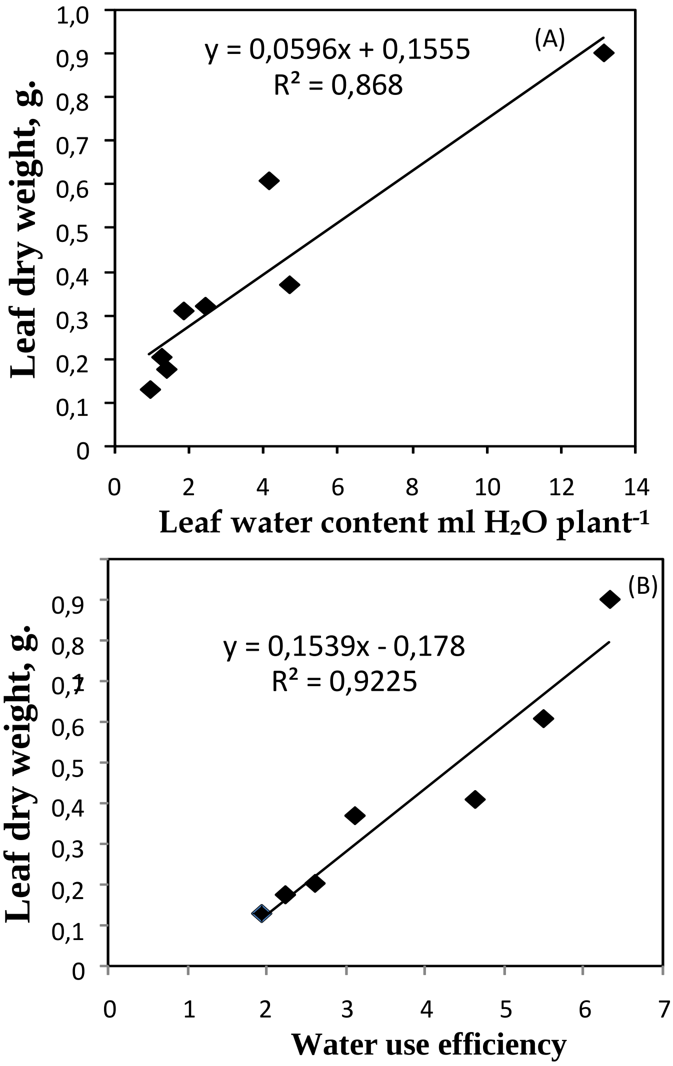

Fig.6.13. Correlation between leaf dry weight and WUE (A), leaf dry weight and leaf water content ml H_2O plant^{-1} (B).

Luo et al. (2016) analyzed the differential adaptation of two *M. truncatula* genotypes (Jemalong A17 and R108) to water deficit showing that Jemalong A17 exhibited higher drought resistance compared to R108. This was attributed to less water loss and higher relative water content (RWC), linked to the reduction in stomatal apertures. In the same way, it has been reported that, a drought sensitive pea genotype experienced a more pronounced decline in RWC under drought conditions than more resistant genotypes (Upreti et al., 2000). Moreover, Naidu et al, 1992 examined the behavior of seven pasture species of *Medicago spp.* to drought finding that *M. polymorpha* had the lowest RWC, likely due to its larger leaf area and higher transpiration rate , while *M. truncatula* maintained the highest RWC. Besides, *M. truncatula* growth under drought conditions depends largely on its capacity to maintain suitable tissue hydration and root development (Rouached et al., 2013). In our study, the higher biomass production of *M. ciliaris* was closely linked to its more balanced water consumption and nutrition uptake, which likely helped limit ROS production. The resistant line of *M. ciliaris* TNC 1.8 originating from Enfidha, exhibited no significant changes in leaf MDA levels after being subjected to 100 mM NaCl for two mouths (Salah et al., 2011).

Several studies have shown that drought can amplify the adverse effects of salinity on plant nutrient status. However, our results showed that the interactive effects of salinity and soil drying on nutrient content were not additive. Chen et al. (2007) suggested that the selection of plants with lower Na^+/K^+ ratios in their tissues may be sufficient to pick up salt-resistant genotypes. In our study, the Na^+/K^+ ratio increased significantly under both S and D+S treatments. This increase was due to the increase in leaf Na^+ content and a decrease in leaf K^+ content.

Under S treatment, *M. ciliaris* exhibited a higher Na^+/K^+ ratio compared to M. *polymorpha*. According to Blumwald, plants maintain lower cytosolic Na^+/K^+ ratios under hyperosmotic salinity by either excluding Na^+ from the cytosol to the outer medium through the plasma membrane Na^+/H^+ antiporter or by sequestering it into vacuoles via the tonoplastic Na^+/H^+ antiporter (Blumwald, 2000). Our results showed that *M. ciliaris* had a lower increase of Na^+ and a less decrease of K^+ at salinity leading to an overall lower Na^+/K^+ ratio compared to *M. polymorpha*..

Furthermore, our findings revealed that in *M. ciliaris* leaf Ca^{2+} and Zn^{2+} content were not affected by D, S, or combined D+S. Notably, *M ciliaris* accumulated higher amounts of Zn^{2+} compared to *M. polymorpha* under D, S and D+S. This Zn^{2+} accumulation in *M. ciliaris* leaves may contribute to its superior biomass production compared to *M. polymorpha*.

In conclusion, this comparative study reveals that *M. ciliaris* retains significantly more water than *M. polymorpha*, demonstrating higher water-use efficiency and superior potassium regulation under saline conditions. These traits contribute to the increased biomass production in *M. ciliaris*, making the TNC1.11 line a promising forage candidate for rehabilitating saline soils in arid and semi-arid regions.

The physiological and biochemical traits examined here are essential for initial screening of resistant species. Water-use efficiency, in particular, has proven valuable across various species for selecting the most tolerant candidates. However, further research into the combined effects of drought and salinity on *Medicago* species is necessary. This can be achieved by using advanced biochemical tools, such as chlorophyll fluorescence, osmolyte accumulation, and antioxidant enzyme assays, alongside molecular techniques. Such studies would help to clarify the mechanisms that underlie the superiority of *M. ciliaris* under combined soil drying and salinity, strengthening its potential as a candidate for saline soil rehabilitation.

CONCLUSIONS AND PERSPECTIVES

Optimizing plant performance in extreme environments requires improving the complex interactions within the plant–soil continuum, a system influenced by both biotic and abiotic factors. Farmers are facing the big challenge of producing crop yield with water supply and soil quality below optimum. In this context, optimization of environmental conditions by improving soil properties with the application of organic amendments such as biochar may be a good strategy to overcome these adverse cues.

The results presented in the first study supported our assumption that soil amendment with Bc enhances the performance of *M. ciliaris* during times of limited water supply. Indeed, biochar application under water-limited conditions helped to moderate the effects of water deficiency, thereby supporting enhanced and adaptive plant responses. This treatment alleviated the negative impacts of drought on growth and water relations, promoting balanced regulation of photosynthesis and effective buffering of reactive oxygen species through photoprotective mechanisms.

In latter case, the results impressively confirmed the agreement with the enzymatic ROS defense by the Halliwell–Asada pathway enzymes. Bc application significantly reduced the negative impact of reactive oxygen species on *M. ciliaris* plants. This aspect is evidenced by the fact that the Bc-mediated moderate change of the AsA/DHAsA ratio also lead to only the minor stimulation of abscisic acid production. This interpretation is also confirmed indirectly by the response of the CO_2/H_2O gas exchange parameters.

We conclude that soil amendment with biochar (Bc) may be an effective strategy to enhance the performance of *Medicago ciliaris* and improve its productivity in arid regions. This species shows great potential as a cash pasture crop, making it well-suited for agricultural development in dry wasteland areas, particularly in a future where water scarcity is a growing concern

The results of the second study revealed that seed priming with ascorbic acid increased plant biomass production in *M. polymorpha* L., by 66%, 100% and 98% in control, NaCl and PEG treated plants respectively. This significant enhancement improved photosynthetic activities, water relations and proline accumulation (Fig. 7.1). In *M. polymorpha* L., seed priming with ascorbic acid constitutes a straightforward and cost-effective solution for farmers aiming to boost plant productivity and resilience in salt, arid, and semi-arid regions, thereby contributing to ecosystem stability.

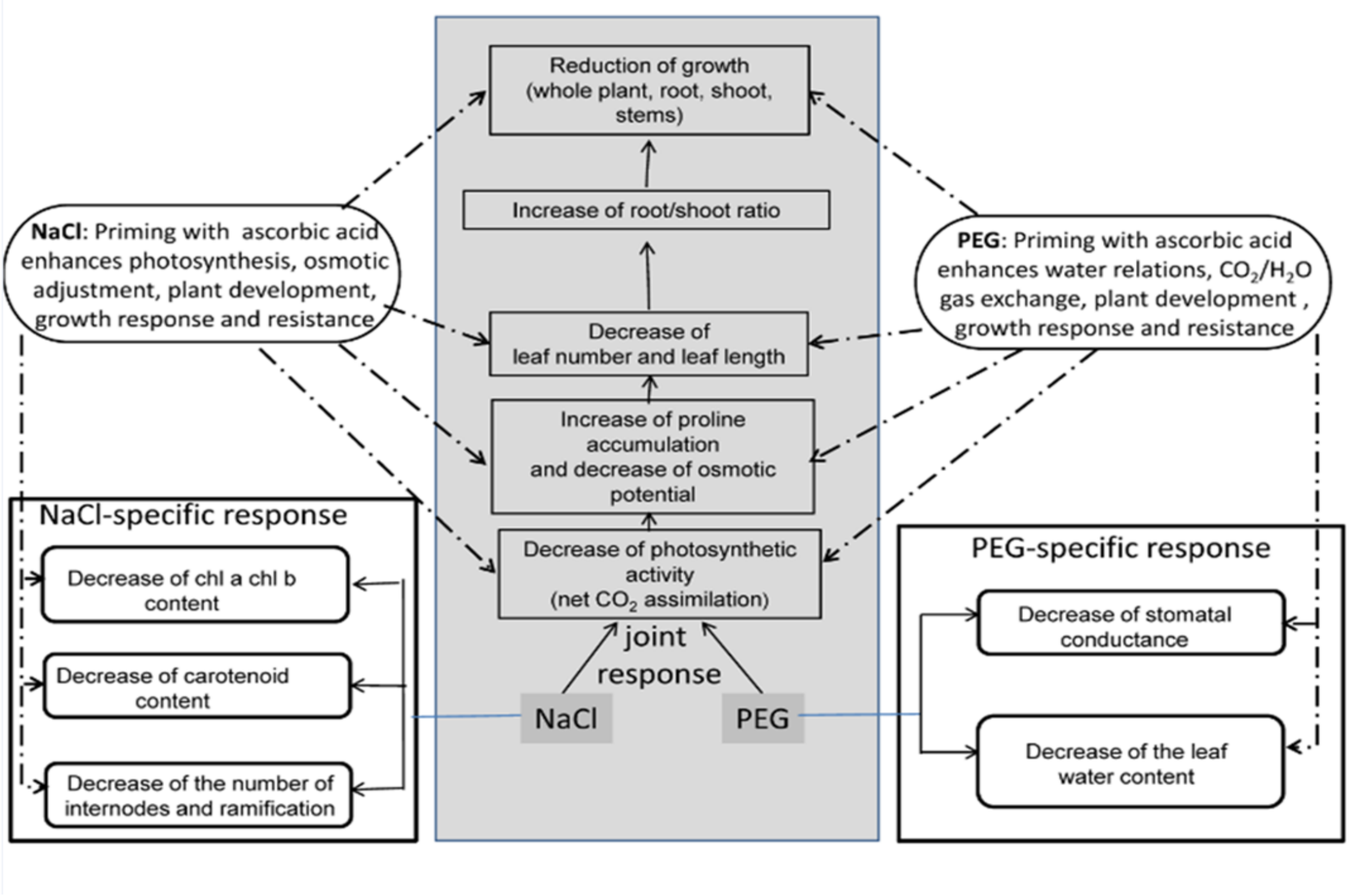

Figure 7.1 Osmotic stress and ascorbic acid priming effects on *Medicago polymorpha* seedlings.

Under all applied constrains both soil amendment with biochar and ascorbic acid seed priming enhanced plant biomass production in *M. ciliaris* and *M. polymorpha.* This improvement was correlated to increased net CO_2 assimilation in both experiments.

These two studies demonstrate that both soil amendment with biochar and ascorbic acid seed priming significantly enhanced the resilience of *M. ciliaris* and *M. polymorpha* under osmotic stress condition induced by (PEG) and salinity (NaCl) as well as soil drying. Biochar application improved WUE under water deficit, likely mitigating oxidative stress which may explain the decrease of proline accumulation. In contrast, ascorbic acid seed priming increased proline content, contributing to osmotic adjustment, increasing leaf water content (LWC), and ensuring cell turgor essential for photosynthesis and metabolic activity. These complementary strategies highlight the capacity of *M. ciliaris* and *M. polymorpha* to balance water conservation with photosynthetic activity while maintaining growth under challenging conditions .

Seed priming and biochar application are two methods that can improve plant resilience through both physiological and soil-level adaptations. Future researchs should focus on integrating these methods, investigating the combined effects of biochar and ascorbic acid seed priming to further enhance drought resistance. Additionally, understanding the molecular and physiological mechanisms underlying these adaptive responses will provide deeper insights into improving *Medicago* performance in marginal environments. Such studies could pave the way for developing sustainable agricultural practices to address the challenges posed by climate change and resource scarcity.

The third study aimed to investigate the effects of salinity, soil drying, and their interaction on two forage species, *M. ciliaris* and *M. polymorpha*. Our findings showed that salinity and drought, when applied individually, negatively impacted growth, photosynthesis, and water and potassium leaf content in both species. Interestingly, the combined effects of these two constraints were not significantly additive, suggesting a distinct interaction that warrants further exploration.

While salinity similarly affected the growth of both species, *M. ciliaris* demonstrated a clear advantage under dry or saline conditions due to its higher water use efficiency (WUE). This trait likely helped mitigate oxidative stress by reducing the accumulation of reactive oxygen species (ROS), and their negative impact. As a result, *M. ciliaris* exhibited superior biomass production under drought, salinity, and combined constrains compared to *M. polymorpha*. These findings highlight the potential of *M. ciliaris* as a promising candidate for use in the rehabilitation of soils in arid and semi-arid salinized regions.

In light of these results, further studies are needed to explore the mechanisms driving *M. ciliaris*' superior performance under extreme conditions and to evaluate its long-term suitability for forage in areas impacted by salinity and drought.

The study's scope could be further expanded through the following avenues :

(i) Molecular studies: Investigating candidate genes associated with photoinhibition and the regulation of reactive oxygen species (ROS) accumulation in chloroplasts under water deficit stress. This approach could uncover molecular mechanisms driving stress tolerance in *Medicago* species.

(ii) Priming effects of NaCl: Examining the effects of NaCl priming on seedlings to enhance Medicago productivity under salinity, drought, and their combined stresses. Preliminary results from this experiment have shown promising outcomes, warranting further exploration.

(iii) Stress recovery assessment: Evaluating the recovery capacity of plants following stress periods through rehydration experiments. This approach would simulate natural conditions in arid and semi-arid regions, where plants frequently encounter alternating dry and rainy episodes.

(iv) Nutritional value analysis: Assessing the nutritional profile of the studied species, including protein, lipid, sugar, vitamin, and fiber content in both aerial parts and seeds. The evaluation would also consider digestibility and antinutritional factors such as oxalate and nitrate, providing a comprehensive understanding of their forage value.

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Impact of Biochar Application at Water Shortage on Biochemical and Physiological Processes in Medicago ciliaris

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Impact of Biochar Application at Water Shortage on Biochemical and Physiological Processes in *Medicago ciliaris*

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Abstract: The application of biochar is mostly used to improve soil fertility, water retention capacity and nutrient uptake. The present study was conducted in order to study the impact of biochar at water deficiency conditions on the physiological and biochemical processes of *Medicago ciliaris* seedlings. Seedlings were cultivated under greenhouse conditions in pots filled with a mixture of soil and sand mixed in the presence or absence of 2% biochar. Plants of uniform size were subjected after a pretreatment phase (72 days) either to low (36% water holding capacity, water potential low) or high soil water potential (60% water holding capacity, water potential high). Pots were weighed every day to control and maintain a stable water holding capacity. In *Medicago ciliaris*, drought led to a significant reduction in plant growth and an increase in the root/shoot ratio. The growth response was accompanied by a decreased stomatal conductance and a reduction of the net CO_2 assimilation rate and water use efficiency. The associated higher risk of ROS production was indicated by a high level of lipid peroxidation, high antioxidant activities and high proline accumulation. Soil amendment with biochar enhanced the growth significantly and supported the photosynthetic apparatus of *Medicago ciliaris* species by boosting chlorophyll content and A_{net} both under well and insufficient watered plants and water use efficiency in case of water shortage. This increase of water use efficiency was correlated with the biochar-mediated decrease of the MDA and proline contents in the leaves buffering the impact of drought on photosynthetic apparatus by increasing the activity of enzymatic antioxidants SOD, APX, GPOX and GR and non-enzymatic antioxidants, such as AsA and DHAsA, giving the overall picture of a moderate stress response. These results confirmed the hypothesis that biochar application significantly reduces both the degree of stress and the negative impact of oxidative stress on *Medicago ciliaris* plants. These results implied that this species could be suitable as a cash pasture plant in the development of agriculture on dry wasteland in a future world of water shortages.

Keywords: drought; *M. ciliaris*; biochar; growth; photosynthesis; antioxidant capacity

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1. Introduction

In the next few decades, experts estimate that there will be a rise in global temperature of around 1.5 to 2 °C [1]. The cold seasons will become shorter and the warm seasons will become longer and heatwaves will occur more often, according to the report released by the Intergovernmental Panel on Climate Change [1]. Warmer temperatures enhance evaporation, which reduces surface water and dries out soils and vegetation. Nevertheless, the intensity of drought usually depends on many factors, such as the occurrence and distribution of rainfall, shifting seasons, evaporative demands and the moisture-storing capacity of the soil, especially in semiarid climates [2,3]. Facing fluctuating and unstable environmental conditions, plants need to adjust to these changes by relying on their resilience (exposure, sensitivity, adaptive capacity) and vulnerability [4]. This includes a response to water and nutrient shortage, temperature variations, UV radiation, fungal pathogens and insects, as well as other biotic and abiotic factors [5,6].

Drought impacts are not confined to arid and semi-arid regions but are increasingly spreading to more temperate and humid areas [7]. Although droughts affect a range of economically important sectors, their impacts are usually more evident within agriculture, including crop failure and reduced yields, abandoned farmland, increased soil degradation and reduced mortality [7,8]. This situation creates an urgent need for attaining agricultural sustainability regarding building resilience and adaptive capacity. Agricultural strategies are required, such as the efficient use of irrigation (=increasing water productivity (WP)), increasing livestock production relative to crops and the selection of alternative crop varieties. This may also open the possibility of enhancing productivity and food security in marginal environments (e.g., deserted or salinized regions) through the contribution of alternative crops [9–11].

Preferably, endemic plants should be chosen to ensure sustainability and environmental compatibility on dry wasteland. Some representatives of the family Fabaceae and genus *Medicago* are suitable candidates. They are high-quality, protein-rich food sources with a low demand for nutrients, a distinct resistance to saline conditions and the ability to grow on low-quality sandy soils [11,12]. *Medicago* is already in use as an alternative crop because of its ecological adaptability, morphological diversity, resistance to pests, high forage quality, high biomass production and ability to reduce soil erosion and to improve soil fertility and pasture in arid Mediterranean regions [13–16]. The latter species is caused by symbiotic nitrogen fixation, leading to a rise of the nitrogen content in plant and soil [17,18]. However, there is a high variation of drought resistance in this genus [19]. Badri [20] studied the variation in tolerance to water deficit in 47 lines of *Medicago truncatula Gaertn., M. polymorpha L.* and *M. ciliaris* (*L.*) All. The latter was the latest-flowering and produced the highest biomass at low soil water availability. Therefore, we selected *Medicago ciliaris* as the test species.

The suitability of *Medicago ciliaris* depends on the efficiency of its strategies during the adaptation to water shortage. Volaire [4] proposed the existence of three primary plant eco-physiological strategies: avoidance, tolerance and escape.

Desiccation tolerance is relatively seldom in dicot plants and *Medicago ciliaris* does not have this ability. In dicots, only resurrection plants are a small polyphyletic group of plant species capable of desiccation tolerance in their vegetative tissues while being able to recover full metabolic competence within hours to days following rehydration [21]. Therefore, *Medicago ciliaris* seems to use the escape or avoidance strategy or a mixture of both [22]. Instead of desiccation tolerance, and as a first feedback reaction during transient periods of drought stress, both avoider and escaper plants respond as follows: the reduction of transpiration, the limitation of vegetative growth, the enhancement of root growth and the avoidance of dehydration [23].

Both remaining strategies also have in common a large energy demand; the necessity to optimize water uptake and to minimize water loss by a high water use efficiency of photosynthesis and during osmotic adjustment; the osmo-protection of macromolecules; the limitation of temperature rise in the leaf; the control of the respiration rate; and the protection against reactive oxygen species (ROS).

In fact, the decreased diffusion of CO_2 and metabolic constraints affect photosynthesis as one of the key phenomena of water deficiency [24]. Photosynthesis is a decisive point of attack and, at the same time, the key process controlling plant growth and development and thus crop yield. Water shortage, for example, can reduce photosynthesis in plants through stomatal and non-stomatal limitations [25], but the coordinated regulation of photosynthesis in plants can increase biomass production and resistance to environmental stress [26].

Water shortage can harm photosynthesis directly through the restriction of CO_2 uptake, which leads to damage in photosynthetic machinery and, as a final consequence, to the development of reactive oxygen species (ROS) [26,27]. As oxygen is produced by the water-splitting system located adjacent to PSII, ROS formation is a real risk. Therefore, the degree of ROS damage and the efficiency of the ROS detoxification system in the chloroplasts are of crucial importance for survival.

In fact, ROS has a high affinity to react with macromolecules, such as lipids, proteins and nucleic acids, and cause the malfunctioning of these macromolecules [28]. For this reason it is very helpful that the extent of ROS damage can be estimated by measuring the peroxidation rate of membrane lipids with the malondialdehyde method [29,30].

However, in order to cope with an increased ROS production, plant cells display a complex and high-energy consuming array of both enzymatic and non-enzymatic detoxification mechanisms [31]. The latter group includes the production of low-molecular weight compounds, such as AsA (ascorbate, vitamin C), glutathione (GSH), carotenoids or flavonoids [32,33]. AsA is one of the most abundant water-soluble reducing compounds present in plant tissues, serving also as an electron donor in numerous reactions [34], such as the effective quenching of H_2O_2 [35]. In addition, the roles of non-photochemical fluorescence quenching parameters (NPQ), cycling electron flow (CEF), Foyer–Halliwell–Asada cycle and the repair cycle for damaged PSII reaction centers in photoprotection are well established [36–38].

In order to carry out a successful recultivation at low soil water availability, it is necessary to improve soil quality and thus the ability of plants species to resist this harsh environment.

A means to improving soil quality is the amendment of biochar (Bc). Bc can significantly increase organic matter content, water holding capacity and the plant-available water in poor-quality sandy soil, the latter characteristic due to Bc's porous nature [26,39]. The addition of Bc to the soil proved to be beneficial to plants in many ways, such as enhancing water retention capacity, nutrient uptake [40–42] water infiltration [43,44], soil aeration and respiration [42,45]. Moreover, Bc-stimulated root growth and thus water uptake from fine Bc pores. It also provided at low water supply better conditions for the synthesis of organic solutes, prevented desiccation with improved turgidity and reduced oxidative stress through high water use efficiency [46,47].

The soil improvements with Bc jointly contribute to the increase on the physiological and biochemical performances of plants and consequently promote plant biomass production. These beneficial effects of Bc were reported in several species, such as maize and rice [48].

However, it should be noted that the effects of biochar under drought conditions on water relations are contradictory. For instance, in soybean [49], authors reported that Bc application significantly enhanced crop growth rate and increased total biomass production approximately twice. Bc also improved WUE but did not improve relative water content (RWC), water retention or uptake capacity in leaves. The authors explained the enhanced biomass production by an improvement in plant nutrition rather than by increasing water uptake. Indeed, Bc application increased soil-available potassium (K) and enhanced its uptake, which lead to an increase in the stress tolerance of soybean.

Lyu reported that the plant defense mechanism is strengthen by Bc application through the increase in protective enzymatic activities and the electron transfer chain, thereby minimizing the deleterious effects of drought on the photosynthetic apparatus [50].

Currently, no data in the literature are available regarding the effectiveness of Bc on the response of *Medicago ciliaris* seedlings to drought stress. It is our hypothesis that Bc biochar application significantly reduces both the degree of stress and the negative impact of oxidative stress on *Medicago ciliaris* plants. It was our intension to study the impact of drought and Bc on the photosynthesis, water use efficiency, oxidative stress damage, ROS defense and growth performance of this promising species. Moreover, regarding the study of the physiological and biochemical mechanisms implied in the response of *Medicago ciliaris* to water shortage, our aim is to improve the response of this species to water deficit through the addition of Bc to the culture medium and to increase the productivity of alternative crops through the development of agriculture on dry wasteland to transform unproductive areas.

2. Materials and Methods

2.1. Plant Material and Growth Conditions

The *Medicago ciliaris* line used in this work was kindly provided by the Laboratory of Extremophile Plants in the Center of Biotechnology at The Technopole of Borj Cedria in Tunisia. This line originated from a local Tunisian population of Enfitha (TNC1.11). In *Medicago ciliaris*, germination is strongly limited by the presence of a hard seed coat; thus, to obtain a maximal rate of germinated seeds, scarification with liquid nitrogen was necessary.

Scarified seeds were germinated in Petri dishes in dark at 25 °C for 3 days then transferred into black pots with a 1.55 L capacity (pot 13 × 13 cm; dimensions at top: 13 × 13 cm; dimensions at the bottom: 9.5 × 9.5 cm, height: 12.5 cm) filled with a mixture of soil (70%) and sand (30%), either mixed or not mixed with 2% Bc. Coniferous wood and hardwood chips (1:4 ratio by weight) were mixed to produce Biochar through pyrolysis in a 36-h cycle at 750 °C using a Schottdorf-type reactor (Carbon Terra, Augsburg, Germany). The experiment was carried out at the University of Giessen, Germany, in a controlled environment greenhouse equipped with an automated greenhouse climate control system (including air conditioner) at a temperature of 24 °C/15 °C (day/night), a relative humidity of 55–60% and a photoperiod of 16/8 h. The emerged seedlings of uniform size were subjected to pretreatment phase (72 days). Two irrigation modes were retained in this study at 60 and 36% WHC. The selection of this two percent of water-holding capacity was based on a preliminary experiment carried out on *Medicago ciliaris* watered with 100, 75, 60 and 36% WHC, showing that for our line and soil type, 60% corresponded to the optimal conditions for growth and that 36% WHC led to a significant decrease of growth parameters. Thus, plants irrigated with 60% WHC corresponded to control plants; however, plants irrigated with 36% WHC correspond to stressed ones. Pots were weighed every day to control and maintain a stable WHC

For all treatments, water was enriched with diluted nutrient solution [51]. Independently of the procedure for watering (60 or 36% WHC), plants received the same quantity of nutrients.

WHC measured according to the technique of Bouyoucos [52] was estimated at around 13.33%.

After 42 days of treatment, a final harvest was carried out and plants were separated into shoots and roots.

2.2. Growth Parameter

Upon harvest, we measured root, shoot and nodule fresh weight (FW) separately. Dry weight (DW) was determined after drying the samples at 60 °C in the oven until a constant weight was reached.

Sensitivity index (SI) was also determined by measuring the difference between the DW of plants subjected to water deficit stress and control plants and the DW of the controls according to the following equation expressed in percent [53]:

$$\mathbf{SI_{stress} = [100 \times (DW_{stressed} - DW_{control})/DW_{control}]} \quad (1)$$

2.2.1. Chlorophyll Fluorescence

Chlorophyll fluorescence was measured in the third fully expanded leaf from starting in the morning from 08:30 am to 15:00 am, using a portable chlorophyll meter (JUNIOR PAM, WALZ GmbH, Effeltrich, Germany). Before measuring, leaves needed to be adapted to the darkness for 40 min to evaluate maximum quantum efficiency of PSII photochemistry [54]. Fluorescence parameters characterizing either the dark-adapted state or light-adapted state were measured at four plants from each of the four applied treatments.

The following chlorophyll fluorescence parameters were calculated using WINCONTROL software (2.133/03.00) with standard settings for rapid light curves (Heinz Walz Gmbh, Effeltrich, Germany; [55,56]), which are the potential maximal efficiency of PSII (Fv/Fm), electron transport rate (ETR), photochemical quantum yield of photosystem II (Y(II)); the quantum yield of regulated non-photochemical energy loss in PS II (Y(NPQ)), quantum yield of non-regulated non-photochemical energy loss in PS II, equivalent to Y(NO); and photosynthetic photon flux density (PPFD) (µE m^{-2} s^{-1}).

2.2.2. CO_2/H_2O Gas Exchange

CO_2/H_2O gas exchange was determined using a Li-Cor LI-6400XT portable photosynthesis system (Li-Cor Biosciences; Lincoln, NE, USA) with a 6400-02(B) LED light source attached to the leaf chamber.

Temperature in the leaf was set at 22.0 °C. Carbon dioxide levels in the leaf chamber were controlled by using CO_2 cartridge and a fixed flow rate of 300 µmol s^{-1}. CO_2 concentration within the leaf chamber (C_a) was fixed at 400 µmol mol^{-1}. Intercellular CO_2 concentration (C_i) [µmol CO_2 m^{-2} s^{-1}], net CO_2 assimilation rate (A_{net}) [µmol CO_2 m^{-2} s^{-1}], dark respiration (R_D) [µmol CO_2 m^{-2} s^{-1}], transpiration rate (E) [mol H_2O m^{-2} s^{-1}] and stomatal conductance (S_C) [mol H_2O m^{-2} s^{-1}] were determined on the third fully expanded leaf from 08:30 to 15:00 am. Water use efficiency (WUE) was calculated as a A_{net}/E ratio. Photorespiration (R_L) [µmol(CO_2) m^{-2} s^{-1}] was estimated as 1/12 (ETR − 4 (A_{net} + RD)) [57]. Gross CO_2 assimilation (A_{gross}) [µmol (CO_2) m^{-2} s^{-1}] was calculated as the sum of A_{net}, R_D and R_L. The slope in the linear range of the light response curve represents the photosynthetic efficiency (V_c) and was calculated as described in [58]. All measurements were carried out in the greenhouse at light saturation conditions with 750 or 1500 µE m^{-2} s^{-1} photosynthetic photon flux density (PPFD) (high water potential: 1500 µmol $m^{-2}s^{-1}$ PPFD and low water potential: 750 µmol $m^{-2}s^{-1}$ PPFD) with 25 ± 15 °C air temperature and 60 ± 10% relative air humidity.

2.2.3. Chlorophyll Content

Leaf SPAD readings (SPAD 502; Minolta Co., Osaka, Japan) provide a nondestructive surrogate method for determining leaf chlorophyll (Chl) concentration [59]. Leaf chlorophyll (Chl) concentrations were measured in the third fully expanded leaf in the morning. The mean of three SPAD readings for each leaf was recorded.

2.2.4. Proline Content

Free proline was qualified spectrophotometrically according to Bates [60].

An amount of 0.2 g of plant fresh material was homogenized in 4 mL of sulphosalicylic acid (3% *w*/*v*), then mixed with 2 mL of acid ninhydrin solution and 2 mL of glacial acetic acid. The mixture was heated at +100 °C for 1 h in a water bath. The reaction was stopped by transferring the mixture to an ice bath. Proline was extracted by adding 4 mL of toluene to each tube, and the absorbance of toluene fraction (aspired from the liquid phase) was measured at λ 520 nm using a UV/VIS spectrophotometer CAMSPEC M550 double beam (Spectronic CamSpec, Leeds, UK). Proline concentration was determined using calibration curve as µmol proline g^{-1} FW.

2.2.5. Lipid Peroxidation

The extent of lipid peroxidation was estimated by determining the concentration of malondialdehyde (MDA) according to Rao and Sresty [61]. Leaf material (50 mg FW) was homogenized with a prechilled mortar and pestle in 2 mL of ice-cold trichloroacetic acid TCA (0.1%, *w*/*v*) and centrifuged at 15,000× *g* for 15 min and at 4 °C. Assay mixture containing 2 mL aliquot of supernatant and 2 mL of 0.67% (*w*/*v*) thiobarbituric acid (TBA), was heated at 95 °C for 20 min and then rapidly cooled in an ice bath. The samples were centrifuged (10,000× *g* for 10 min at 4 °C) and the supernatant absorbance was measured at λ 532 and λ 600 nm using UV/VIS spectrophotometer CAMSPEC M550 double beam (Spectronic CamSpec, Leeds, UK). The concentration of MDA was calculated from the extinction coefficient 155 mM^{-1} cm^{-1}.

2.2.6. Hydrogen Peroxide Content

The hydrogen peroxide (H_2O_2) concentration was measured according to the method previously described by Loreto and Velikova [62]. Frozen leaf samples (500 mg) were homogenized in 5 mL of 1% (*w*/*v*) ice-cold trichloroacetic acid (TCA) and centrifuged at 14,000× *g*, for 20 min at 4 °C. Subsequently, 0.5 mL of supernatant was mixed with 0.5 mL of potassium phosphate buffer (10 mM, pH 7.0) and 1.5 mL of potassium iodide (1 M) in a ratio 2:1 (*v*/*v*). The absorbance was measured at λ 390 nm using a UV/VIS spectrophotometer CAMSPEC M550 double beam (Spectronic CamSpec, Leeds, UK). The hydrogen peroxide content was calculated using a standard curve using different concentrations of H_2O_2.

2.3. Protein Quantification and Antioxidant Enzyme Assay

Fresh leaves (100 mg) were homogenized with ice-cold sodium phosphate buffer (50 mM, pH 7.2) containing 1 mM ascorbic acid, 1mM dithiothreitol (DTT), 0,1% of triton, 10 mM ethylene diamine tetra acetic acid (EDTA, disodium salt) and 10% (*w*/*v*) Polyvinylpolypyrrolidone (PVPP). The homogenate was centrifuged at 12,000× *g* for 20 min at 4 °C. The supernatant was collected and stored in small Eppendorf at −80 °C.

Protein content was determined after mixing the supernatant with an acid solution of Coomassie–Brillant–Blau G-250 and subsequent incubation in the dark for 10 min (see Bradford, 1976). The absorbance was measured at λ 595 nm using a UV/VIS spectrophotometer CAMSPEC M550 double beam (Spectronic CamSpec, Leeds, UK). Soluble protein concentration in the enzyme extracts were estimated using a standard curve of different concentrations of bovine serum albumin (BSA).

Superoxide dismutase activity was assayed by its ability to inhibit photochemical reduction of nitroblue tetrazolium chloride (NBT) at 560 nm. According to Beyer and Fridovich [63], we prepared a reagent containing 10 mM of L-methionine, 0.1 mM of nitroblue-tetrazolium chloride (NBT) and 0.75% of Triton X-100 in 50 mM potassium phosphate pH 7.8 in a dark bottle. Of this reagent, 1 μL was added to the reaction mixture (3 mL) containing 40 μL of enzyme extract followed by 10 μL of 0.12 mM riboflavin. The mixture was prepared twice, one of them was incubated under fluorescent lamps (40 W) for 7 min and the second was kept in the dark to be used as blank for the measurements. The absorbance of the mixture was measured at λ 560 nm. The enzyme activity was calculated as the percentage inhibition per minute.

Ascorbate peroxidase (APX, EC 1.11.1.11) activity was assayed according to Nakano and Asada [64]. The reaction mixture (3 mL) consisted of 50 mM of potassium phosphate buffer (pH 7.0), 0.2 μM of EDTA, 0.5 mM of ascorbate, 2 mM H_2O_2 and 50 μL of enzyme extract. The reaction was initiated by the addition of H_2O_2. Ascorbate peroxidase was assayed by monitoring the decrease in absorbance at 290 nm. The molar extinction coefficient was 2.8 $mM^{-1}cm^{-1}$.

Guaiacol peroxidase (GPX) activity was measured by recording the increase of the absorbance at λ 470 nm due to a tetra-guaiacol formation (ε = 26.6 L mol^{-1} cm^{-1}) according to Tatiana [65]. The reaction mixture (3 mL) contained 50 mM of potassium phosphate buffer (pH 7.0), 2 mM H_2O_2, Guaiacol 2.7 mM and 50 µL of enzyme extract. The enzyme activity was calculated as the percentage of inhibition per min. The molar extinction coefficient was 26.6 L mol^{-1} cm^{-1}.

According to Foyer and Halliwell [66], Glutathione reductase (GR, EC 1.6.4.2) activity was determined by the oxidation of β-NADPH at λ 340 nm (ε = 6.2L mol^{-1} cm^{-1}). The reaction mixture (3 mL) contained 100 mM Tris-HCl (pH 7.8), 0.5 mM GSSG, 0.03 mM β-NADPH, 5mM EDTA and 100 µL of enzyme extract. The molar extinction coefficient was 6.2 L mol^{-1} cm^{-1}.

2.4. Extraction and Determination of Non-Enzymatic Antioxidant Ascorbate (AsA) and Dehydro-Ascorbate (DHAsA)

Frozen leaf samples (400 mg) were ground in liquid nitrogen and homogenized in 2 mL of ice cold 6% TCA. The mixture was centrifuged at 16,000× *g* for 20 min at 4 °C and supernatant was collected. Ascorbate (AsA) and dehydro-ascorbate (DHAsA) were determined with a dipyridyl assay based on the reduction of Fe^{+3} by reduced ascorbate, followed by complex formation between Fe^{+2} and bipyridil, which absorbs at λ 525 nm. Total ascorbate was determined after the reduction of DHAsA to AsA by reacting with dithiothreitol. A standard curve was prepared for the estimation of total ascorbate (with pretreatment DTT) and DHAsA (subtracting AsA from total ascorbate). This method was described by [67].

2.5. Statistics

Between four and five replicates were used for data analyses. Statistical analyses were carried out by two-way analysis of variances using SigmaPlot software. A two-way analysis of variance (ANOVA) was performed to test the independence of variation among conditions (equal variance test) and normal distribution of data of each variable (Shapiro–Wilk). The Holm–Sidak method (all pairwise multiple comparison procedures) was used to check whether the means of the posterior homogeneous subgroups differed significantly at $p < 0.05$.

3. Results

3.1. Growth

The dry weight of control plants (0% Bc WPh) was about 10 g at the time of harvest. Biochar amendment significantly increased the biomass (~24%), (Figure 1A). Instead, water deficit led to a significant reduction. However, this reduction was more pronounced in 0% Bc (~45%) than in 2% Bc.

Shoot and root dry weight variations were in the mean similar to those of the whole plant dry weight (Figure 1B,C).

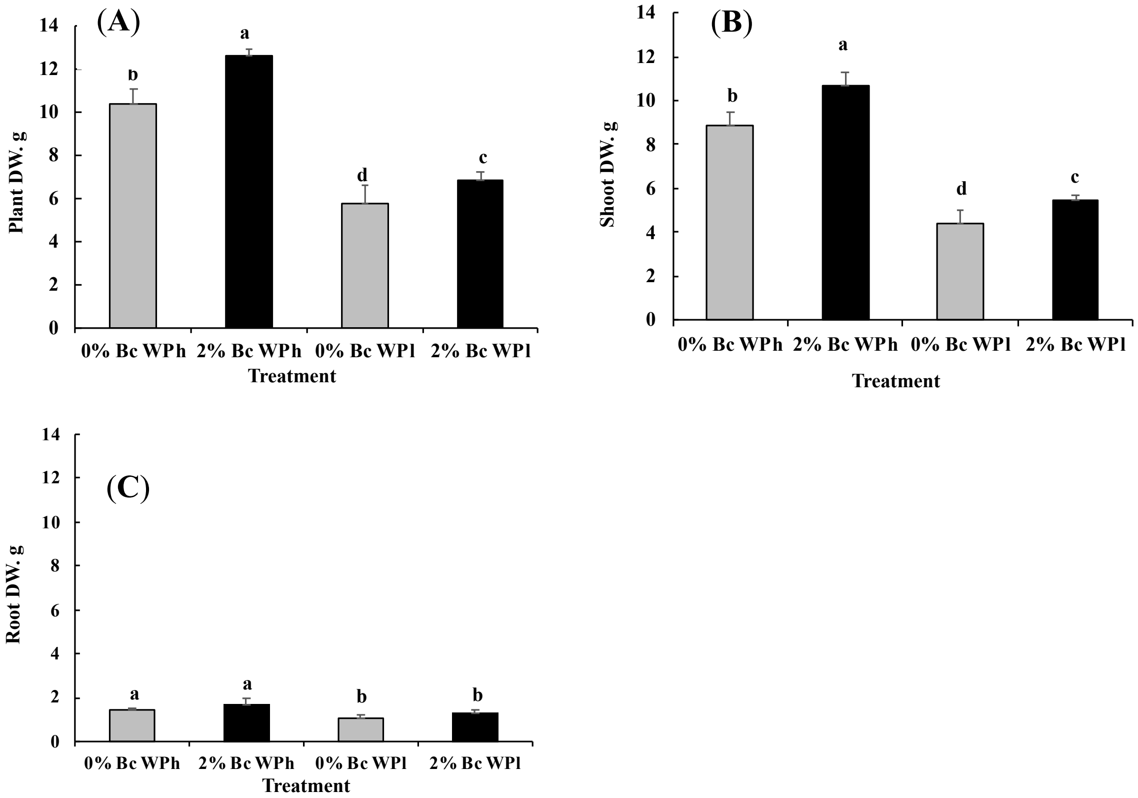


Figure 1. Varietal differences in plant growth parameters; dry weight of the whole plant (**A**), shoot (**B**), and the root (**C**), in *Medicago ciliaris* after 3 weeks of drought treatment. Values represent mean ± SE (*n* = 5) and the different letters a to d indicate significant differences between the treatments. Low soil water potential (WPl), high soil water potential (WPh), Biochar (Bc).

The negative values of the sensitivity index (SI) under water deficit conditions reflect a growth-reduction (Table 1) and the positive values of SI at 2% Bc reflect a growth stimulation even under water deficit conditions.

Table 1. Relative impact (SI in %) of drought and biochar on the dry weight of *Medicago ciliaris* plant, shoot and root. Low soil water potential (WPl), high soil water potential (WPh), Biochar (Bc).

Sensitivity Index (SI)	WPl	Bc WPh	Bc WPl
Plant	−46.81%	17.61%	−37.2%
Shoot	−50.47%	40.29%	−37.77%
Root	−24.34%	16.38%	−9.00%

As shown in Figure 2, the root/shoot ratio was significantly higher in plants subjected to water deficit stress than in controls. The biochar amendment caused a significant increase of the root/shoot ratio with sufficient water supply but not under drought conditions.

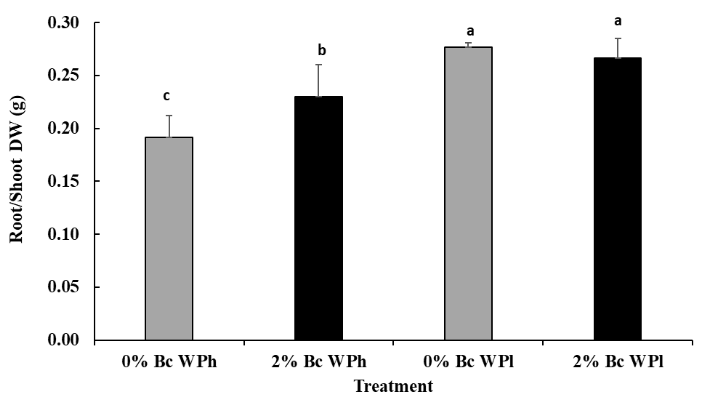


Figure 2. Root–shoot ratio in *Medicago ciliaris* after 3 weeks of drought treatment. Values represent mean ± SE (n = 5) and the different letters a to c indicate significant differences between the treatments. Low soil water potential (WPl), high soil water potential (WPh), Biochar (Bc).

3.2. Tissue Water Status

As shown in (Figure 3A), shoot and root water content were significantly reduced by water deficit by 55% and 27%, respectively, indicating the depressive effects of drought on water status in *Medicago ciliaris*. Biochar had hardly any effect on the water status with the exception of a further reduction in water content in the roots (Figure 3B).

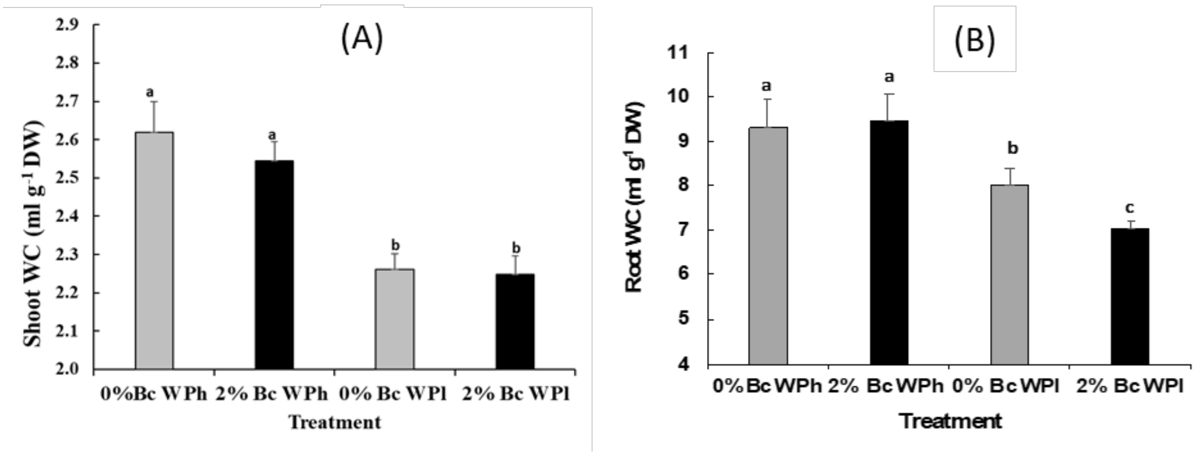


Figure 3. Shoot water content (**A**) and root water content (**B**) in *Medicago ciliaris* after 3 weeks of drought treatment. Values represent mean ± SE (n = 5) and the different letters a to c indicate significant differences between the treatments. Low soil water potential (WPl), high soil water potential (WPh), Biochar (Bc).

3.3. Chlorophyll and Protein Content

Water deficit stress significantly reduced chlorophyll content (Figure 4). The biochar amendment caused a significant increase in chlorophyll content at low and high soil water potential.

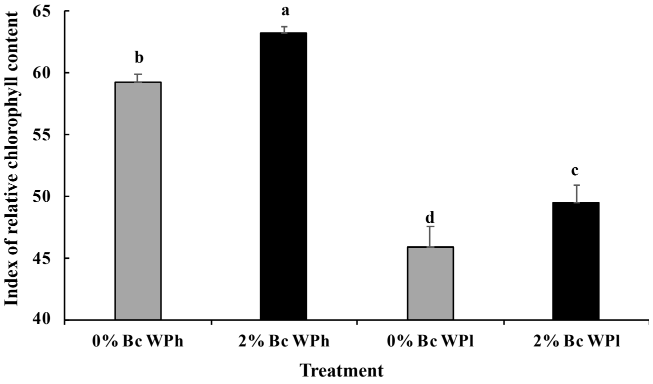


Figure 4. Chlorophyll concentration in *Medicago ciliaris* after 3 weeks of drought treatment. Values represent mean ± SE ($n = 5$) and the different letters a to d indicate significant differences between the treatments. Low soil water potential (WPl), high soil water potential (WPh), Biochar (Bc).

The development of the protein content was nearly reciprocal to the chlorophyll content (Figures 4 and 5). Drought led to a significant increase of the protein content and biochar to a non-significant decrease (in the mean).

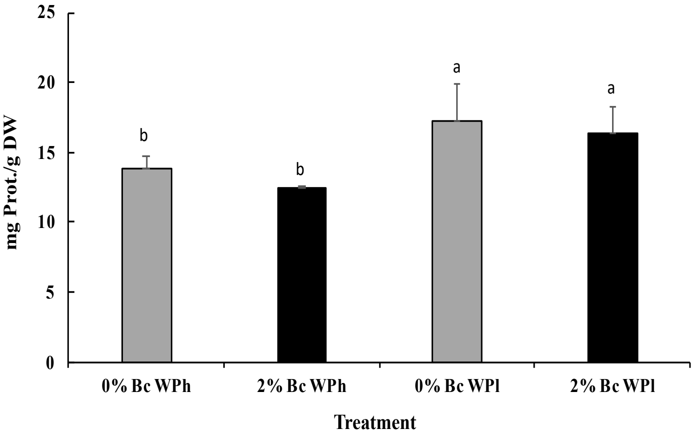


Figure 5. Leave protein content in *Medicago ciliaris* after 3 weeks of drought treatment. Values represent mean ± SE ($n = 5$) and the different letters a and b indicate significant differences between the treatments. Low soil water potential (WPl), high soil water potential (WPh), Biochar (Bc).

3.4. Proline and MDA Accumulation

Drought led to a significant increase of the proline content (Figure 6A) and of the MDA content (Figure 6B). However, biochar caused a significant decrease of the proline and MDA content at low and high soil water potential.

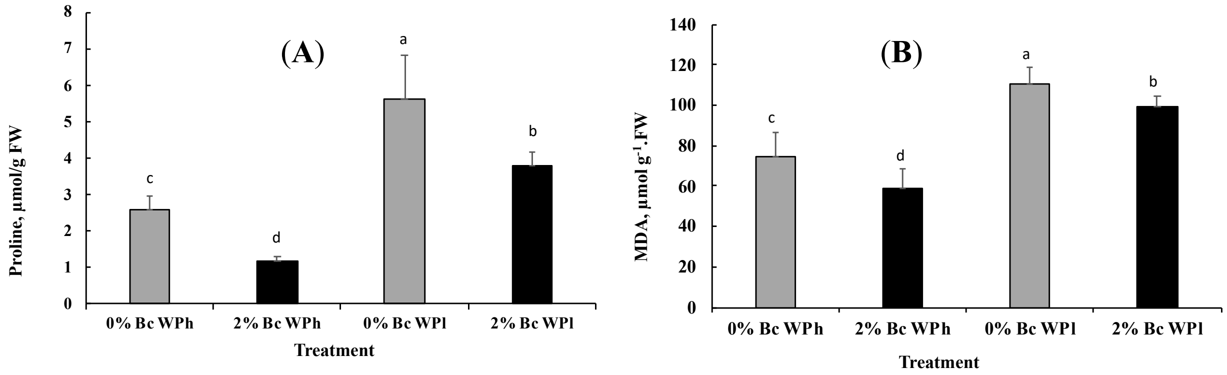


Figure 6. Changes in Proline (**A**) and MDA (**B**) content in *Medicago ciliaris* leaves after 3 weeks of drought treatment. Values represent mean ± SE (*n* = 5) and the different letters a to d indicate significant differences between the treatments. Low soil water potential (WPl), high soil water potential (WPh), Biochar (Bc).

3.5. Leaf CO_2/H_2O Gas Exchange

The highest A_{net} was reached at a high water potential without biochar amendment (10.66 µmol m^{-2} s^{-1}) (Table 2). Drought led to a significant decrease in A_{net} (2513 µmol m^{-2} s^{-1}). The Biochar amendment significantly buffered the drought-induced reduction of A_{net}. The differences in A_{net} correlated well with the photosynthetic efficiency (V_c).

There was a clear correlation between the drought-induced reduction of A_{net} and ETR under the incorporation of dark and light respiration rates (the latter not shown) and a homeostatic and stable ETR/A_{gross} ratio in all four treatments.

There was a clear direct correlation between the drought-induced reduction of A_{net} and the stomatal conductance, with the logical consequence of low C_i/C_a ratios in both generously watered treatments and high C_i/C_a ratios in both water deficient treatments.

The application of biochar did not have any effect on C_i/C_a and Sc. However, biochar had a significant positive impact on the water use efficiency at low water potential. This effect was reached mainly by the maintenance of high A_{net} rates.

Table 2. CO_2/H_2O gas exchange and chlorophyll fluorescence parameters (A_{net}, V_c, SC, C_i/C_a ratio, WUE, R_L, R_D, ETR, ETR/A_{gross} and Y(NPQ)) of *Medicago ciliaris* leaves at a saturating light intensity after 3 weeks of drought treatment. Values represent mean ± SE (*n* = 5) and different letters indicate significant differences between treatments. Net CO_2 assimilation rate (A_{net}) [µmol(CO_2) m^{-2} s^{-1}], photosynthetic efficiency (V_c), stomatal conductance (S_c) [mol(H_2O) m^{-2} s^{-1}], ratio of intercellular and atmospheric CO_2 concentration (Ci/Ca ratio) [µmol(CO_2) m^{-2} s^{-1}], ratio of net CO_2 assimilation rate and transpiration (A/E) (µmol/mmol), Photorespiration (R_L) (µmol(CO_2)*m^{-2}* s^{-1}), dark respiration (R_D) (µmol CO_2 m^{-2} s^{-1}), electron transport rate (ETR) (µmol electrons m^{-2} s^{-1}), gross CO_2 assimilation (A_{gross}) [µmol(CO_2) m^{-2} s^{-1}], quantum yield of regulated non-photochemical energy loss in PS II (Y(NPQ)), electron (e^-).

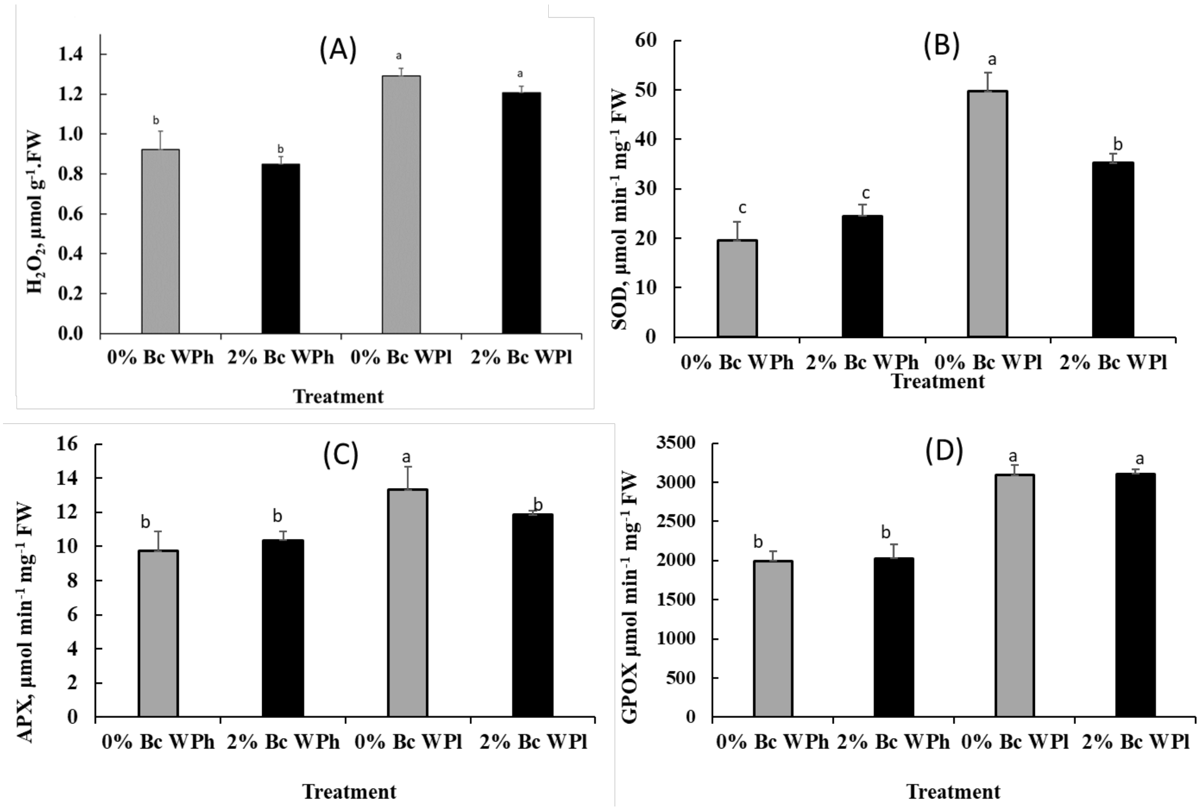
Treatment Parameter	**WPh (at 1500 µE m^{-2} s^{-1} PPFD)**		**WPl (at 750 µE m^{-2} s^{-1} PPFD)**	
	0% Bc	2% Bc	0% Bc	2% Bc
A_{net} (µmol CO_2 m^{-2} s^{-1})	10.667 a ± 0.566	8.029 b ± 0.803	2.513 d ± 0.294	4.495 c ± 0.801
V_c (µmol CO_2*m^{-2}*s^{-1})	0.057 a ± 0.008	0.048 ac ± 0.012	0.030 b ± 0.001	0.045 bc ± 0.012
Sc (µmol CO_2 m^{-2} s^{-1})	0.07 a ± 0.008	0.05 b ± 0.004	0,021 c ± 0,008	0.03 c ± 0.007
C_i/C_a ratio	0.367 a ± 0.05	0.323 a ± 0.06	0.510 b ± 0.09	0.422 b ± 0.01

WUE (A/E)	9.476 b ± 1 27	6.595 c ± 0.21	6.4 d ± 1.85	11.04 a ± 0.61
R_L (μmol(CO_2)*m^{-2}*s^{-1})	11.88 b ± 0.73	13.59 a ± 0.95	9.05 c ± 0.56	8.01 c ± 0.72
R_D (μmol CO_2 $m^{-2}s^{-1}$)	1.189 ab ± 0.141	0.640 b ± 0.157	1.675 a ± 0.600	0.900 b ± 0.452
ETR (μmol e^- m^{-2} s^{-1})	182.23 b ± 7.71	195.73 a ±8.15	112.38 c ± 3.9	109.6 c ± 6.42
ETR/A_{gross} ratio (e^-/CO_2)	7.75 a ± 0.2	8.65 a ± 0.26	8.43 a ± 0.63	8.4 a ± 0.68
Y(NPQ)	0.382 a ± 0.04	0.301 b ± 0.06	0.329 a ± 0.03	0.371 a ± 0.029

3.6. Enzymatic Antioxidant Assays

Drought stress caused an increased accumulation of H_2O_2 in the leaves of *Medicago ciliaris* treated with and without biochar (Figure 7A). It is noticeable that higher H_2O_2 values correlate with lower chlorophyll content (see Figure 4) and lower photosynthetic activity (see Table 2). The drought induced an increase in reactive oxygen species (ROS), such as H_2O_2, making it necessary to also measure the antioxidant enzyme activities.

The drought-induced adjustment of *Medicago ciliaris* to an enhanced attack by reactive oxygen species (ROS) is reflected by increased activities of SOD, APX, GPOX and GR (Figure 7B–E). However, biochar reduced the APX and SOD activities in plants. Both enzymes constitute first line of defense against oxidative stress. Their reduced activities might be an indicator of a reduced demand for an adaptive response to ROS. The non-significant reduction of H_2O_2 content and GR activity points in the same direction.



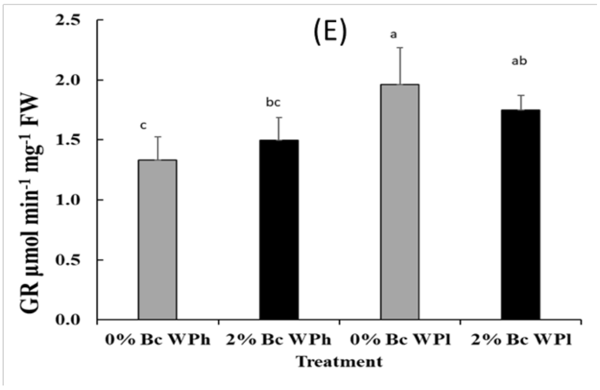


Figure 7. Varietal differences in the content of H_2O_2 (**A**) and the enzymatic activities of SOD (**B**), APX (**C**), GPOX (**D**), and GR (**E**) in *Medicago ciliaris* leaves after 3 weeks of drought treatment. Values represent mean ± SE ($n = 5$) and the different letters a to c indicate significant differences between the treatments. Low soil water potential (WPl), high soil water potential (WPh), Biochar (Bc).

3.7. Non Enzymatic Antioxidant Assays: Ascorbate Determination

Both factors water deficiency and biochar induced a significant increase in the total ascorbate concentration in the leaves of *Medicago ciliaris* (Figure 8A). Drought reached this effect by a joint increase of the reduced ascorbate (AsA, Figure 8B) and oxidized ascorbate (DHAsA, Figure 8C) concentration. However, biochar had a significantly higher impact on the concentration of DHAsA in drought, leading to an overall significant decrease in the AsA/DHAsA ratio in both biochar treatments (Figure 8D)

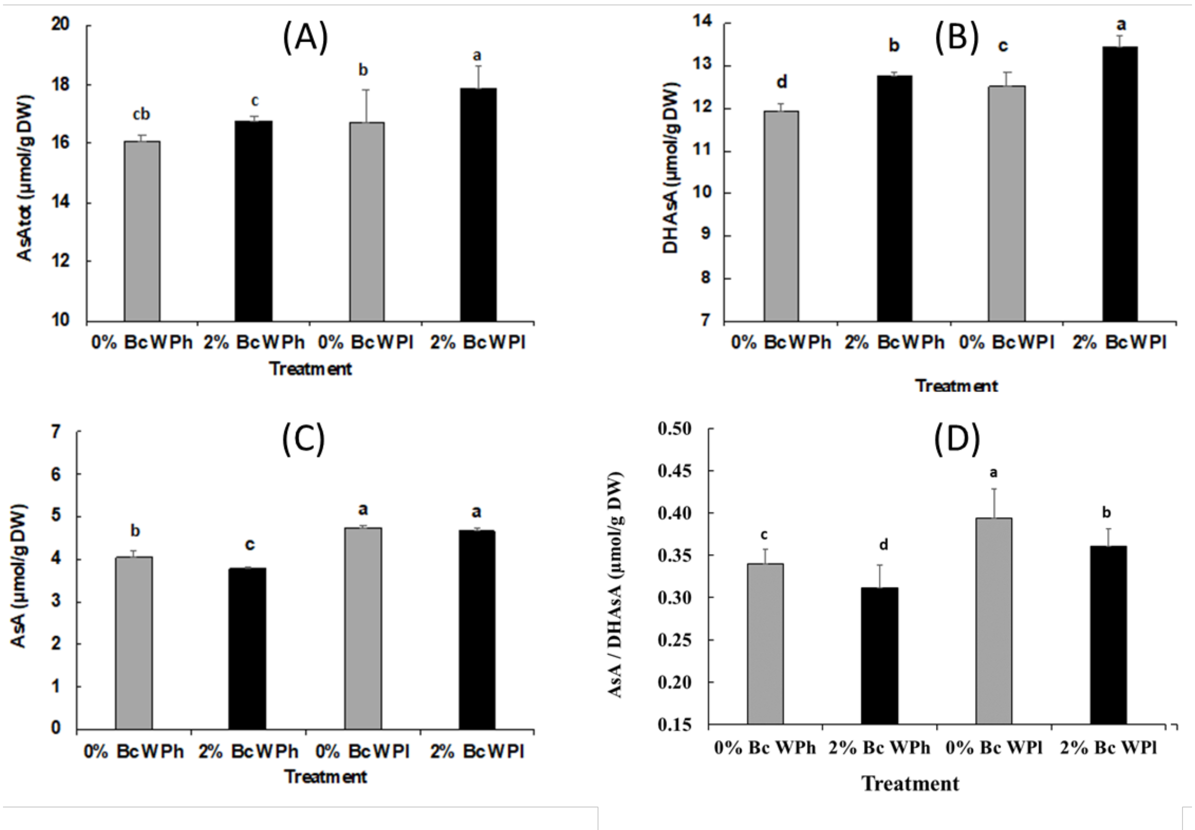


Figure 8. The content of total ascorbate (AsA + DHAsA) (**A**), AsA, (**B**), DHAsA (**C**), and AsA/DHAsA ratio (**D**) in *Medicago ciliaris* leaves after 3 weeks of drought treatment. Values represent mean ± SE ($n = 5$) and the different letters a to d indicate significant differences between the treatments. Low soil water potential (WPl), high soil water potential (WPh), Biochar (Bc).

4. Discussion

In agreement with the present study, scientists worldwide are exploring possibilities in order to create the best possible growing conditions for drought-resistant crops that are able to maintain high productivity even in dry wastelands [68,69]. We decided to select *Medicago ciliaris* because it is a relatively salt-resistant alfalfa species.

4.1. Adjustment of Growth and Water Relations

However, it was obvious that alfalfa reduced biomass production by 45% in cases of water deficit (Figure 1). This result matches with results from [70], showing that the reduction of plant biomass production of medic plants ranged between 12 and 73%. However, it could be shown that the addition of Bc (biochar) to the soil substrate led to a significant increase in the biomass production of M. *ciliaris* in both water regimes (see also Sensitivity index in Table 1). Similar Bc effects were reported for rice and maize grain yields (increase of 12.1% and 28% respectively) [71–73].

Independent of the presence of Bc in the culture medium and in line with our findings, drought-stressed plants exhibit a higher root/shoot DW ratio than plants growing under adequate water supply (Figure 2). This may be related to the preferential allocation of dry matter to roots [70] and may facilitate adaptation to drought by limiting the transpiring leaf area and extracting water residuals [74].

The drought-induced increase of the root/shoot ratio may be also a consequence of decreasing RWC in both organs (Figure 3). The decrease of the RWC in case of water shortage seems to be a typical response of alfalfa. For instance, the exposure of several annual *Medicago spp*. (*M. rugosa, M. scutellata, M. littoralis, M. truncatula, M. murex, M. polymorpha, M. intertexta, M sativa*) to five days of drought led to a reduction of RWC up to 40% [75]. In the cases of *Medicago ciliaris* varieties, the decrease amounted to around 60% in severely dehydrated plants [76].

From this perspective, it is surprising that Bc amendment led not only to a further reduction of the root RWC during water shortage but also to higher growth rate. This apparent contradiction can be explained by the enhanced osmotic adjustment with organic osmoprotectants as a strategy to tolerate the adverse effects of drought conditions [74]. Furthermore, Bc improved soil quality by producing higher organic matter that enhanced growth-regulating substances and plant functioning [77].

Similar results were also found for soybean [49]. The authors reported that Bc application significantly enhanced crop growth rate, increased total biomass production approximately twice and improved WUE but did not improve RWC, water retention or uptake capacity in leaves. The assumption seems obvious that the enhanced biomass production after addition of Bc to the soil substrate may be caused by an improvement in plant nutrition rather than by increasing water uptake. Indeed, biochar application increased soil-available potassium (K) and enhanced its uptake and the stress resistance in soybean.

4.2. Regulation of Photosynthesis

The establishment of a new, suitable equilibrium and a high efficient use of resources is the main strategy during adaptation to water shortage in *M. ciliaris*. The following adaptation of photosynthesis to a new optimum is a good example of coordinated regulation in *M. ciliaris*: The suppressive impact of drought on photosynthesis (A_{net}, Table 2) contributed, together with a reduction of the chlorophyll content (Figure 4), leaf area and photosynthetic electron transport rate (ETR), to the maintenance of a constant ETR/A_{net} ratio (Table 2). This mechanism reduced the generation of ROS and counteracted the otherwise possible destruction of chloroplasts [78,79].

Usually, a reduced chlorophyll concentration would imply a reduced ability for light harvesting and thus reduced photosynthesis [80]. *M. ciliaris* seems to actively use this correlation in case of drought or be forced into backwards regulation and the protection of the plants against oxidative stress, as confirmed in the current study. The latter process could happen because of the limited stomatal conductance and CO_2/H_2O gas-exchange during water shortage [81]. Indeed, our studies showed that A_{net} and S_C but not C_i (or the C_i/C_a ratio, Table 2) increased and decreased simultaneously. Stomatal conductance was higher in plants receiving normal irrigation than in drought-treated plants [78]. The increase of the C_i/C_a ratio in the leaf intercellular is a very common response during times of limited water supply because of reduction of flow through the closing stomates [82]. However, the opposite happened in *M. ciliaris* (Table 2). This can be explained by the fact that the photosynthetic carbon assimilation capacity decreased under water shortage to a higher degree (76%) than the leaf conductance (70%), which nicely illustrates the resultant photosynthetic shifts from stomatal to non-stomatal limitations. These results are similar to some earlier findings published by He et al. [83], where drought stress was also accompanied by increasing intercellular CO_2 concentrations of bamboo leaves (*D. minor var. amoenus).* In this context it was shown that photosynthesis is primarily affected during mild and moderate stress conditions by stomatal limitation, but under severe water deficiency, it is affected by non-stomatal limitation in chloroplast CO_2 fixation abilities, rather than CO_2 diffusion resistance [84–87].

It is well known that Bc improves plant performance in the form of higher organic matter production, an increased synthesis in growth-regulating substances and an improved plant functioning [77]. Bc amendment also supports at water shortage, increasing the activity of anti-oxidant enzymes and the maintenance of high leaf chlorophyll content [88,89]. A number of previously published reports [46,90,91] showed that Bc application can improve soil water availability in general and buffer the effect of reduced water supply on plant photosynthetic carbon assimilation capacity. This is in line with our findings that soil amendments with Bc boost chlorophyll content both under well-watered and insufficiently watered plants. In comparison to the results of the water shortage treatment (0% Bc WP_l, see above) and in agreement with the above-cited literature was the Bc-induced higher maintenance (2% Bc) of net photosynthesis (A_{net} only 40% less as WP_h, Table 2) accompanied by a proportionally higher chlorophyll content (Figure 4, [78,92,93]) and a less pronounced decrease in stomatal conductance (S_c, only 44% less as WP_h). In order to survive in dry arid zones, optimizing photosynthesis as well as stomatal conductance is essential for plant species with the aim of preserving net CO_2 assimilation and reduce evaporation [94]. Under these unfavorable conditions it seems to be beneficial that Bc application enhanced WUE in cases of water shortage (Table 2). The high WUE correlated with the maintenance of a high A_{net} and the reduction of non-stomatal limitation. By increasing WUE, oxidative stress is most likely reduced and, consequently, resistance against drought stress is increased (H_2O loss per net CO_2 uptake) [46]. This interpretation is in line with some previous studies in which soil amendment with Bc alleviated drought stress symptoms by significantly enhancing the water use efficiency, stomatal conductance, chlorophyll contents and photosynthesis of tomato, cowpea and okra leaves during water shortage [95,96].

A remarkable feature of the photosynthetic apparatus is its ability to adapt to changes in environmental conditions by sensing light quality and quantity, CO_2 levels, temperature and nutrient availability [97]. The water shortage and the resulting low A_{net} and low WUE led in *Medicago ciliaris* (0% Bc) to a reduced demand and the necessity of the regulation of light energy coming through the chloroplast electron transport chain. While light is essential for photosynthesis, it can also lead to light-induced damage when the absorbed light energy exceeds the capacity of the photosynthetic machinery. To avoid that, the excess photons and electrons need to be dissipated. This occurs through photoinhibition or a rapidly inducible non-photochemical quenching process Y(NPQ) in which the absorbed excess light energy is dissipated as heat [98,99]. Chlorophyll fluorescence is an important photosynthetic parameter that reflects the absorption and utilization of light energy from Photosystem II (PSII). However, *M. ciliaris* does not respond to water shortage as expected with higher Y(NPQ) (Table 2) but rather with a significant decrease in ETR ($p < 0.05$) together with an increase in dark-respiration (R_D) and decrease in light-respiration (R_L), leading finally to no significant change in the ETR/A_{gross} ratio. The reduction in ETR correlates in *Medicago ciliaris* with a reduction of the chlorophyll content, indicating that PSII had been damaged to varying degrees, photosynthetic organs had been altered, and the effects of excessive light energy could not be disposed through heat dissipation (Y(NPQ)) but instead through photochemistry by producing large amounts of reactive molecules, causing oxidative damage to photosynthetic organs [100] and the resultant photosynthetic shifts from stomatal to non-stomatal limitation. In the current study, we applied Bc treatment in order to increase productivity and escape ROS damage. Indeed, biochar application during water shortage led to significant higher chlorophyll concentrations, higher A_{net} and also lower R_D and R_L (Table 2), giving the overall picture of a moderate stress response.

4.3. Indicators of Oxidative Stress

The results of CO_2/H_2O gas exchange and PSII chemistry confirms the acceptance of a possible increasing of oxidative stress during water deficiency in soil without Bc amendment, resulting in malfunctioning and eventually the death of the affected cells [101]. In general, several photoprotective mechanisms exist, such as plastid antioxidant enzymes and molecules [98] and repair processes for damaged PSII [102] and lipid peroxidation [97]. In this study we used MDA, proline and H_2O_2 as biomarkers for oxidative stress (MDA, Figure 6) [103], non-enzymatic photo-protection (proline, Figure 6) [104] and redox regulation (H_2O_2, Figure 7) [105].

In agreement with our expectations, water shortage led to a significant increase in MDA content in *M. ciliaris*, indicating a substantial stress by reactive oxygen species, leading to lipid peroxidation, fatty acid saturation and consequently damage to the membranes [106,107]. The formation of MDA is actually the consequence of enzymatic breakdown in cells. *M. ciliaris* plants grown with Bc amendment had lower MDA content than non-treated ones. This effect can be explained by the coordinated activation of protective enzymes (see Figure 7) which leads to attenuate ROS production, hence oxidative stress [90,108]. Our results are similar to some earlier findings in which soil amendment with Bc decreased the MDA content of *Phragmites karka* and *Brassica olerecae* (Cabbage seedling) under drought stress conditions [109,110].

We got similar responses of both proline and MDA as to Bc amendment during water stress. Proline, produced under stressful conditions, can act as a free radical scavenger for photo-protection but also a compatible solute in osmotic adjustment [111]; a metal chelator; an activator of ROS detoxification pathways; a cell redox balancer; a cytosolic pH buffer; a source of energy; a source of nitrogen and carbon; a stabilizer of subcellular structures and membranes, including photosystem II [112]; and can act as a signaling molecule [104]. Water shortage in *M. ciliaris* leaves (0% Bc) led to a considerable increase in leaf proline accumulation, which shows the importance of proline as photoprotectant and osmoprotectant (see also [113,114]. In agreement with our findings, Yildirim [110] reported that Bc treatment lowered proline content in the plants. Our results suggest reduced osmotic and oxidative stress in Bc-treated plants.

The last used biomarker, H_2O_2, regulates plant growth, development and acclimatory and defense responses [115]. Moreover, among oxidative species, H_2O_2 is a very vigorous metabolite that deteriorate the structure of biological membranes during abiotic stresses [116]. The increased production of ROS (such as H_2O_2) in drought-stressed plants of *M. ciliaris* (Figure 7) is a common phenomenon taking place under stress conditions [117]. Plants use antioxidant defense (enzymatic or non-enzymatic) to deal with oxidative stress [90]. Plants employ diverse defensive adaptive mechanisms to survive under adverse cues such as the activation of a signaling pathway, expression of genes and accumulation of stress-related proteins [118] and enzymes. The latter effect may also be used to explain the increase in protein content in *Medicago ciliaris* plants subjected to water shortage (Figure 5).

4.4. Photoprotective Mechanisms: Enzymatic Oxidants

In the present investigation, water shortage boosted enzymatic and non-enzymatic antioxidant activity in *M. ciliaris* plants. Enzymes like superoxide dismutase (SOD), ascorbate peroxides (APX), guaiacol peroxidase (GPOX) and glutathione reductase (GR) are directly engaged in catalyzing ROS degradation reactions by directly scavenging ROS [119,103] and indirectly reducing membrane lipid peroxidation and alleviating the damage in PSII structure and function.

The first enzyme in the antioxidant pathway is SOD, which removes superoxide radical by catalyzing its dismutation to H_2O_2 and another oxidized to O_2 [120]. The increase in SOD activity observed in the leaves of *M. ciliaris* (Figure 7) as a function of the applied water stress levels might be correlated to the enhanced protection from damages, among them lipid peroxidation, associated with oxidative stress. In *M. ciliaris*, water shortage led to increased concentrations of APX, GPOX and GR (see Figure 7), suggesting the involvement of the Halliwell–Asada pathway, where APX reduces H_2O_2 to water and MDHA using ascorbic acid as substrate [121] at the expense of NADPH [26,122,123]. Khaleghi [103] reported that APX activity increased in drought-stressed *Maclura pomifera*, *Picea aspertata* and *Nicotiana tabacum*, respectively. The stimulation of APX activity might be correlated to the increased H_2O_2 generation by the observed enhanced SOD activity (both Figure 7). Wang [124] reported that peroxidase activity is closely related to PSII electron transport properties and PSI, but the activity of the latter could be inhibited with the increase in SOD activity.

SOD and APX showed maximum activity in the leaves of untreated plants and minimum activity in the leaves of Bc-treated plants (Figure 7). These results correlate with the Bc-mediated decrease in MDA and proline contents in the leaves and an improvement of photosynthetic parameters. It was previously shown that Bc application can buffer the impact of drought on photosynthetic apparatus by regulating the activity of protective enzymes and affecting electron transfer [86,90]. Our results are similar to some earlier findings in water shortage conditions where Bc application lowered enzymatic activity and lipid peroxidation and enhanced photosynthesis in *Pyrus ussuriensis* Maxim [90] *Brassica olerecae* [110] and *Phragmites karka* [109].

4.5. Photoprotective Mechanisms: Non-Enzymatic Antioxidants

Besides enzymatic antioxidants, there are non-enzymatic antioxidants, such as reduced (AsA) and oxidized (DHAsA) ascorbate, glutathione (GSH) and carotenoid, which can play a role in the antioxidant system in two ways, either directly interacting with ROS or functioning as substrates in enzyme-catalyzed ROS-degrading reactions [101,119,125]). Ascorbate (AsA, Vitamin C) is one of the universal non-enzymatic antioxidants, as it has the ability to donate a hydrogen atom and form a relatively stable ascorbyl-free radical. It protects plants against oxidative damage by environmental stresses, such as drought [34,126]. It participates in diverse redox and ROS neutralization reactions in the chloroplast and can be a facultative electron donor for the photosynthetic electron transport chain [127]. DHAR is responsible for regenerating AsA from the oxidized state and regulates the cellular AsA redox state, which is crucial in the response to abiotic stresses. Ascorbic acid (AsA) and its oxidized form dehydroascorbate (DHAsA) play a key role in redox state-based signaling mechanisms by the detoxification of ROS and its products, as well as the transmission of redox signals [128]. To prevent levels exceeding the anti-oxidative capacity of cells, ROS formation has to be closely regulated.

The increased activity of APX at water shortage correlates with the increase of AsA and DHAsA and can be explained by the high demand and capacity to eliminate H_2O_2 in the leaves of *M. ciliaris* (s.a.). An increased AsA–GSH cycle enables chloroplast to prevent photoinhibition by maintaining the NADP+/NADPH ratio so that ETR is least affected [129].

Water shortage causes a significant increase in DHAsA content (Figure 8). Several plant studies revealed that the upregulation of the AsA–GSH pathway enzymes and the enhancement of the DHAsA and GSSG levels gave plants better tolerance to abiotic stresses by reducing the ROS [130]. DHAsA is supposed to be involved in zeaxanthin biosynthesis by dissipating excess light energy in the thylakoid membranes of chloroplast and preventing oxidative stress by maintaining the activity of antioxidant enzymes. In accordance with our expectations, water shortage also led to a significant increase in AsA (reduced form of ascorbate) content and the AsA/DHAsA ratio. Alterations in the AsA/DHA ratio is involved in stress sensing, and redox homeostasis is one of the most important factors for protecting cells from ROS toxicity [131]. The leaf apoplast redox status specifically modulates plant growth and their response to hormones, antioxidant enzyme activities, expression patterns of catalase, glycolate oxidase and some other genes, and MAPK activity and the regulation of transcripts associated with calcium channels [132]. For instance, the AsA/DHAsA ratio is a prominent modulator of the enzymes responsible for carbon assimilation under stress conditions [28] and usually calculated to evaluate AsA availability and, thus, used as an indicator of oxidative stress and initiators of the plant defense system [28]. Several authors mentioned that the increase in the AsA/DHAsA ratio above a distinct limit lead to an enhanced production of abscisic acid in plants [133] and could cause, in accordance with the available data shown in this paper, the closing of stomata, the reduction of CO_2 fixation, cell expansion and plant growth (see [134]). Moreover, the increase of the AsA/DHAsA ratio at 0% Bc and water shortage up to the highest level of all four treatments deepens the impression that the increase in SOD, APX, GPOX and GR activities were not high enough to hinder extensive ROS damage in *M. ciliaris* leaves.

However, soil amendment with biochar (2% Bc) during water shortage led to a significant reduction of the AsA/DHAsA ratio down to the level of well-watered control plants (0% Bc). This Bc-mediated effect was mainly reached by the significant higher increase in both the total ascorbate AsA_{tot} (oxidized and reduced) and DHAsA content, which optimize *M. ciliaris* chances in buffering oxidative stress by directly scavenging ROS [66,131].

5. Summary and Conclusions

The results presented in this study support our assumption that Bc application enhances the performance of *M. ciliaris* during times of limited water supply. Indeed, biochar application during water shortage led to a more moderate exposure to water deficiency and in this way supported an improved and interactive plant adjustment. It led to a reduced impact of limited water supply on growth and water relations and included a balanced regulation of photosynthesis and the buffering of reactive oxygen species with photoprotective mechanisms. In latter case, the results impressively confirmed the agreement with the enzymatic ROS defense by the Halliwell–Asada pathway enzymes (Figure 7), corresponding to our previous statement that Bc application significantly reduces the negative impact of reactive oxygen species on *M. ciliaris* plants. This aspect is evidenced by the fact that the Bc-mediated moderate change of the AsA/DHAsA ratio also lead to only the minor stimulation of abscisic acid production [133]. This interpretation is also confirmed indirectly by the response of the CO_2/H_2O gas exchange parameters (Table 2).

We conclude that amendment through Bc may be a helpful approach to improve the performance of *Medicago ciliaris* during water shortage and to increase plant productivity in the arid land regions. This species seems to be suited to use a cash pasture plant in the development of agriculture on dry wasteland in a future world of water shortage. However, further field trials should be carried out under corresponding conditions as an intermediate step before agricultural use to verify the presented results on an agro-ecosystem level.

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Abbreviations

Biochar (Bc), fresh weight (FW), dry weight (DW), water-holding capacity (WHC), net CO_2 assimilation rate (Anet), photosynthetic efficiency (Vc), stomatal conductance (Sc), ratio of intercellular and atmospheric CO_2 concentration (Ci/Ca ratio), ratio of net CO_2 assimilation rate and transpiration (A/E), photorespiration (RL), dark respiration (RD), electron transport rate (ETR), gross CO_2 assimilation (Agross), quantum yield of regulated non-photochemical energy loss in PS II (Y(NPQ)), electron (e-), photosystem 2 (PSII), non-photochemical quenching (NPQ), cycling electron flow (CRF), reactive oxygen species (ROS), ascorbate (reduced form) (AsA), dehydroascorbate (oxidized form) (DHAsA), glutathion (GSH), sensitivity index (SI), hydrogen peroxide (H_2O_2), malondialdehyde (MDA), superoxide dismutase (SOD), ascorbate peroxidase (APX), guaiacol peroxidase (GPOX), glutathione reductase (GR).

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Seed priming with ascorbic acid improves response of *Medicago polymorpha* L. seedlings to osmotic stress induced by NaCl and PEG solutions

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ABSTRACT

This study assessed to which extent seed priming with ascorbic acid (0.2 mM) may improve *Medicago polymorpha* L. performance under water shortage induced by irrigation with either 50 mM NaCl or 100 g/L polyethylene glycol (PEG). Parameters related to plant morphology, CO_2/H_2O leaf gas exchanges, osmotic adjustment, pigment content, and proline accumulation were specifically determined. Both NaCl and PEG solutions induced osmotic stress and reduced plant biomass (−30% and −40%, respectively), number of leaves and ramifications, stem length, net CO_2 assimilation (−31% and 63%, respectively), and leaf water content. However, both treatments and especially PEG led to increased root/shoot ratios and leaf proline content. Interestingly, seed priming using ascorbic acid improved CO_2/H_2O gas exchange and plant biomass production (+66%, +100%, and +92% in control, NaCl-, and PEG-treated plants, respectively). It also improved the water relations as reflected by the decrease of leaf osmotic potential and higher leaf proline accumulation (+67% and +120% in PEG- and NaCl-treated plants, respectively) and water content (especially under PEG treatment). Besides, seed priming with ascorbic acid increased leaf carotenoid and chlorophyll contents (+65 and +45%, respectively, for chlorophyll *a* and chlorophyll *b*), thereby contributing to the better photosynthetic activity, and hence plant performance under salinity. We conclude that seed priming with ascorbic acid is an easy, cost-effective and promising approach to mitigate the impact of osmotic stresses like drought and salinity, by especially improving plant water relations and photosynthetic activity.

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Introduction

Drought and salinity are among the major factors limiting crop production in the arid and semi-arid areas. Not only they strongly reduce crop productivity of vegetable crops

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but also affect food security. Intensity and frequency of these two global issues are expected to be higher in the future because of the global climatic changes (Jiao et al. 2021). Plant responses to salinity and drought are often similar, multifactorial, and complex. Still, the physiological and biochemical backgrounds of plant resistance to both stresses is poorly understood.

Seed priming before germination can induce drought and salinity resistance and offset the damages associated with biotic and/or biotic stresses. It is one of the most promising, authentic, and affordable approaches to mitigate the deleterious effect of osmotic stress and is considered as an effective technique for achieving successful germination and for the improvement of the nutrient acquisition from nutrient-poor soils (Arun et al. 2022). During the early developmental stages, seed priming improves the germination rate and uniformity but also the later behavior of seedlings in terms of plant productivity and resistance to drought, salinity, low temperature, and chilling (Arun et al. 2022; Ben Youssef et al. 2021; Shah et al. 2019; Peyvast et al. 2010). Balmer et al. (2015) reported that plants grown from primed seeds react more efficiently and rapidly to a stress. It is thought that exposure of plants to a primary constraint triggers a set of temporary metabolic adaptations inducing a stress memory that allows plants to adapt more efficiently to subsequent episodes of stress (Siva Devika et al. 2021). However, the underlying physiological and biochemical background of this process is still poorly understood.

Ascorbic acid (AsA) is one of the important metabolites involved in cell division and osmotic adjustment (De-Gara et al. 2003). AsA application *via* seed priming may thus be helpful in improving the stand establishment and resistance to water shortage and salinity in plants (Farooq et al. 2013; Baig et al. 2021). Priming with this compound lowers the impact of salt by changing protein abundance, destination, and storage (Ali et al. 2019). Over-production of reactive oxygen species (ROS) is one of the key responses of plants to environmental stresses (Farooq et al. 2013). Ascorbic acid is one of the most important antioxidants at cellular processes such as cell division and expansion, and at metabolism activity in germination stage (Arrigoni et al. 1992), cell detoxification and protecting from ROS and preventing cell death (Conklin and Barth 2004).

Using different seed priming approaches, including hydropriming, low temperature, scarification, urea, KNO_3, KCl, NaCl, and ascorbic acid, Sadeghi and Robati (2015) found that ascorbic acid priming is the most effective tool to trigger water stress tolerance. Similar results were reported by Qamar et al. (2021) who revealed that seed priming with ascorbic acid and salicylic acid proved to be effective in enhancing growth characteristics and yield attributes of *Praecitrullus fistulosus* (Stocks) Pangalo.

Medicago polymorpha L. is an important local legume due to its fodder value. Indeed, it is considered as a potential species for pasture enhancement in the Mediterranean regions (Sheaffer et al. 2002). *M. polymorpha* L. is also used in traditional Chinese medicine for treating indigestion, urinary tract calculi, jaundice and intestinal inflammation (Guo et al. 2020). However, *M. polymoroha* L. is particularly salt-sensitive. According to Ibrar and Hussain (2003), the germination of this plant is completely inhibited at 15 dS/m (175 mM NaCl), whereas at the vegetative stage, parameters including plant height, leaf and branching number, and shoot dry weight (DW) are severely inhibited at 5 dS/m (50 mM NaCl). Besides, Cherifi et al. (2016) showed that reduction of growth in four populations of *M. polymorpha* L. ranged between 40% and 60% under 50 mM NaCl in plants cultivated in sand filled pots. Based on these data, we chose 50 mM NaCl concentration for our study.

The present study was conducted to evaluate the potential of ascorbic acid (0.2 mM) in improving the drought (polyethylene glycol [PEG]-induced) and NaCl resistance of *M. polymorpha* L. It was hypothesized that seed priming with ascorbic acid might improve key traits (osmotic adjustment, morphological parameters, water relations, pigment content, photosynthetic activity, and antioxidant potential) responsible for plant productivity. Our strategic aim is to encourage farmers to use seed priming technique as an easy and affordable approach that can improve *M. polymorpha* L. performance under changing environments.

Material and methods

Healthy and uniform seeds of *M. polymorpha* L. (Mateur origin, north Tunisia) were used for the experiment. The seeds were disinfected with 10% (w/v) sodium hypochlorite for 5 min and then washed three times with distilled water. For priming, seeds were soaked during 12 h in 0.2 mM (35 mg/L) ascorbic acid solution. Ascorbic acid priming protocol is simple and does not require any expensive chemicals and sophisticated equipment. Seeds were primed in the ascorbic acid solution or transferred to distilled water (seed weight to solution volume 1:5) at room temperature in the dark. Subsequently, seeds were rinsed thoroughly with distilled water, spread on thin layer of filter paper and dried to their original weight. Finally, primed (P) as well as non-primed (NP) (control) seeds were germinated in Petri dishes. After three days, germinated seeds (germination rate is about 85%) were transferred to plastic pots (3.5 L) for hydroponic culture and subjected to osmotic stress by incubation in solutions with an osmotic potential of −0.3 MPa. The low osmotic potential was reached by either PEG (100 g/L PEG 6000) or alternatively by NaCl solutions (50 mM, having electrical conductivity of 5 dS/m). Six treatments were used, such as primed seeds incubated with water, primed seeds incubated with NaCl solution, primed seeds incubated with PEG solution, NP seeds incubated with water, NP seeds incubated with NaCl solution, and NP seeds incubated with PEG solution. Overall, 300 plants were used in this experiment. For each treatment we had 50 plants (5 pots, every pot contained 10 plants). Pots are rectangular and its length, width, and height are 24 cm, 18 cm, and 8.1 cm, respectively. Eight homogenous plants served for each physiological test. The culture solution in the pots was permanently aerated to prevent anoxia in the roots of the plants. After 30 days of treatment the final harvest was carried out.

The culture experiment was carried out in the greenhouse of the Center of Biotechnology in Borj Cedria at 30 ± 5 °C/16 ± 2 °C (day/night) temperature, 60 ± 5%/90 ± 5% (day/night) relative humidity, and 14 h light/10 h dark regime.

Growth parameters and water relations

Plants were harvested and divided into roots, leaves, and stems, and their respective fresh weights (FW) were determined. DW was obtained after oven drying at 60 °C in an oven until constant weight was reached. Leaf water content (LWC) was determined as LWC ($\mathrm{mL\ g^{-1}\ DW}$) = (FW − DW)/DW.

With respect to plant morphology, leaf number, root and shoot length, and number of ramifications and internodes were determined. In addition, fresh leaf, stem, and root samples from each plant were stored at −80 °C. The osmotic potential was determined from third leaf from the top of eight plants per treatment with a vapor pressure osmometer (Wescor 5500). For this purpose, fresh leaves were cut into small segments, placed in Eppendorf tubes and immediately frozen in liquid nitrogen (Martínez-Ballesta, Martínez, and Carvajal 2004). Finally, leaves were ground with a mortar and sap was collected after centrifugation for analyses.

Leaf gas exchange determination

Measurements of net CO_2 assimilation (A), transpiration rate (E), and stomatal conductance (gs) were determined on fully expanded leaves of each plant using a LCi portable photosynthesis system (ADC BioScientific Inc., Hoddesdon, UK). Data were automatically collected every minute after the stabilization of photosynthesis rate.

Pigment analysis

Leaf chlorophyll and carotenoid concentrations were determined spectrophotometrically according to Lichtenthaler and Wellburn (1983). Briefly, 5 mL of 80% acetone was added to 120 milligrams of fresh leaf samples. The extraction time was 72 h at 4 °C in the dark at 4 °C. The extract was centrifuged for 5 min at 14,000 g. The absorbance of supernatant was recorded at 663, 646, and 470 nm for chlorophyll *a*, chlorophyll *b*, and total carotenoids, respectively.

Proline determination

Proline was determined following the ninhydrin method (Bates, Waldren, and Teare 1973), using proline as a standard. Leaf samples (100 mg FW) were homogenized in 1.5 mL of 3% (w/v) aqueous sulfosalicylic acid and centrifuged for 30 min at 14,000 g. To the supernatant, 2 mL of acid ninhydrin and 2 mL of glacial acetic acid were added and the mixture was boiled for 1 h. After extraction with toluene, the free proline was quantified at 520 nm from the organic phase.

Statistical analysis

The program AV1W MSUSTAT was used for statistical analysis with orthogonal contrasts and mean comparison procedures to detect differences between treatments. Mean comparisons were carried out using the multiple range tests with Fisher's least significant difference (L.S.D.) ($p < 0.05$).

Results

Growth and morphological parameters

Biomass production

The total plant DW as well as leaf and stem biomass were significantly reduced by PEG or NaCl salinity (−30 and −40%, respectively) (Figures 1 and 2). No significant differences were observed regarding root development between plants subjected to NaCl or to PEG solution. Seed priming with ascorbic acid improved whole plant biomass production by 66%, 100%, and 98% in the control, NaCl-, and PEG-treated plants, respectively. A similar trend was observed for growth of the different plant organs (leaves, stems, and roots) (Figure 2). However, the increase in root DW of primed plants under control, NaCl or PEG solution treatments was less pronounced when compared to leaves. Indeed, leaves of control plants produced three-fold more biomass than roots (Figure 2). Root/shoot ratio was significantly higher in plants challenged with osmotic stress, as compared to the control, but the highest values were observed in plants cultivated in the presence of PEG. Independently of osmotic stress treatment, seed priming with ascorbic acid led to a significant decrease in root shoot ratio (Figure 3(a)).

Leaf number

Osmotic stress treatments led to a significant reduction of leaf number (Figure 3(b)) (PEG −40%, NaCl −26%). Seed priming with ascorbic acid led to a significant increase of the number of leaves of plants in either NaCl or PEG solution treatment and reached similar values as control plants.

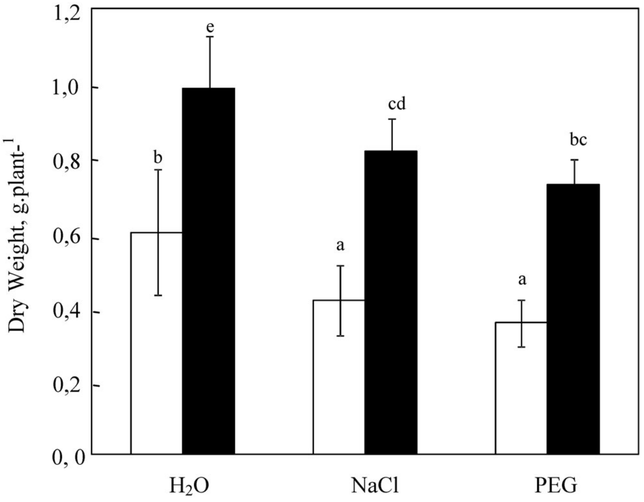

Figure 1. Whole plant biomass production of *Medicago polymorpha* seedlings derived from non-primed (*open columns*) or 0.2 mM ascorbic acid primed (closed columns) seeds and subjected to three incubation treatments during one month, such as Control, NaCl 3 g/L, and PEG 100 g/L. Values are the means of eight replicates, vertical bars are SEs. Values sharing a common letter are not significantly different at $p < 0.05$.

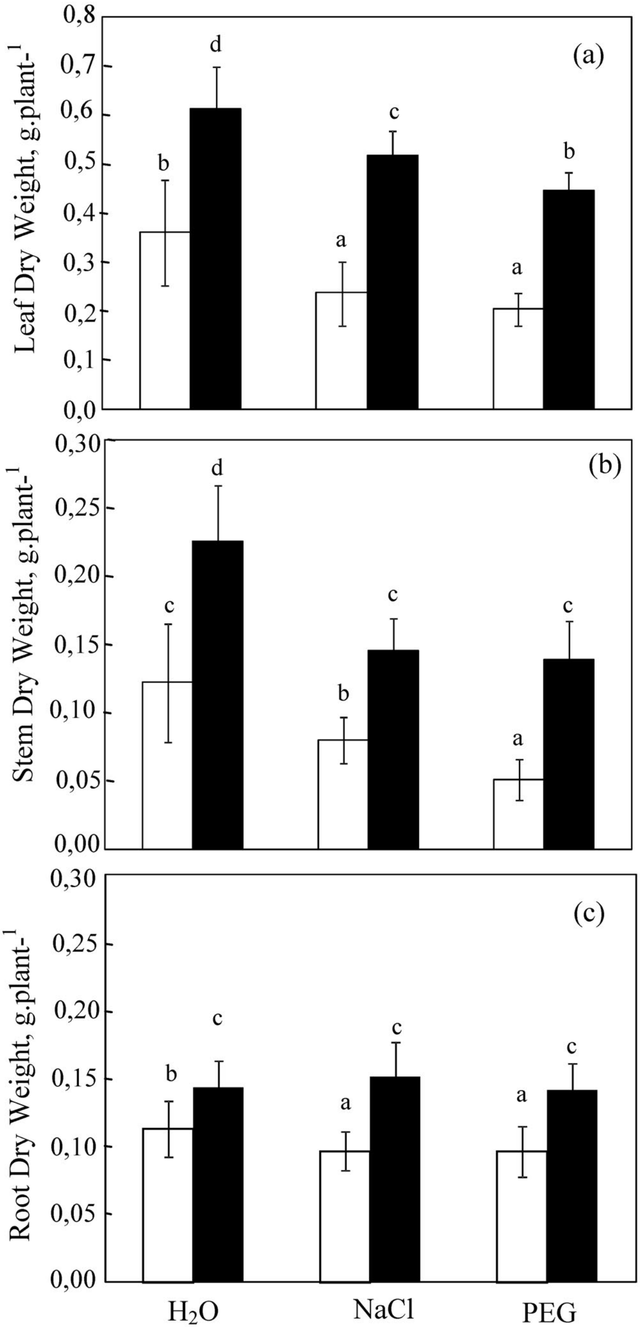

Figure 2. Leaf (a), shoot (b), and root (c) dry weights of *Medicago polymorpha* seedlings derived from non-primed (*open columns*) or 0.2 mM ascorbic acid primed (closed columns) seeds and subjected to three incubation treatments during one month, such as Control, NaCl 3 g/L, and PEG 100 g/L. Values are the means of eight replicates, vertical bars are SEs. Values sharing a common letter are not significantly different at $p < 0.05$.

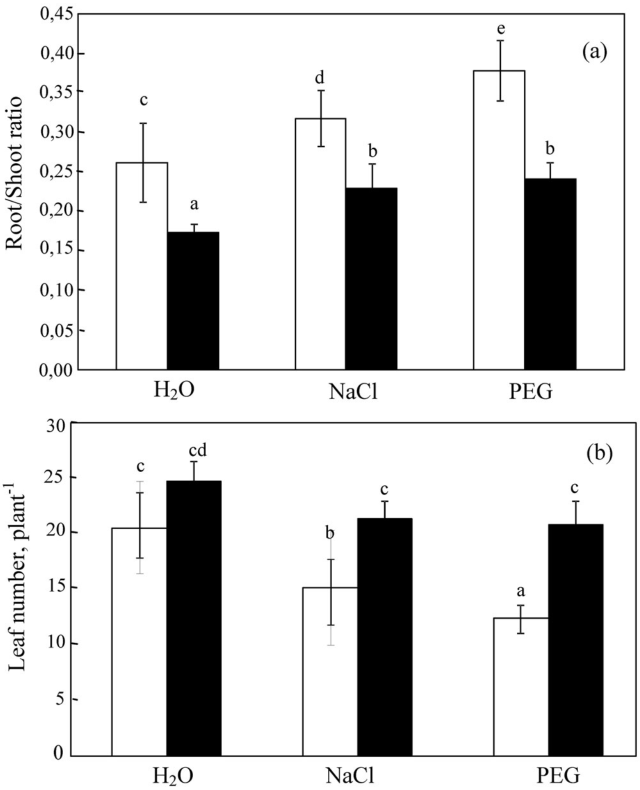

Figure 3. (a) Root/shoot dry weight ratio and (b) leaf number of *Medicago polymorpha* seedlings derived from non-primed (*open columns*) or 0.2 mM ascorbic acid primed (closed columns) seeds and subjected to three incubation treatments during one month, such as Control, NaCl 3 g/L, and PEG 100 g/L. Values are the means of eight replicates, vertical bars are SEs. Values sharing a common letter are not significantly different at $p < 0.05$.

Numbers of ramifications and internodes, root, and stem length

Incubation in NaCl solution led to a marked reduction of the number of ramifications and internodes (−28% and −26%, respectively) (Figure 4(a,b)), whereas ascorbic acid priming significantly mitigated this negative effect of both parameters under saline conditions. Control and PEG treatments showed no significant impact on both parameters, however, stem length was significantly reduced by both osmotic stress treatments (Figure 4(c,d)) (−52% and −44%, respectively, for NaCl and PEG). Ascorbic acid seed priming caused increase in stem length only in NaCl solution treatment. Root length was neither affected by osmotic stress treatments nor by priming with ascorbic acid (Figure 4(c,d)).

Photosynthetic parameters

The leaf net CO_2 assimilation reached 19 μmolm^{-2}s^{-1} under control conditions (Figure 5). Osmotic stress treatments led to a significant reduction of this parameter (−31% and −63%, respectively, for NaCl and PEG solutions). Interestingly, irrespective of treatment, seed priming with ascorbic acid led to a significant increase of net CO_2

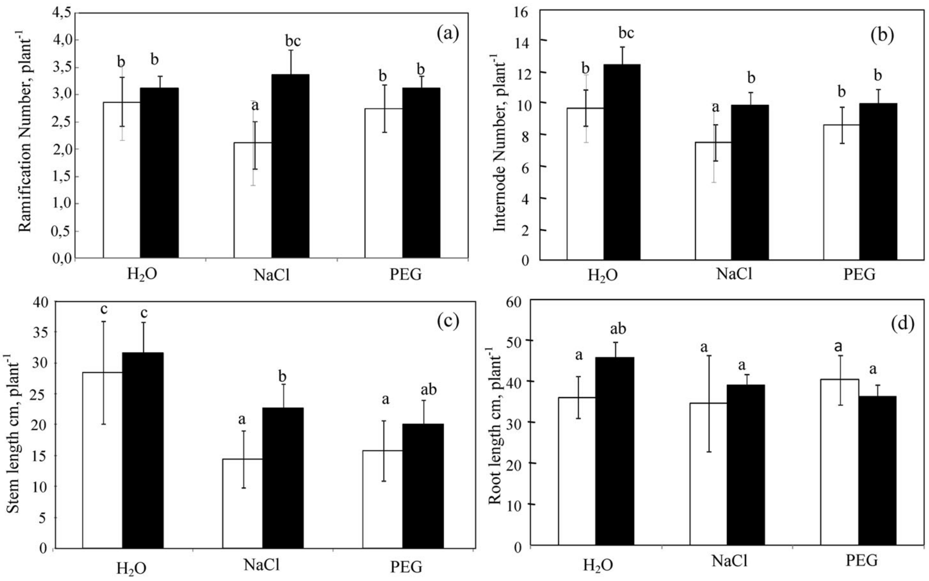

Figure 4. Number of ramifications (a), internodes (b), shoot (c), and root (d) length of *Medicago polymorpha* seedlings derived from non-primed (*open columns*) or 0.2 mM ascorbic acid primed (closed columns) seeds and subjected to three incubation treatments during one month, such as Control, NaCl 3 g/L, and PEG 100 g/L. Values are the means of eight replicates, vertical bars are SEs. Values sharing a common letter are not significantly different at $p < 0.05$.

assimilation. This was also true for transpiration (Figure 5(b)) in the three treatments. The stomatal conductance was only reduced in primed or NP seedlings exposed to PEG treatment, whereas seed priming with ascorbic acid had no effect on stomatal conductance under both control or osmotic stress conditions.

Water relations

LWC was comparable in both control and NaCl solution treatments (about 5 mL g^{-1} DW) (Figure 6(a)), but PEG treatment led to a significant reduction of LWC (−53%). This effect was buffered by priming with ascorbic acid. Both osmotic stress treatments caused a significant decrease of the leaf osmotic potential, but this effect was more pronounced when plants were incubated in PEG solution compared to NaCl solution. Priming with ascorbic acid further reduced osmotic potential under both osmotic stress treatments (Figure 6(b)).

Pigment concentration

NaCl solution caused a strong reduction of the chlorophyll *a*, chlorophyll *b*, and carotenoid contents (−53%, −46%, and −50%, respectively) (Figures 7 and 8(a)). Priming with ascorbic acid significantly counteracted this effect for the three pigments. PEG solution had no effect on photosynthetic pigments in both primed and unprimed seedlings.

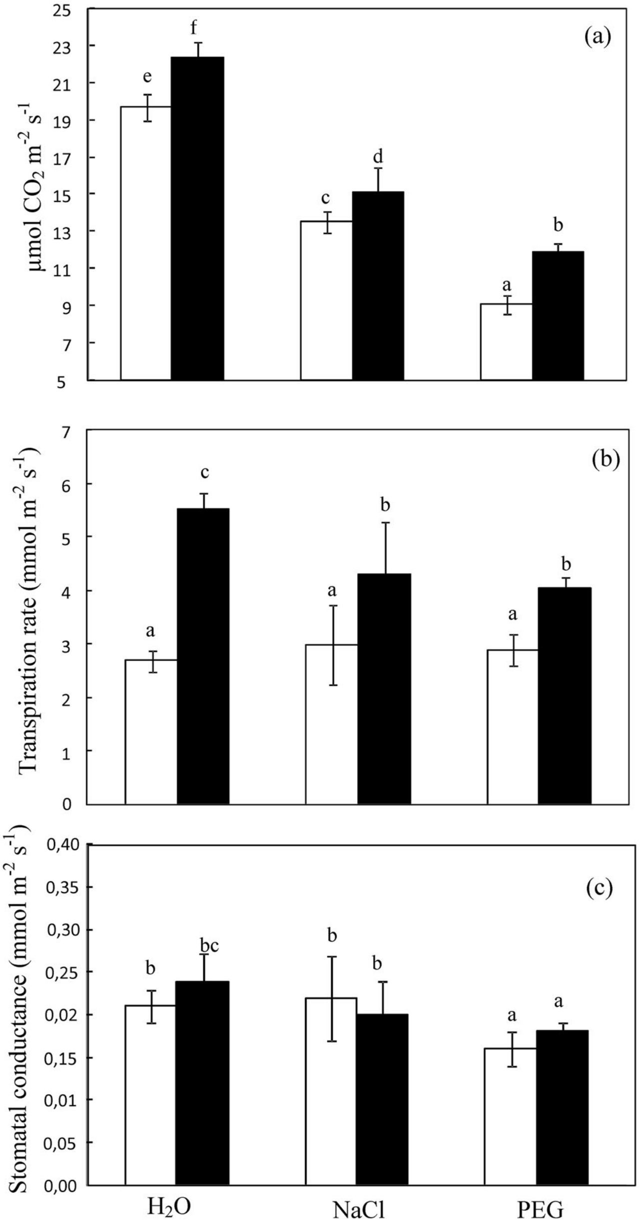

Figure 5. Net CO_2 assimilation (a), transpiration (b), and stomatal conductance (c) of *Medicago polymorpha* seedlings derived from non-primed (*open columns*) or 0.2 mM ascorbic acid primed (closed columns) seeds and subjected to three incubation treatments during one month, such as Control, NaCl 3 g/L, and PEG 100 g/L. Values are the means of eight replicates, vertical bars are SEs. Values sharing a common letter are not significantly different at $p < 0.05$.

Proline accumulation

Leaf proline content was about 0.7 mmol g^{-1} FW (Figure 8(b)) under control conditions. Both osmotic stress treatments led to a significant increase of proline content.

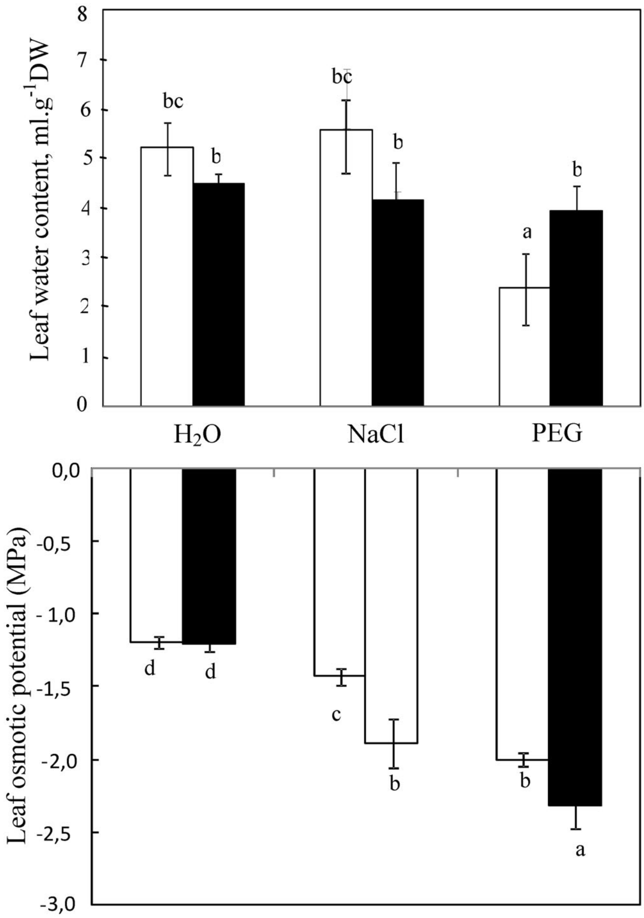

Figure 6. Water content (upper graph) and osmotic potential (lower graph) of *Medicago polymorpha* seedlings derived from non-primed (*open columns*) or 0.2 mM ascorbic acid primed (closed columns) seeds and subjected to three incubation treatments during one month, such as Control, NaCl 3 g/L, and PEG 100 g/L. Values are the means of eight replicates, vertical bars are SEs. Values sharing a common letter are not significantly different at $p < 0.05$.

Seed priming with ascorbic acid led to a further significant increase in leaf proline content.

Discussion

The present study investigated the impact of priming on growth, physiological, and biochemical *of M. polymorpha* L. plants growing in osmotic stress treatments caused by incubation in 50 mM NaCl or 100 g/L PEG solutions. We hypothesized that priming with ascorbic acid can lead to a pre-adjustment to osmotic stress characterized by enhanced plant productivity, morphological parameters, photosynthetic activity, water relations, pigment content, and proline accumulation.

Our results showed a reduction in biomass in *M. polymorpha* by 40% and 30% when plants were subjected to osmotic stress induced by PEG and by NaCl solutions. In

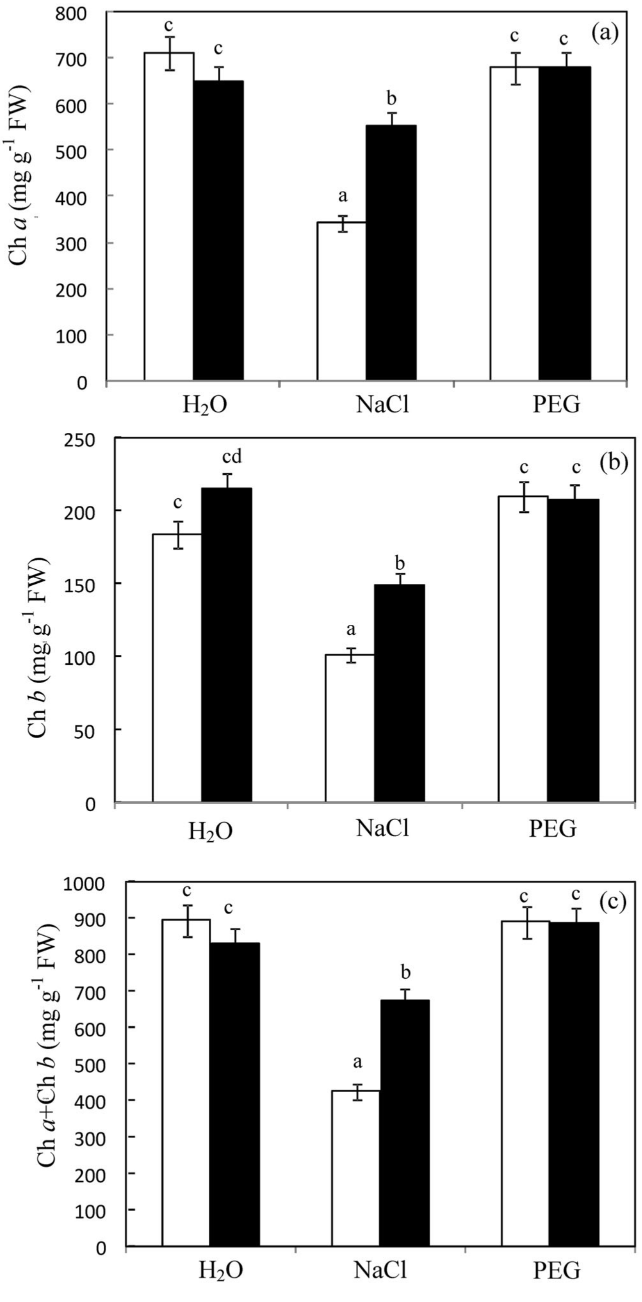

Figure 7. Chlorophyll *a* (a), chlorophyll *b* (b), and chlorophyll total (c) of *Medicago polymorpha* seedlings derived from non-primed (*open columns*) or 0.2 mM ascorbic acid primed (closed columns) seeds and subjected to three incubation treatments during one month, such as Control, NaCl 3 g/L, and PEG 100 g/L. Values are the means of eight replicates, vertical bars are SEs. Values sharing a common letter are not significantly different at $p < 0.05$.

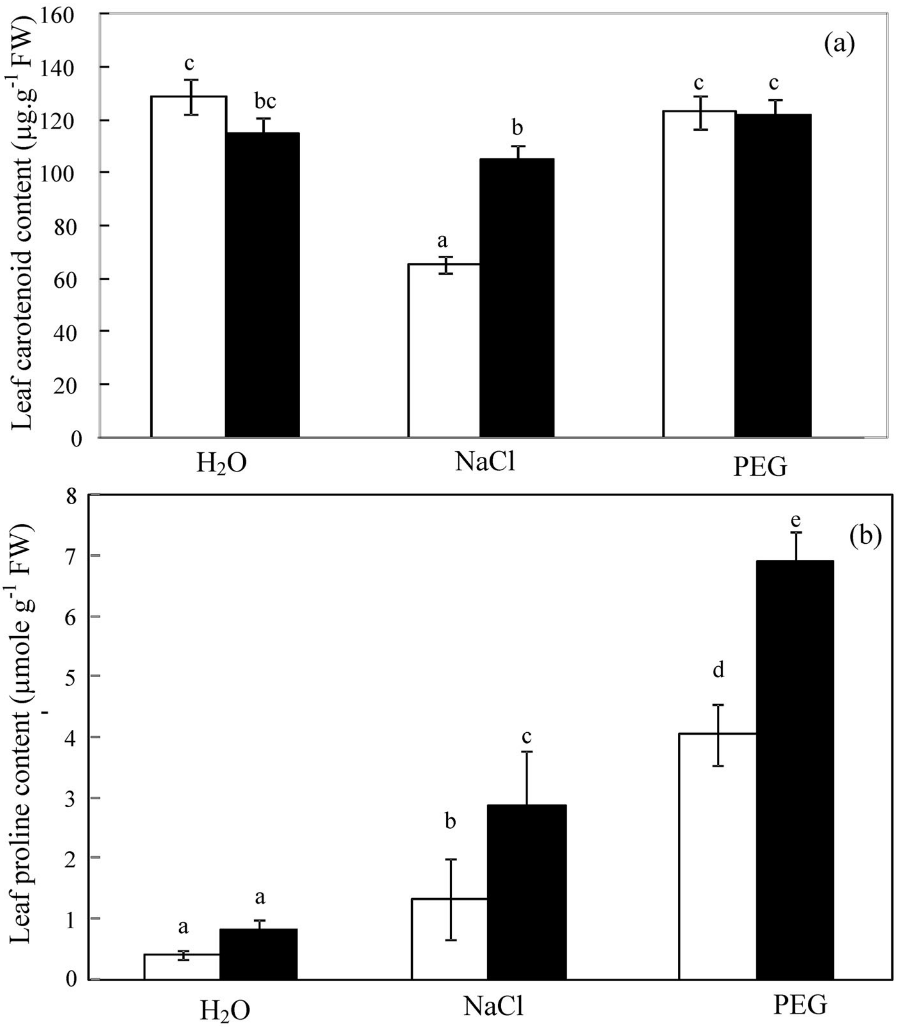

Figure 8. (a) Leaf carotenoid content and (b) leaf proline content of *Medicago polymorpha* seedlings derived from non-primed (*open columns*) or 0.2 mM ascorbic acid primed (closed columns) seeds and subjected to three incubation treatments during one month, such as Control, NaCl 3 g/L, and PEG 100 g/L. Values are the means of eight replicates, vertical bars are SEs. Values sharing a common letter are not significantly different at $p < 0.05$.

agreement with our findings, Slama et al. (2022) reported that the plant biomass reduction of *Medicago* can range between 12 and 73% under water deficiency conditions. Cherifi et al. (2016) also showed that reduction of growth in four populations of *M. polymorpha* L. can range between 40 and 60% at 50 mM NaCl salinity level.

Whereas drought is a pure reduction of the water potential, salinity is characterized by an additional ionic impact (Li et al. 2022). Hence, NaCl salinity differs from drought by the further constrains of Na and Cl toxicity and ion homeostasis (Ma, Celeste, and Freitas 2020). In our case, osmotic stress, whether induced by NaCl or by PEG solution is responsible for several similar and deleterious effects on plant growth and development, which were attenuated to a large extent by seed priming with ascorbic acid.

Indeed, the latter improved plant biomass production by 66%, 100%, and 98% in control, NaCl-, and PEG-treated plants, respectively. This confirms previous studies carried out on rice, wheat, chickpea, sunflower, cotton, and sesame (Nakao et al. 2018; Bhusal and Thakur 2020; Sharma, Pandey, and Verma 2021; Catiempo et al. 2021; Zhao et al. 2020).

Both osmotic stress treatments applied in the present study (Na and PEG solutions) led to an increase of the root/shoot ratio, independent of seed priming. Recently, Slama et al. (2022) documented that the increase of root/shoot ratio is a reliable criterion of adjustment and resistance to drought. This parameter is high in the most tolerant species to drought like *Medicago sativa*, *Medicago truncatula*, and *Medicago laciniata*. Root/shoot ratio decreased following priming in control or osmotic stress treatments, due to the beneficial effects of priming on shoot biomass production when compared to roots. We interpret this result as an improved water influx capacity and stress defense (Bacher et al., 2022). Optimal quality and physiological activity of the roots is a necessary condition for optimal shoot development and also for subsequent development of the seeds with good quality (Bláha 2019). The root to shoot ratio is strongly associated with plant integrity during growth and development in each vegetation period and an important parameter for the production of *M. polymorpha* L.

Our data indicated that both osmotic stress treatments affected leaf net CO_2 assimilation, the most fundamental physiological process in plants. Water deficiency can induce photo-inhibition and oxidative stress and thus, alter the activities of enzymes involved in photosynthesis such as RuBisCO. Besides, drought and salinity affect different types of pigments, photosystems, and components of electron transport system (Scheibe and Beck 2011). Our results showed a decrease (about −70%) in chlorophyll *a* and *b* in plants exposed to osmotic stress induced by NaCl solution.

Seed priming with ascorbic acid led to an improvement of CO_2 fixation in all three studied treatments. In plants subjected to salinity, this effect was associated with an increase of the contents of photosynthetic pigments (chlorophyll *a*, chlorophyll *b*, and carotenoids). Similarly, Dai et al. (2017) found that in soybean, seed priming with $CaCl_2$ and $ZnSO_4$ increased photosynthesis due to higher accumulation of photosynthetic pigments. Ramani et al. (2006) reported that carotenoids are involved in the adaptation of photosystem II to salinity. Carotenoids act as accessory pigments, harvesting light for photosynthesis, and as photoprotective agents against oxidative stress (Slama et al. 2017). Carotenoids are also key protective compounds against lipid membrane peroxidation (Johnson et al. 2007). Another study conducted in ZnO priming in wheat seed revealed that priming improves the photosynthetic efficiency through the improvement of light absorption efficiency, energy trapping by pigment and transport followed by lower production of ROS. It has been documented (Khan et al. 2019) that seed priming improves photosynthesis rate through the improvement of stomatal length, width, and number. However, in our study seed priming had no effect on stomatal conductance in osmotic stress treatments.

In *M. polymorpha* L., leaf number was significantly impacted by osmotic stress treatments, but this effect was alleviated by seed priming. In cowpea, water deficit was shown to affect not only the production of grain but also the whole process of growth of all organs of the plant including the size of individual leaves and leaf number (Blum

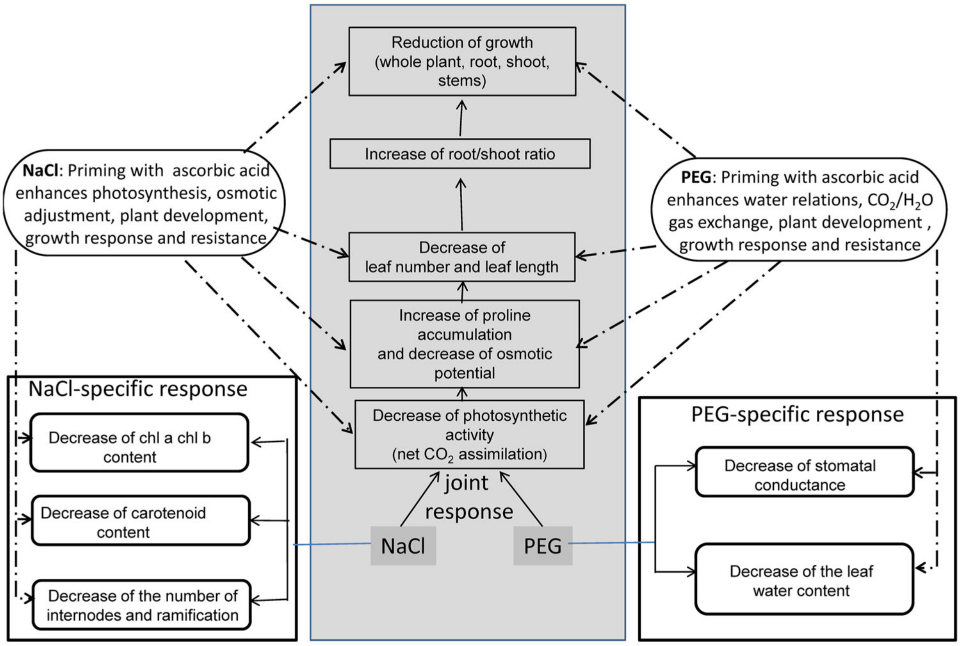

Figure 9. Osmotic stress and ascorbic acid priming effects on *Medicago polymorpha* seedlings.

2017). Arun et al. (2016) explained beneficial effects of seed priming on leaf number and area by the advantage of rapid seedling emergence. The authors explained this effect by changes of cell number and division, cell wall plasticity and cell membrane permeability, cell enlargement, and the rapid cell wall elongation (Salisbury and Ross 1992). Our results showed that besides changes in the leaf number, several morphological parameters (such as number of leaf internodes, ramifications, and stem length) were affected by both osmotic stress treatments and that this effect could be reduced by priming. These findings are in agreement with previous studies on chickpea, rice, and pepper crop grown in saline conditions (Kaur et al. 2017; Zheng et al. 2016; Aloui, Mohamed Aymen, and Chérif 2017).

Tissue hydration is an important process to assess osmotic stress resistance in plants. In our study, seed priming with ascorbic acid improved the water status of plants treated with PEG solution through the increase of the LWC and the simultaneous decrease of the osmotic potential. Seed priming in wheat also positively affected water status leading to a significant yield enhancement (Meena et al. 2014). Seed priming-mediated drought mitigation occurs by cytoskeletal restructuring, as well as aquaporin formation (Ge et al. 2014). Our results showed that seed priming increased root biomass production in *M. polymorpha* L. and that this behavior may have supported the increase of water uptake capacity.

To counteract the negative effects of unfavorable environmental conditions, plants also evolved an osmotic adjustment and oxidative stress defense mechanism that implies increased synthesis of proline, secondary metabolites such as carotenoids and xantophylls and carbohydrates (Serraj and Sinclair 2002). These compounds are involved in maintaining the structure and the integrity of cell wall (Kaur et al. 2017). Serraj and

Sinclair 2002 reported that proline is one of the most common osmolytes, which helps in alleviating the negative effect of drought on plants. Hossain et al. (2014) showed that proline, when produced under stressful conditions, can act as a compatible solute in osmotic adjustment, a free radical scavenger, a metal chelator, an activator of ROS detoxification pathways, a cell redox balancer, a cytosolic pH buffer, a source of energy, a source of nitrogen and carbon, a stabilizer of subcellular structures and membranes including photosystem II, and can act as a signaling molecule. Seed priming can protect plants and stimulate growth by accelerating the accumulation of osmolytes or non-enzymatic antioxidants such as proline under drought or saline conditions. In our case when cultivated in the presence of PEG, priming of seeds with ascorbic acid led to a drastic increase in leaf proline content, this behavior was associated with a protection of proteins, increasing antioxidative activity and an increase in leaf water relation in plants.

Conclusion

The results of the current study revealed that in *M. polymorpha* L., seed priming with ascorbic acid increased plant biomass production by 66%, 100%, and 98% in control, NaCl-, and PEG-treated plants, respectively. This positive effect was reached following the improved photosynthetic activities, water relations, and proline accumulation (Figure 9). In *M. polymorpha* L., seed priming with ascorbic acid constitutes a simple and cheap alternative that can be used by farmers in order to increase plant productivity and performance in saline, arid and semi-arid regions, and to achieve ecosystem stability.

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ORIGINAL PAPER

Combined effects of salinity and soil drying on growth, water, status, and photosynthesis in *Medicago ciliaris* and *Medicago polymorpha*

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Abstract

Drought stress along with soil salinity are the most common and frequently co-occurring abiotic stresses and threaten plant productivity, especially in arid and semi-arid regions of the world. The aims of this study were to investigate the interaction between soil drying (D) and salinity (S) in two forage species *Medicago ciliaris* TNC1.11 line and *M. polymorpha* TNP1.11 line. Plants were cultivated for 1 month in silty-sandy soil under two irrigation modes: 80% and 33% of field capacity (FC). The amount of the evapotranspirated water was replaced by a nutrient solution containing either 0 or 50 mM NaCl. Morphological parameters, photosynthesis, whole plant growth, water, and nutrient status (Na, K, Ca, Zn) were investigated. Under control or stressed conditions, *M. ciliaris* produced more biomass when compared to *M. polymorpha*. The whole-plant biomass production represented 237%, 230%, 175%, and 172%, respectively, in treatment C, S, D, and D + S in *M ciliaris* when compared to *M polymorpha*. The higher productivity correlates under all studied treatments in *M. ciliaris* with (i) a higher photosynthetic activity and water-use efficiency, (ii) more leaves per plant, higher number of ramifications and a longer stem, (iii) a high root water content under C, S, D + S treatments, and (iv) a high Zn accumulation in leaves. Salinity and drought applied individually negatively affect growth, photosynthesis, water, and potassium leaf content in both studied species. The effects of both constraints were not significantly additive. Salinity affects growth of the two studied species by the same manner (reduction by 64% as compared to control). The major advantage of *M. ciliaris* under dry or saline conditions seems to be the general high water-use efficiency and the corresponding positive impact on the ROS risk. Besides, *M ciliaris* showed a lower increase of Na^+ and less decrease of K^+ at salinity leading to an overall lower Na^+/K^+ ratio as *M. polymorpha*. This shows that the selectivity of *M. ciliaris* is higher as the one of *M. polymorpha*. When compared to *M. polymorpha*, TNC1.11 line of *M. ciliaris* produced more biomass under drought, salinity, and combined stresses; therefore, we recommend its multiplication and use by farmers as a forage candidate for the rehabilitation of soils in arid and semi-arid salinized regions.

Keywords Combined stresses · Growth · Photosynthesis · Rehabilitation · Salinity · Soil drying · Water-use efficiency

Introduction

Salinity is one of the major environmental factors repressing the agricultural production in the world after drought and threatens crop production in arid and semi-arid regions, where soil salt content is naturally high, and precipitation can be insufficient for leaching (Mubarak et al. 2021; Shaaban et al. 2023a, b).

The osmotic effect is characteristic at low level of salinity and in the initial phase of salt exposure, while the ionic effect occurs during long-term exposure and at high level of salinity (Arzani and Ashraf 2016). In this case, leaf Na^+ content can reach a toxic level and can generate the production

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of reactive oxygen species (ROS) that damage DNA, protein, chlorophyll, and membrane function. Moreover, high contents of Na^+ and Cl^- under salinity stress repress nutrient-ion activities as it disturbs the nutrient ratios by producing extreme ratios of Na^+/Ca^{2+} and Na^+/K^+ (Abd El-Mageed et al. 2022; Ali et al. 2024. Adaptation to stress is a very complex process in plants and needs the elimination, compartmentalization, and optional ion uptake to preserve an appropriate Na^+, K^+, and Cl^- balance (Lasheen et al. 2024; Saudy et al. 2023b).

Drought alone causes more annual losses in crop yield than all pathogens combined (Gupta et al. 2020). Drought's impact on agriculture depends on the degree and duration of the reduced precipitation and soil water gradients, as well as on plant species and developmental stages (Tardieu et al. 2018). The areas affected by drought are approximately 40% of the world's available lands. This constraint continues to be an important challenge to agricultural researchers. Drought induces morpho-physiological and biochemical changes in plants Ramadan et al. 2023a, b; Saudy et al. 2023a; Shaaban et al. 2023b). Drought often leads to stomatal closure that restricts the diffusion of CO_2 into the leaf or due to non-stomatal limitations, which leads to decrease of carbon assimilation and other processes of photosynthesis (Paul et al. 2019; Cui et al. 2022). During drought spells, plant can maintain physiological activity by (i) increasing root water uptake from the soil (higher root/shoot ratio), (ii) increasing water-use efficiency (closing stomata), (iii) adjusting osmotic processes within tissues, and, (iv) activating antioxidant and metabolite production (Gupta et al. 2020).

Under natural conditions, plants are often subjected to a combination of different types of abiotic stresses that contribute considerably to reducing plant growth and productivity (Shahin et al. 2023; Abou El-Enin et al. 2023; Doklega et al. 2024). Individually, salt and drought stress conditions have been the subject of intense research (Paul et al. 2019).

Studies dealing with the combination of drought and salt demonstrated that responses to combined stresses cannot be simply extrapolated from the responses of plants to these different stresses when applied individually (Zandalinas et al. 2018). Some salt-resistant plant species do not survive drought stress and vice versa (Makhlouf et al. 2022; El-Metwally et al. 2022). Salinity shares many similar features with drought stress, particularly in the early stress responses due to the osmotic effect (Munns 2002). However, the long-term plant responses to both stresses may behave differently, because sodium toxicity and nutrient imbalances can add to the initial stress due to its transport within plant tissues via the transpiration stream (Salem et al. 2021; Saudy et al. 2021; El-Bially et al. 2018). It has been reported that when combined, high salinity and soil drying may interfere with nutrient accumulation, thereby further contributing to growth inhibition. Indeed, it has been reported that when salt and drought are combined, a decrease in potassium phosphorus and calcium contents in shoot tissues was associated with an increase in sodium level (Brown et al. 2006).

However, the combination of both stresses may even open chances to resist above their single resistance level. When drought and salinity occur simultaneously, plants can employ crosstalk and exhibit strategic defense responses which could be distinctive from the response to either individual stress (Ma et al. 2020). Thus, the molecular and metabolic responses to a combination of stresses are unique and cannot be extrapolated from plant response to the individual stress (Mittler and Blumwald 2010; Mittler 2006).

Moreover, it is imperative to keep a suited water potential and appropriate nutrient ratios in saline conditions because a low soil moisture in saline conditions would further reduce water availability, accentuate salt toxicity, and suppress root growth, which eventually results in restricting water absorption from the subsoil and alters plants ability for survival (Abd-Elrahman et al. 2022; Salem et al. 2022). Therefore, experiments on the combination of these stresses have a considerable practical and ecological significance for ensuring sustainability of plants performance to abiotic stresses (Goharrizi et al. 2020).

Besides, it is very important to consider the selection and the use of suited salt and drought-resistant species for soil revegetation and preservation purposes (Morales et al. 1998). Morphological and physiological changes of plants under stress conditions such as plant biomass, leaf area, and plant height, in combination with photosynthetic activity measurements, can provide valuable information on the extent and mechanisms of stress induced crop yield loss, which can be utilized in the selection of resistant lines or species (Badri et al. 2016a).

Physiological and biochemical characteristics play a crucial role in primary screening of resistant genotypes and can be determined at initial stages of plant development (Goharrizi et al. 2020). Besides El-Metwally et al. 2021 suggested that producing a high level of drought tolerance requires the ability of a cultivar to increase its water-use efficiency.

The specific effect of combined salt and drought stress on halophytic plants has been the topic of several studies which indicates that salinity and drought can interact and may ameliorate each other's effects (Chakraborty et al. 2018; Slama et al. 2007). However, for non-halophilic species, water deficit and salinity are often considered as additive stress factors that depend on the species, stress duration, and its severity. Indeed, growth responses of many plants under different intensities of combined drought and salinity stresses still need to be unveiled.

Data on plant growth, biochemical, and physiological responses of *Medicago ciliaris* and *Medicago polymorph* caused by the combination of salinity and drought stress are lacking. Considering these aspects, the aims of the study are to investigate the interacting effects of salinity and drought stress in two forage species *Medicago ciliaris* and *M. polymorpha* and to determine if these effects are additive or not on growth, photosynthesis, water, and nutrient status. Our strategic aim is to select the most tolerant species by the co-occurrence of salinity and drought, which conditions are expected to be more common in the future due to the ongoing climate changes, to encourage its multiplication and use by farmers for the rehabilitation of soils in arid and semi-arid salinized regions.

Material and methods

Plant material and growth conditions

Healthy and uniform seeds of *Medicago ciliaris* (TNC1-11) and *M polymorpha* (TNP1-11) were disinfected with 10% (w/v) sodium hypochlorite for 5 min and then washed three times with distilled water. After that, seeds were germinated (at the beginning of May) in 3.5-L pots filled with sifted limono-sandy soil in a greenhouse, at our experimental station near the sea shore, 35 km north-east of Tunis (10 °10′ E, 36 °48′ N; mean annual rainfall and temperature were 19.4 °C and 456 mm, respectively). Soil used in this experiment is taken from a plot located at the center of Biotechnology of Borj Cedria. It contains 0.25, 0.95, 0.65, and 0.05 meq $(100\ g)^{-1}$of dry soil of Na^+, K^+, Ca^{2+}, and Cl^-, respectively, and 0.24 and 0.45 g kg^{-1} of dry soil of P_2O_5 and total N, respectively. The pH and the electrical conductivity of the aqueous extract (1/10) were 6.65 and 0.05 mmhos cm^{-1}, respectively. One week after sowing, seedlings were thinned to one per pot to achieve homogeneous plants. The soil field capacity (FC) was determined according to the technique of Bouyoucos (1983) and was11.5%. Following a period (3 weeks) of ample watering (80% FC), plants were divided into four groups: the first group was irrigated with tap water at 80% FC (control plants) (C), and the second at 33% FC (water-deficit stressed plants) (D). The third group was irrigated with tap water at 80% FC added with 50 mM NaCl (S) and the forth at 33% FC supplemented with 50 mM NaCl (D + S). Regular weightings (every 3 days) enabled to restore the moisture of soil at 80 or 33% FC. For all treatments, tap water was enriched with diluted nutrient solution (Hewitt 1966). A final harvest was carried out after 1 month of treatment (2 months after sowing, at the end of June).

Two irrigation modes were retained: 80% and 33% FC. The latter was selected to represent water-deficit stress on the basis of a previous experiments carried out in our laboratory on *Medic* plants (Gharred et al. 2023; Slama et al 2022, 2011; Yousfi et al 2010, 2012; Badri et al 2016a, b; Rouached et al 2013), showing that severe water-deficit stress led to a significant decrease of growth. Beside, 50% FC is a moderate stress for medic plants; in our study, we try to simulate natural conditions indeed in their natural biotopes and plants were exposed to severe drought stress. Based on these data, we chose 33% field capacity for this study.

Medicago polymorpha L. is an important local legume due to its fodder value. Indeed, it is considered as a potential species for pasture enhancement in the Mediterranean regions (Sheaffer et al. 2002). This species is particularly salt-sensitive (Gharred et al. 2022). According to Ibrar and Hussain (2003), at the vegetative stage, parameters including plant height, leaf, and branching number, and shoot dry weight are severely inhibited at 50 mM NaCl. Besides, Cherifi et al. (2016) showed that reduction of growth in four populations of *M. polymorpha* L. ranged between 40% and 60% under 50 mM NaCl in plants cultivated in sand filled pots. Based on these data, we chose 50 mM NaCl concentration for our study.

Growth parameter and water relations

Plants were harvested and divided into roots, leaves, and stems, and their respective fresh weights (FW) were determined. Dry weight (DW) was obtained after oven drying at 60 °C in an oven until a constant weight was reached. Leaf water content (LWC) was determined as LWC (ml g^{-1} DW) = (FW − DW)/DW (Talbi Zribi et al. 2024).

With respect to plant morphology, leaf number, root and shoot length, and number of ramifications and internodes were determined. In addition, fresh leaf, stem, and root samples from each plant were stored at − 80 °C.

Leaf gas exchange determination

Measurements of net CO_2 assimilation (A), transpiration rate (E), and stomatal conductance (g_s) were determined with a portable infrared CO_2/H_2O gas exchange system (LCpro+, ADC Bio-Scientific, Great Amwell, Herts, UK) at the end of the treatment period. Measurements were carried out between 10:00 and 12:00 h on the youngest fully emerged leaf ($n = 8$ per treatment). Measurement conditions were: 1200 µmolm^{-2} s^{-1} photosynthetically active radiation (PAR), 380 µmol mol^{-1} CO_2 at 27 ± 2 °C. Data were automatically collected every minute after the photosynthesis rate had stabilized. Water-use efficiency was calculated as A_{net}/g_s (Yousfi et al. 2015).

Nutrient extraction and analysis

Sodium and potassium were analyzed using flame spectrophotometry (Corning 410, UK) after extraction of the finely grounded dry matter in nitric acid (Munns et al. 2010). Ca, Zn concentrations were determined by atomic absorption spectrometry (Perkin-Elmer Analyst 300).

Statistical analysis

Data were analyzed using the statistical software STATISTICA 5.0 (StatSoft France 1998). Pearson coefficients were calculated to assess correlation between different variables. Interaction were determined with two-way analysis of variance. Significant differences between means were separated using the Tukey test ($P = 0.05$). The measurements were done with eight replicates.

Results

Growth

Under control or stressed conditions, *M. ciliaris* produced more biomass when compared to *M. polymorpha*. The whole-plant biomass production represented 237%, 230%, 175%, and 172% in treatment C, S, D, and D + S in *M. ciliaris* when compared to *M. polymorpha* (Fig. 1). In the two species, salinity significantly decreased plant growth with the same manner (64%). In *M. ciliaris*, reductions are 63 and 70% versus 50 and 60% in *M. polymorpha* for treatment D and D + S, respectively. The effects of the two factors were not additive in the two species. Indeed, the same whole-plant biomass production was obtained under salinity, drought or the combination of the two stresses.

In *M. polymorpha*, changes in leaf DW were similar to those observed in the whole plant (Fig. 2). However, in *M. ciliaris*, the effects of drought (D) and soil drying combined with salt (D + S) were significantly more pronounced when compared to the effects of salt (S). Stem biomass production variation was similar to those observed in leaves for the two species (Fig. 2). Root biomass production is low when compared to stems and leaves (50%, 25% under control

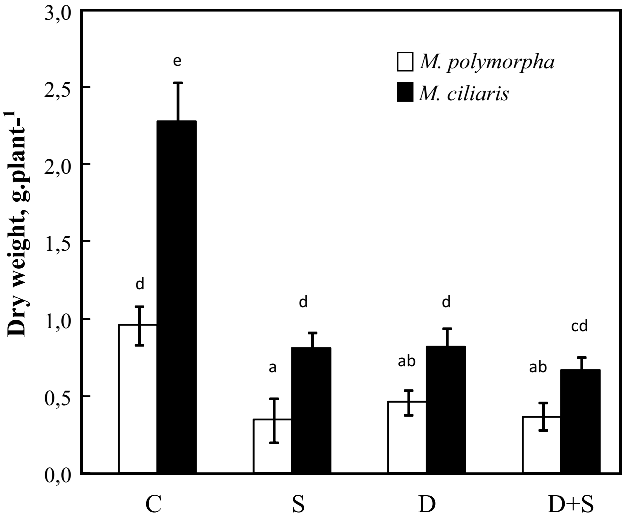

Fig. 1 Whole-plant biomass production of *M. polymorpha* and *M. ciliaris* seedlings subjected to four water treatments during 2 months—control (C): 80% FC, salinity (S): 50 mM NaCl, soil drying (D): 33% FC, and the combination of the two stresses (D + S). Values are the means of eight replicates, vertical bars are SEs. Values sharing a common letter are not significantly different at $P < 0.05$

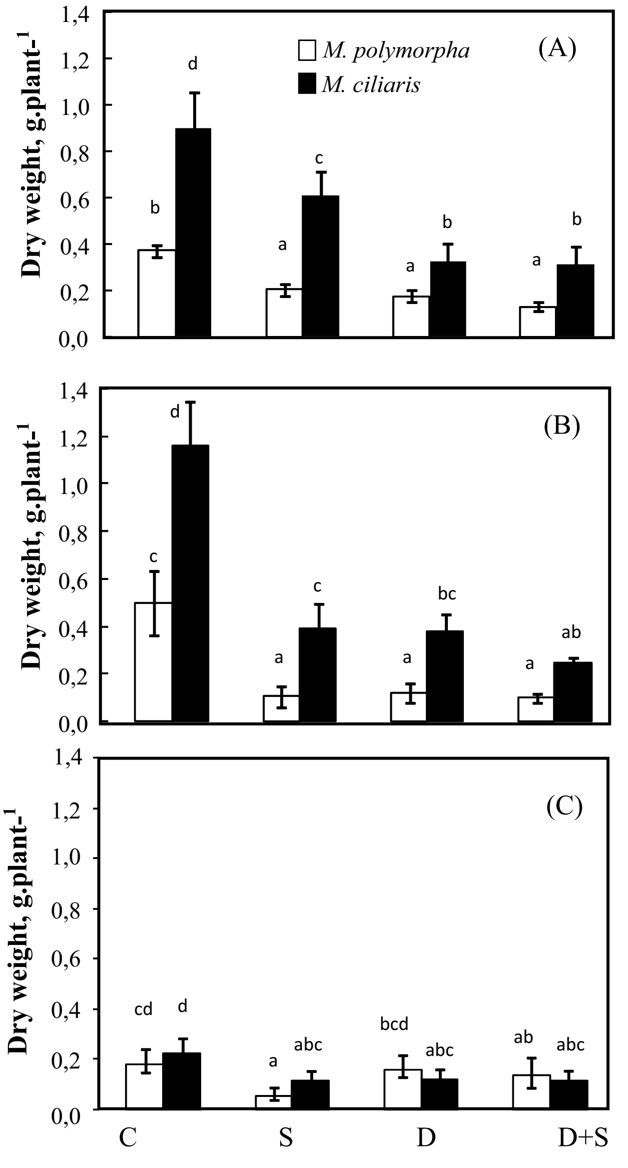

Fig. 2 Leaf (**A**), stem (**B**), and root (**C**) biomass production of *M. polymorpha* and *M. ciliaris* seedlings subjected to four water treatments during 2 months—control (C): 80% FC, salinity (S): 50 mM NaCl, soil drying (D): 33% FC, and the combination of the two stresses (D + S). Values are the means of eight replicates, vertical bars are SEs. Values sharing a common letter are not significantly different at $P < 0.05$

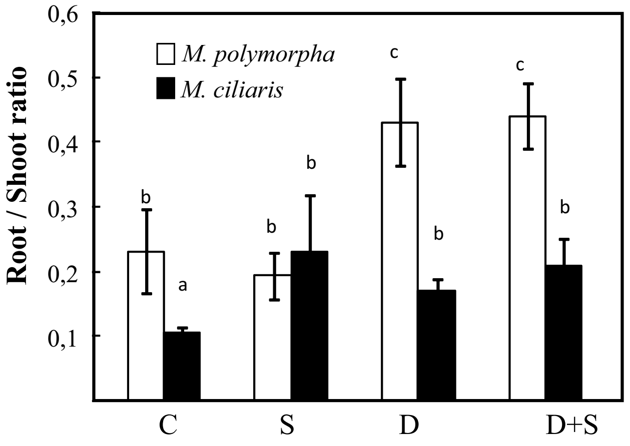

Fig. 3 Root/shoot ratio of *M. polymorpha* and *M. ciliaris* seedlings subjected to four water treatments during 2 months—control (C): 80% FC, salinity (S): 50 mM NaCl, soil drying (D): 33% FC, and the combination of the two stresses (D + S). Values are the means of eight replicates, vertical bars are SEs. Values sharing a common letter are not significantly different at $P < 0.05$

conditions for *M. ciliaris* and *M. polymorpha*, respectively). In *M. polymorpha*, root growth was more sensitive to salt applied alone (S) or in combination with soil drying (D + S) when compared to soil drying (D). However, in *M. ciliaris*, root growth was significantly reduced under (S), (D), and (D + S) treatment.

Root/shoot ratio

In *M. polymorpha*, the root/shoot ratio (Fig. 3) increased by 50% in plants subjected to water-deficit alone or combined with salinity. However, in *M. ciliaris*, this parameter increased significantly in (S), (D), and (D + S) with the same manner by about 45%.

Morphological parameters

In the two species, the highest value of root and stem length, ramification, and leaf number was observed under control conditions (Fig. 4). When compared to *M. polymorpha*, *M. ciliaris* produced more leaves under C, S, and D + S

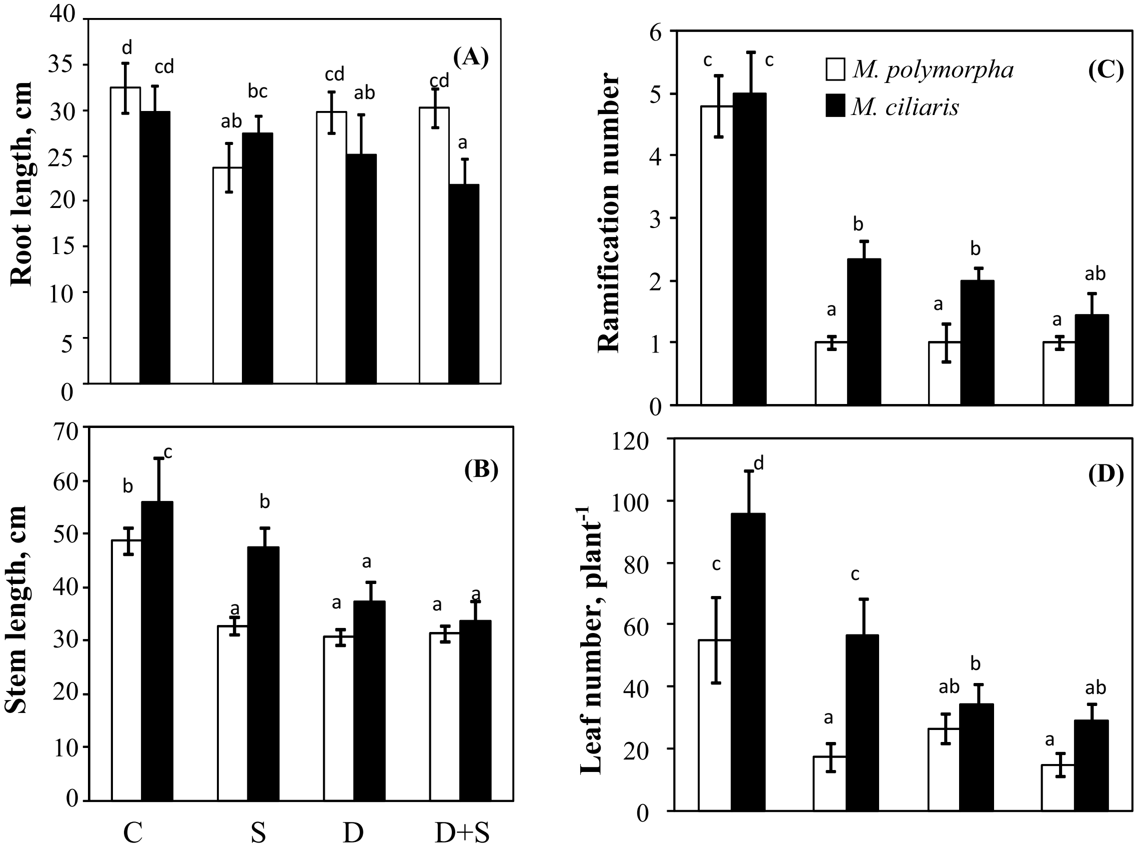

Fig. 4 Root (**A**) and stem length (**B**) ramification (**C**) and leaf number (**D**) of *M. polymorpha* and *M. ciliaris* seedlings subjected to four water treatments during 2 months—control (C): 80% FC, salinity (S): 50 mM NaCl, soil drying (D): 33% FC, and the combination of the two stresses (D + S). Values are the means of eight replicates, vertical bars are SEs. Values sharing a common letter are not significantly different at $P < 0.05$

treatment (55 versus 17 leaves under control conditions) and have the longest stems under C and C + S treatment. In *M. polymorpha*, leaf number was significantly affected under S and D + S treatment in the same manner (reduction by 72%). However, in *M. ciliaris*, the effects of drought alone or combined with salinity were significantly more pronounced when compared to salt. Single stress or the combined effects of the two constraints significantly affected root and stem length, ramification, and leaf number with the same manner in *M. polymorpha*. However, in *M. ciliaris*, the effects of drought alone or combined with salt were more pronounced when compared to salt applied individually on stem length and leaf number (Figs. 4, 5).

Gas exchange

The highest value of CO_2 assimilation was observed in well-watered plants of *M. ciliaris* (8.8 $\mu mol \cdot CO_2\ m^{-2}\ s^{-1}$) (Fig. 6A). Under C or C + S conditions, *M. ciliaris* exhibited a higher net CO_2 assimilation when compared to *M. polymorpha*. However, when plants were subjected to drought alone or combined to salinity, no differences were observed between the two studied species. In *M. polymorpha,* salinity and drought applied individually or all together significantly decreased net CO_2 assimilation. The combined effects of these two constraints were not additive. In *M. ciliaris*, net CO_2 assimilation was significantly more affected in this later specie under D and D + S treatments when compared to S.

Under control treatment, drought, or the combination of the two stresses, *M. polymorpha* exhibited the highest values of stomatal conductance (Fig. 6B). This parameter was significantly reduced under S, D, and D + S treatments in *M. polymorpha*. In *M. ciliaris*, this parameter was more affected in D and D + S than S when compared to the control treatment. Changes in E were similar to those observed in gs (Fig. 6C).

As shown in Table 1, water-use efficiency is higher in *M. ciliaris* when compared to *M. polymorpha* for all treatment.

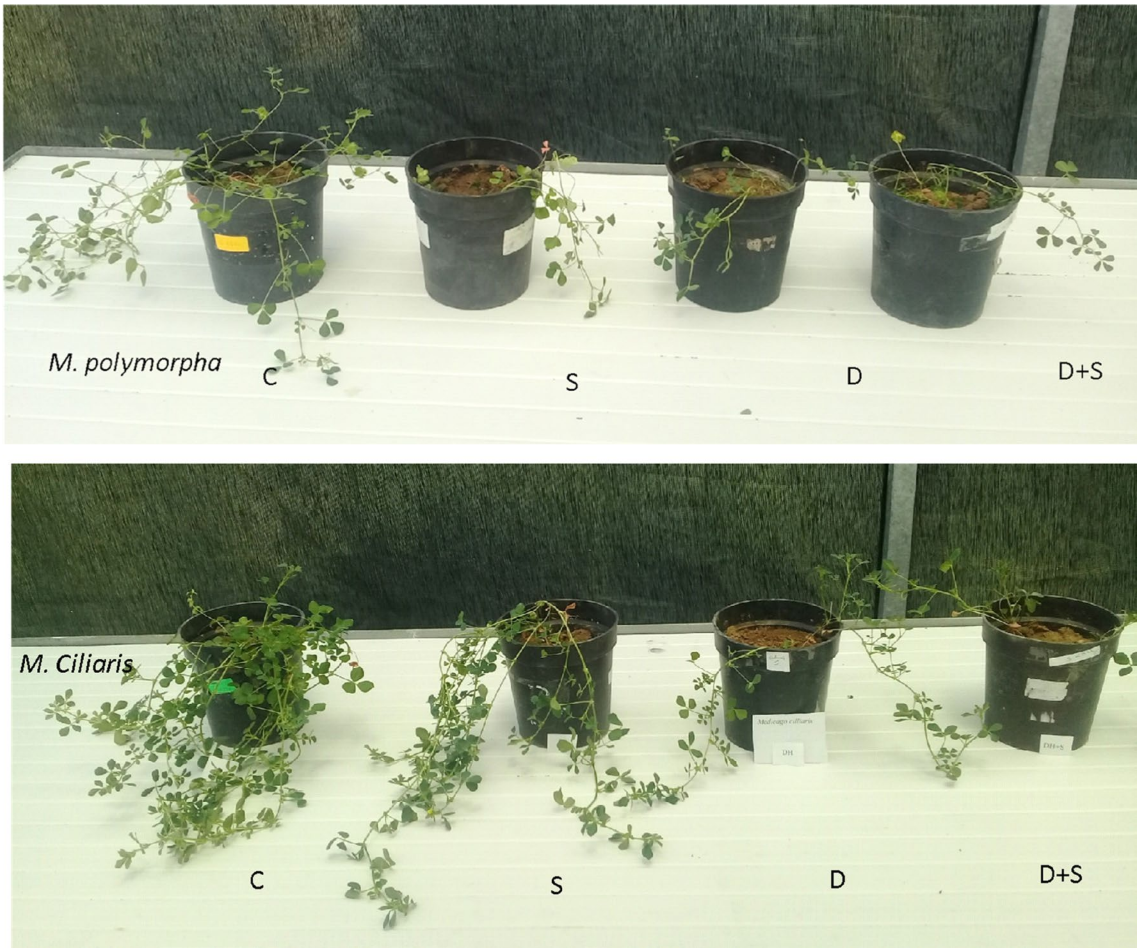

Fig. 5 *Medicago polymorpha* and *ciliaris* aspect. Plants were cultivated under control conditions (C) (80% FC), salinity (S) 50 mM NaCl, soil drying (D) 33% FC and the combination of the two stresses (D + S)

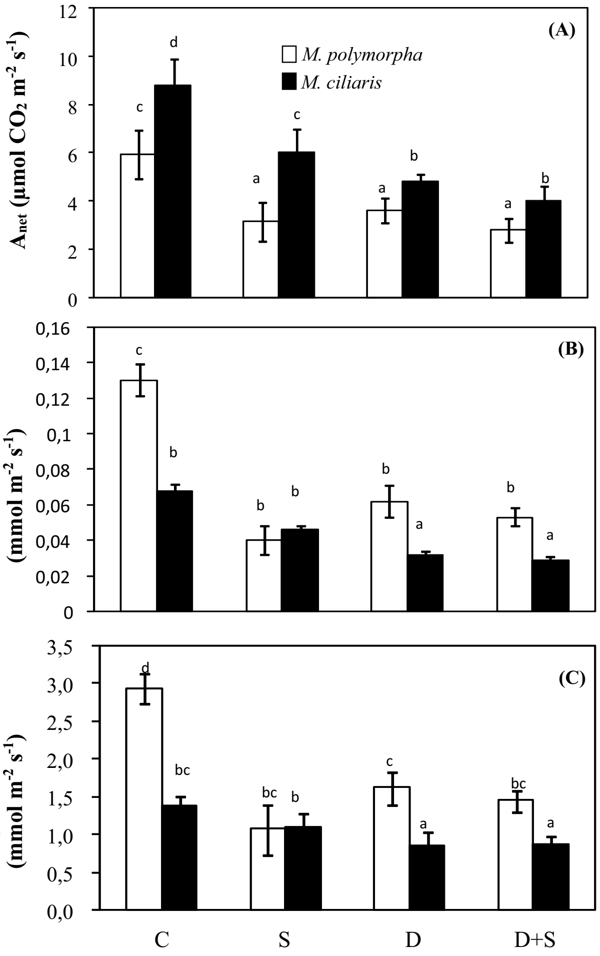

Fig. 6 Anet CO_2 (**A**), gs (**B**), and E (**C**) of *M. polymorpha and M. ciliaris* seedlings subjected to four water treatments during 2 months—control (C): 80% FC, salinity (S): 50 mM NaCl, soil drying (D): 33% FC, and the combination of the two stresses (D + S). Values are the means of eight replicates, vertical bars are SEs. Values sharing a common letter are not significantly different at $P < 0.05$

Table 1 Water-use efficiency of *M. polymorpha and M. ciliaris* seedlings subjected to four water treatments during 2 months—control (C): 80% FC, salinity (S): 50 mM NaCl, soil drying (D): 33% FC, and the combination of the two stresses (D + S)

	M. polymorpha	*M. ciliaris*
C	3.11 c	6.33 f
S	2.61 b	5.49 e
D	2.23 b	5.68 e
D + S	1.93 a	4.63 d

Values are the means of eight replicates. Values sharing a common letter are not significantly different at $P < 0.05$

Water relations

Leaf hydration was higher in *M. ciliaris* when compared to *M. polymorpha* under control condition (Fig. 7A). The two species exhibited the same leaf water content under S, D, and D + S treatments. Both drought and salinity applied alone or in combination significantly reduced the leaf water content. This parameter was more affected in *M. ciliaris* when compared to *M. polymorpha,* for example, reduction was by 45% versus 55% under drought conditions. Stem hydration was

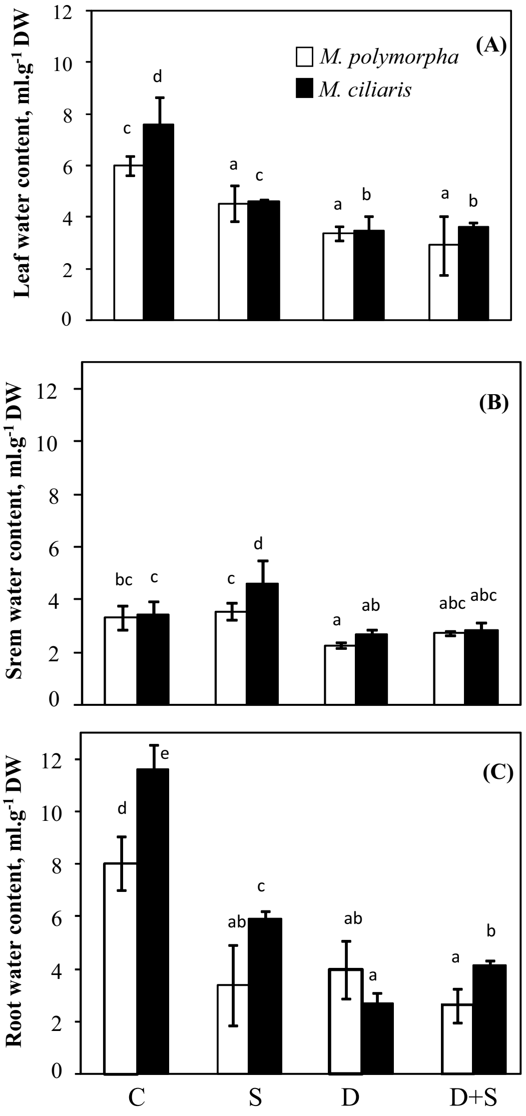

Fig. 7 Leaf (**A**), stem (**B**), and root (**C**) water water content of *M. polymorpha* and *M. ciliaris* seedlings subjected to four water treatments during two months: control (C): 80% FC, salinity (S): 50 mM NaCl, soil drying (D): 33% FC and the combination of the two stresses (D + S). Values are the means of eight replicates, vertical bars are SEs. Values sharing a common letter are not significantly different at $P < 0.05$

affected in *M. polymorpha* under D and D + S treatments (Fig. 7B). Stem hydration was less sensitive to drought, salt, and both stresses combined when compared to leaves and roots in the two studied species. Root water content was affected by all applied constraints (Fig. 7C). The effects of salt and drought are not additive in tissue hydration in the two species. As shown in Table 2, water content per plant is higher in *M. ciliaris* when compared to *M. polymorpha* for all treatments. This parameter is 2.8-, 3.2-, 1.7-, and 1.9-fold in *M ciliaris* when compared to *M. polymorpha* under C, S, D, and D + S treatments, respectively.

Table 2 leaf water content ml H_2O plant^{-1} of *M. polymorpha and M. ciliaris* seedlings subjected to four water treatments during 2 months: control (C): 80% FC, salinity (S): 50 mM NaCl, soil drying (D): 33% FC and the combination of the two stresses (D + S)

	M. polymorpha	*M. ciliaris*
C	4.7 f	13.14 g
S	1.27 b	4.15 e
D	1.4 b	2.44 d
D + S	0.96 a	1.86 c

Values are the means of eight replicates. Values sharing a common letter are not significantly different at $P < 0.05$

Nutrient status

In the two studied species, high sodium concentration was observed in the leaves of salt-treated plants, and those exposed to simultaneously drought and salinity. Under S + D treatment, *M. polymorpha* accumulated significantly more sodium when compared to *M ciliaris* (Fig. 8A).

Potassium content was higher in *M. ciliaris* under C and S treatment when compared to *M. polymorpha*. This parameter was not affected by drought in *M. polymorpha*; however, it decreased significantly under salt applied alone or combined to soil drying. In *M. ciliaris*, there was no significant interaction between the two constraints, potassium content decreased significantly by 25%, 30%, and 45% under S, D, and D + S treatment (Fig. 8B).

When compared to control, Na/K ratio remained unchanged under drought conditions in the two studied species. This parameter increased significantly under S and D + S treatment by 150% and 200% in *M. polymorpha* versus 237% and 275% in *M. ciliaris*. Under salt conditions, Na/K

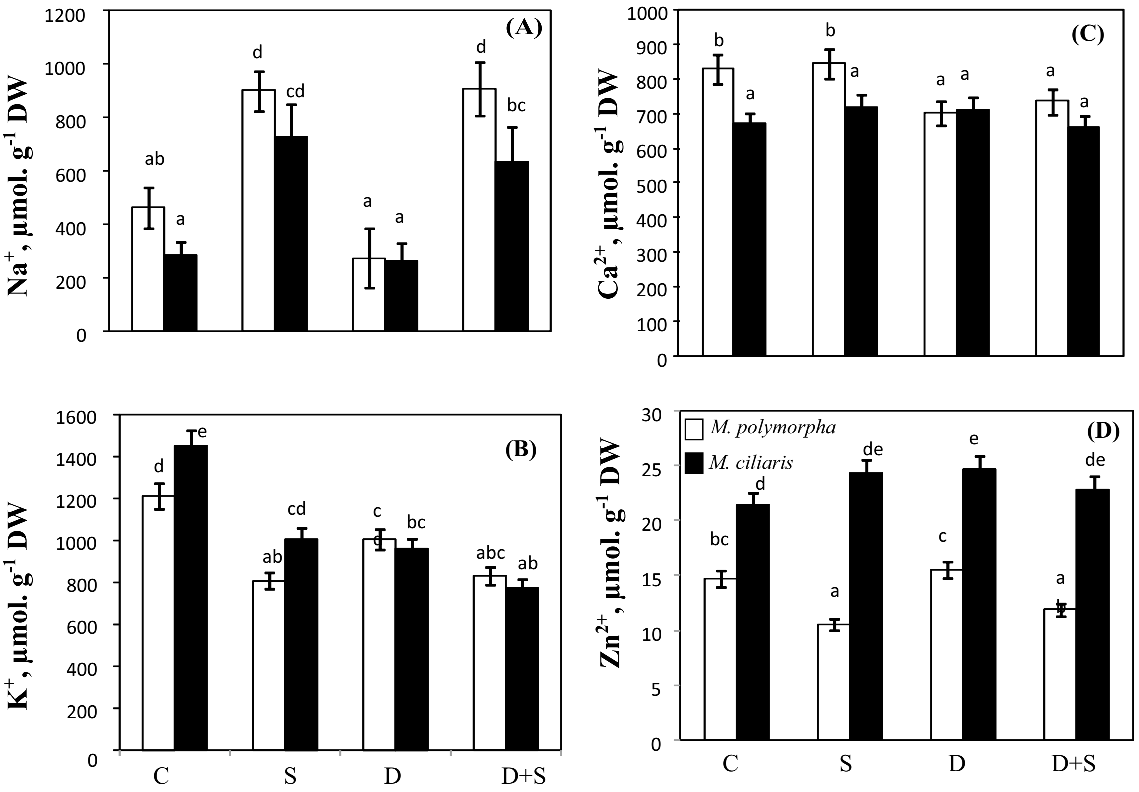

Fig. 8 Leaf sodium (**A**), potassium (**B**), calcium (**C**), and zinc (**D**) contents of *M. polymorpha and M. ciliaris* seedlings subjected to four water treatments during 2 months—control (C): 80% FC, salinity (S): 50 mM NaCl, soil drying (D): 33% FC, and the combination of the two stresses (D + S). Values are the means of eight replicates, vertical bars are SEs. Values sharing a common letter are not significantly different at $P < 0.05$

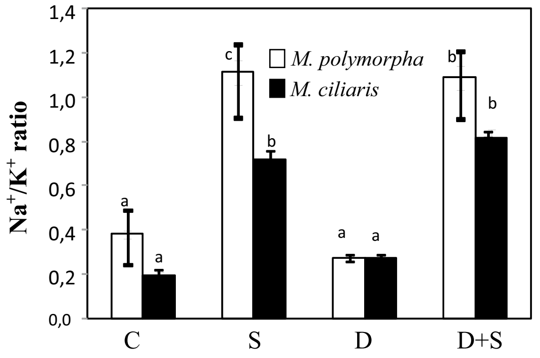

Fig. 9 Na/K ratio in leaves of *M. polymorpha and M. ciliaris* seedlings subjected to four water treatments during 2 months—control (C): 80% FC, salinity (S): 50 mM NaCl, soil drying (D): 33% FC, and the combination of the two stresses (D + S). Values are the means of eight replicates, vertical bars are SEs. Values sharing a common letter are not significantly different at $P < 0.05$

ratio was higher in *M. polymorpha* when compared to *M. ciliaris* (Fig. 9).

Calcium and zinc

Calcium content decreased significantly in *M. polymorpha* under D and D + S in *M. polymorpha*. However, in *M. ciliaris*, leaf calcium content was not significantly affected by all applied treatment (Fig. 8C).

Whatever the treatment, *M. polymorpha* accumulated a high amount of Zn in their leaves when compared to *M. polymorpha*, about x-, y-, z-, and h-fold under C, S, D, and D + D treatment, respectively. In *M. ciliaris*, leaf Zn content was not affected by all applied treatment; however; in *M. polymorpha*, Zn content decreased under salt conditions and remained unchanged under D and D + S treatment. (Fig. 8D).

Correlations and interaction effects of drought and salt

The whole-plant dry weight correlated positively with both, leaf and root hydration, leaf number, root length on the one hand, and with A_{net}, g_s, E, and K^+ on the other hand. However, the whole-plant dry weight correlated negatively with root/shoot ratio, Zn^{2+}, and Na^+/K^+.

A high positive correlation was observed between the whole-plant dry weight, leaf number, and photosynthetic parameters (A_{net}, g_s, E), and leaf potassium content (Figs. 10, 11). Root dry weight and length were positively correlated with leaf Zn^{2+} content and negatively correlated with Na^+ content and Na^+/K^+ ratio.

As shown in Tables 3 and 4, in the two studied species, drought had a high significant effect on leaf and stem dry weight and hydration, leaf number, and photosynthetic parameters (A, g_s and E) and had no effects on Na^+, K^+, and Zn^{2+}.

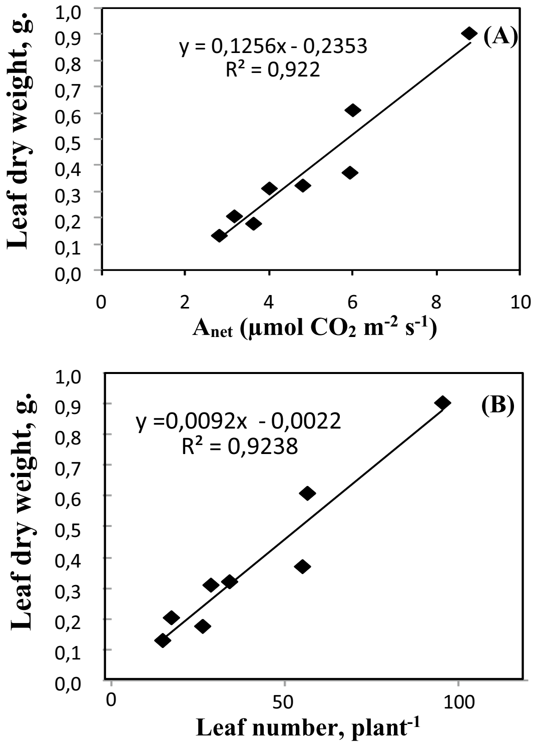

Fig. 10 Correlation between leaf dry weight and net CO_2 assimilation (**A**), and leaf dry weight and leaf number (**B**)

In *M. polymorpha,* leaf calcium content was slightly affected by drought.

A high significant effect of salt was observed on the two species on whole-plant dry weight, root hydration, leaf Na^+ and K^+ content. Salinity highly affected leaf Zn^{+2} content in *M. polymorpha*. In case of *M. ciliaris,* there was no significant effects of salt on leaf calcium and Zn^{+2} contents.

The interaction effects between the two constraints were observed in *M. polymorpha* (Table 3) on the whole plant, leaf, and stem dry weight, on E and g_s. No significant interaction was observed on root dry weight, root/shoot ratio, leaf, and stem hydration nor Na^+, Ca^{2+}, and Zn^{2+} leaf contents. In *M. ciliaris,* the two constraints (Table 4) had no interaction effects on root/shoot ratio, stem hydration, E, g_s, root and stem length, and Na^+, Ca^{2+} and Zn^{2+} leaf contents.

Discussion

The aim of our work was to compare the responses of the two forage species *M. ciliaris* and *M. polymorpha* to drought and salinity applied separately or in combination to select the most tolerant specie and identify some traits involved in this tolerance.

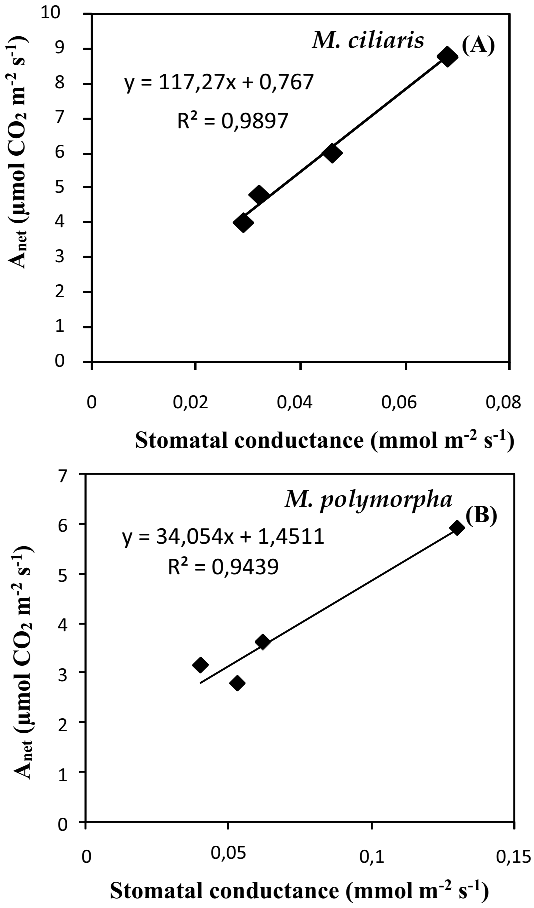

Fig. 11 Correlation between net CO_2 assimilation and stomatal conductance in *M. ciliaris* (**A**) and M. *polymorpha* (**B**)

Table 3 Results of a two-way analysis of variance of the effect of drought (D), salinity (S), and their interaction (D*S) on parameters describing plant growth, gas exchanges, water relations, and nutrition status in *M. polymorpha* plants

M. polymorpha			
	D	S	D*S
	F	F	F
WP DW	13.21*	32.02***	25.38***
Leaf DW	76.51***	120.67***	42.32***
Stem DW	25.84***	30.30***	30.84***
Root DW	0.50 ns	12.65*	2.28 ns
Root/shoot	13.08*	0.01ns	0.23 ns
H_2O leaves	30.82***	6.33*	0.82 ns
H_2O stems	30.94***	8.02*	1.68 ns
H_2O root	20.04**	31.71***	10.59*
A_{net}	16.62**	1.83 ns	11.46*
E	24.01***	45.12***	29.14***
GS	30.06***	45.88***	29.83***
Leaf number	17.82**	43.67***	11.49*
Root length	2.25 ns	9.69*	12.50*
Stem length	21.83**	13.74*	16.32**
Na^+	1.29 ns	41.21***	1.20 ns
K^+	0.09 ns	25.92***	7.00*
Na^+/K^+	1.42 ns	15.77**	0.73 ns
Ca^{2+}	12.07*	0.55ns	0.06 ns
Zn^{2+}	2.41 ns	31.68***	0.13 ns

Our results showed that when subjected to salinity, the two species were affected by the same magnitude (64%) but *M. ciliaris* kept always under all treatments the highest biomass production (absolute value). However, when drought was applied individually or in combination with salt, *M. ciliaris* was more affected, under D and (D + S) treatments when compared to control (relative value). Reduction of plant biomass production in forage species when subjected to stressed conditions was reported by several studies (Badri et al. 2016a, 2016b; Slama et al. 2022; Gharred et al. 2022; Ben Salah et al. 2011).

The two studied species produced more biomass in their leaves and stems when compared to the roots. Leaves and stems seem to be more affected by salt, drought, and the combination of the two stresses when compared to roots. Consistently with our findings, it has been reported that water-deficit stress was more inhibiting for shoot than for root biomass, resulting in an increase of the root/shoot DW ratio. This behavior is considered a criterion of adaptation to drought (Farhat et al. 2019). Our results showed that salinity and water-deficit stress, whether they were separately applied or in combination, caused a significant reduction in the morphological parameters of leaves and ramification numbers, stem length, stomatal conductance, and transpiration. These responses could be considered as an important mechanisms, which minimized water loss (Abrar et al. 2022).

Under salinity condition, *M. ciliaris* exhibited a significant higher stem length, leaf and ramifications number when compared to *M. polymorpha*. Under D + S treatment, no differences were observed between the two studied species in these parameters. The combined effects of abiotic stresses suggest that their interaction depends on both the nature of the constraint and the plant species. It has been reported by Mittler and Blumwald 2010 that some stress combinations might be beneficial for plants. When compared to each of the individual stresses applied separately, salinity could have a beneficial effect for the plant's ability to cope with drought. For non-halophytes, water deficit and salinity are often considered additive stress factors (Saudy et al. 2019; El-Bially et al. 2022). Our results pointed out that drought and salinity had no additive effects on plant growth parameters in the two studied species.

Plant's adaptation to water deficit comprise different strategies: (a) improving water uptake, (b) reducing water consumption, (c) as well as improving water-use efficiency

Table 4 Results of a two-way analysis of variance of the effect of drought (D), salinity (S) and their interaction (D*S) on parameters describing plant growth, gas exchanges, water relations, and nutrition status in *M. ciliaris* plants

M. ciliaris	D	S	D*S
	F	F	F
WP DW	38.48***	39.41***	26.13***
Leaf DW	99.45***	12.28*	10.66*
Stem DW	65.50***	61.78***	30.47***
Root DW	8.57*	10.68*	8.55
Root/shoot	0.48ns	6.88*	1.99 ns
H_2O leaves	75.19***	24.08**	29.82***
H_2O stems	19.94**	6.21*	3.40 ns
H_2O root	334.67***	52.22***	149.75***
A_{net}	121.67***	17.68**	5.59*
E	34.51***	1.22 ns	1.82 ns
GS	62.05***	7.48*	3.65 ns
Leaf number	92.44***	26.13***	7.35*
Root length	3.52 ns	0.34 ns	0.67 ns
Stem length	67.72***	10.63*	0.88 ns
Na^+	0.50 ns	64.03***	1.24 ns
K^+	4.38 ns	25.91**	34.35***
Na^+/K^+	0.005 ns	76.68***	1.90 ns
Ca^2	2.18 ns	0.001 ns	0.05 ns
Zn^{2+}	2.89 ns	0.15 ns	0.41 ns

(WUE) (Kang et al. 2021). Water-use efficiency is an important indicator that exhibits the response of a specific cultivar under different available irrigation water and soil moisture statuses (Hadid et al. 2023; Saudy et al. 2020). Generally speaking, the water-use efficiency of a plant decreases under water deficit and vice versa. However, when plants maintain their physiology and reschedule their water use, they are identified as exhibiting anti-drought behavior (Kilemo 2022). Exhibiting a higher level of drought tolerance requires the ability of a cultivar to increase its water-use efficiency (Hussain et al. 2022). In our case, the major advantage of *ciliaris* is indeed its high water-use efficiency, which leads to the combination of a high *A* to a higher biomass production. In addition, it does help to balance the osmotic stress and hinder a negative water balance. The difference of WUE ratios of both species is even getting higher at harmful conditions.

A high correlation ($R^2 = 0.86$) was observed between leaf dry weight production and WUE in one hand and leaf water content per plant in another hand (Fig. 12A, B). In our case, water content per plant is higher in *M. ciliaris* when compared to *M. polymorpha* for all treatments.

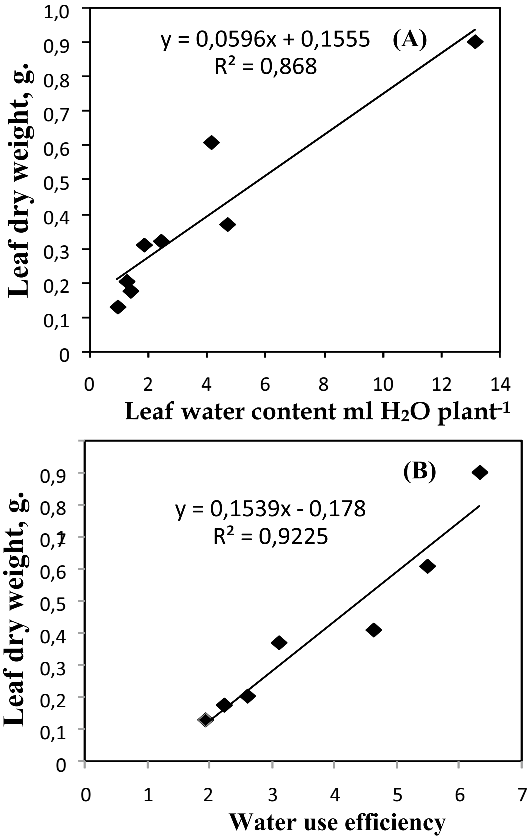

Fig. 12 Correlation between leaf dry weight and WUE (**A**), and leaf dry weight and leaf water content ml H_2O plant^{-1} (**B**)

Luo analyzed the differential adaptation of two *M. truncatula* genotypes (Jemalong A17 and R108) to water stress and showed that the tolerance of Jemalong A17 seedlings, when compared to R108, was associated with less water loss and a higher relative water content due to the reduction in stomatal apertures (Luo et al. 2016). In the same way, it has been reported that sensitive pea genotype was more affected by a decline in relative water content than tolerant ones under drought conditions (Upreti et al. 2000). Moreover, Naidu et al 1992 examined the behavior of seven pasture species of *Medicago spp.* to drought and reported that the lowest value of RWC was found with *M. polymorpha,* whereas the highest one was found with *M. truncatula*. The former species had a greater leaf area when compared to the others, and this may have led to higher transpiration rates, hence lower RWC. Besides, *M. truncatula* growth under drought conditions depends largely on its capacity to maintain suitable tissue hydration and root development (Rouached et al. 2013).

In our study, the higher biomass production of *M. ciliaris* is closely bound to the much better balance of water nutrition and can be seen as advantageous to hinder ROS production. It has been reported that the tolerant line of *Medicago ciliaris* TNC 1.8 originating from Enfidha subjected to 100 mM NaCl for 2 mouths showed no changes in leaf MDA levels (Ben Salah et al. 2011).

Several studies showed that drought may magnify the adverse effects of salinity on plant nutrient status. Our results showed that the interactive effects of salinity and soil drying were not additive on plant nutrient content. It was shown that selection of plants with lower Na^+/K^+ ratios in their tissues may be sufficient to pick up salt-resistant genotypes (Chen et al. 2007). In our case, Na^+/K^+ ratio increased significantly under S and D + S treatments. This increase is due to the increase in leaf Na^+ content and the decrease in leaf K^+ content.

Under (S) treatment, *M. ciliaris* exhibited a higher Na^+/K^+ ratio when compared to *M. polymorpha*. According to Blumwald 2000, plants maintain lower cytosolic Na^+/K^+ ratios under salt stress essentially by excluding Na^+ from the cytosol to the outer medium by the plasma membrane Na^+/H^+ antiporter or by sequestering it into the vacuoles by the tonoplastic Na^+/H^+ antiporter. Our results showed that *M. ciliaris* had a lower increase of Na^+ and a less decrease of K^+ at salinity leading to an overall lower Na^+/K^+ ratio as *polymorpha*. This shows that the selectivity of *ciliaris* is higher as the one of *polymorpha*. Thus, if we compare both species, *ciliaris* responds better to salinity than *polymorpha*.

Furthermore, we found that, in *M. ciliaris*, leaf calcium and Zn^{2+} content were not affected by drought and salt applied alone or in combination. Besides, *M. ciliaris* accumulated a higher amount of Zn^{2+} compared to *M. polymorpha* under D, D + S, and S. Zn accumulation in leaves of *M. ciliaris* could explain the higher plant biomass production in this specie when compared to *M. polymorpha*.

As a conclusion, the comparison of the two studied species behavior showed that *M. ciliaris* contains significantly more water than *polymorpha*. The high water-use efficiency and its great selectivity to potassium under salinity enables *ciliaris* the still absolute higher biomass production. *M. ciliaris* TNC1.11 line can be used as a forage candidate for the rehabilitation of soils in arid and semi-arid salinized regions. Physiological and biochemical characteristics play a crucial role in primary screening of resistant species. Besides, water-use efficiency is used for several species as a target for the selection of the most tolerant species (El-Metwally et al. 2021, Slama et al. 2022). It is necessary to deepen the combined effects of drought and salinity in Medic plants by biochemical tools such as chlorophyll fluorescence, osmolyte accumulation, and antioxidant enzyme activities and by molecular tools to better elucidate the superiority of *Medicago ciliaris* under combined soil drying and salinity.

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Declarations

Conflict of interest The authors declare that they have no known competing personal or financial interests that could have influenced the work reported in this paper. There are no conflicts of interest to declare.

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