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The interaction effect of superabsorbent and biochar on physiological properties of *Satureja rechingeri* under drought-stress

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Abstract

This study investigated the sole effect of cattle manure biochar (B) and Stockosorb superabsorbent (SA) and their combination on the physiological characteristics of *savory (S.rechingeri* Jamzad) under drought stress. A field experiment was conducted in a completely randomized block design as a factorial split plot with three replications for two consecutive years. The treatments included three levels of drought stress (100, 80 and 60 % of FC) as DS_0, DS_1, and DS_2 which were assigned as main plots, and the combination of two biochar rates of 0 and 10 t ha^{-1}(B_0 and B_1), and two super absorbent Stockosorb® rates of 0 and 6 g per $plant^{-1}$ (SA_0 and SA_1) were allocated as subplots. After two years, the results demonstrated that drought stress reduced relative water content (RWC), chlorophyll content, and dry shoot weight. Also, the proline, electrolyte leakage (EL%) rate, flavonoid content, photosynthetic pigments increased in plants. Individual application of biochar, and SA had a significant effect on the amount of RWC%, chlorophyll a, chlorophyll b, total chlorophyll and carotenoid content. Biochar application had no significant impact on proline content and the EL% rate; in comparison to the control treatment, the application of SA resulted in a 15.1% decrease in EL% rate and a 21.4% decrease in proline content. In summary, the application of cattle manure biochar and superabsorbent are suitable amendments to improve the physiological status of savory under drought-stress conditions.

***Keywords*:** Cattle manure, Amendment, Proline, Lamiaceae, Stockosorb

1. Introduction

Recently, about one-third of the drugs used in human societies are of plant origin ((Hadian et al., 2011); In Iran's botanical landscape, the medicinal herb *Satureja rechingeri* Jamzad. (Savory) stands out as a prominent and valuable plant (Hadian et al., 2014). Savory has been proven to have antioxidant and antimicrobial properties (Hadian et al., 2011). Water stress represents the primary limiting factor for crop production, exerting adverse effects on the physiological characteristics of medicinal plants like savory (Bettaieb et al., 2009; Beiranvandi et al., 2022; Momivand et al., 2021). Drought stress negatively affects plant physiological parameters such as turgor, mesophyll conductance, photosynthesis, transpiration rate, and relative leaf water content (Asha et al., 2021). This reduction in plant parameters ultimately results in a decrease in plant height, chlorophyll and carotenoid content, biomass, and yield (Zhu QiaoHong et al., 2014).

Drought stress in the early stages of plant growth causes leaf rot, which reduces photosynthesis and leads to an increase in CO_2 concentration (Gholami Zali et al., 2018). Also, drought stress reduces N and P content, resulting in a decrease in nutrient acquisition (Bista et al., 2018). Plants show various adaptive mechanisms to cope with the adverse effects of drought stress. Oxygen free radicals produced by environmental stress are dangerous for cellular components. Plants have developed several antioxidant systems to deal with these deadly compounds (Yang et al., 2017). Proline and proteins are effective in drought stress mitigation (Sigita et al., 2023), and proline acts to maintain the osmotic balance between the cytoplasm and the cell vacuole in the plant cells. In addition, proline plays an osmotic role as a carbon and nitrogen reservoir and protects the plant from free radical damage (Khatun et al., 2020; Hosseinifard et al., 2022).

Management practices (e.g., application of amendments) play an essential role in soil water and nutrient storage (Baronti et al., 2014). Recently superabsorbent and biochar are used to increase soil water retention, water use efficiency and dry matter production (Fouladidorhani et al., 2020), increase nutrient efficiency (Ippolito et al., 2012) and improve soil quality (Yang, 2018), increase the plant photosynthesis, transpiration rate, chlorophyll content (Trupiano et al., 2017; Speratti, 2018), biomass (Zhaoxiang et al., 2020), nitrogen content in okra leaf (Jabborova et al., 2021). Biochar amendment (woody biomass) with a particle size of less than 2 mm significantly increased the total chlorophyll content in Okra (*Abelmoschus esculentus*) plants than the control under water stress conditions (Jobborava et al., 2021). The effectiveness of superabsorbents in improving plant growth and water retention depends on the properties of soils, and plant species (Farrell et al., 2013).

In addition, various medicinal plants [e.g., Fennel (Gholami Zali et al., 2018), Tarragon (Momivand et al., 2021), Milk thistle (Keshavarz Afshar et al., 2016), Sage (Aslani et al., 2023)] have been extensively studied for their response to drought stress, but little work has been done on the impact of drought stress on savory (*Satureja rechingeri*) after amending with biochar in field studies. Hence, the objective of this study was to investigate the physiological response of S. rechingeri to various treatments of drought stress and to evaluate the potential impact of cattle manure biochar and Stockosorb superabsorbent in mitigating the adverse effects of drought.

2. Materials and methods

2.1. Study area and weather conditions

The savory (*S. rechingeriJamzad*) field experiment was conducted at the medicinal plant research farm of Lorestan University, Khorramabad (32°3′ 35″ N, 46° 27′18″ E and its height is 1100 m above sea level, and the average annual rainfall is 461 mm), Iran for two growing seasons in the period between 2017 and 2019.

2.2. Experimental design

The experiment was conducted as a factorial split plot with a randomized complete block design with three replications. Three levels of drought stress [100±5% FC, (no stress) DS_0, 80±5% FC, (moderate stress) DS_1, and 60±5% FC, (severe stress) DS_2], two rates the application of biochar(B); [B_0 (Non- biochar), B_1(10 ton/ ha)], and two rates the application of Stockosorb® superabsorbent(SA); [SA_0 (Non- SA) and SA1(6g/ plant)] were considered. Before the experiment, the land was fallow, and random soil sampling was conducted from a depth of 0-30 cm. The chemical properties of the investigated soil are shown in Table 1.

Table 1. Some chemical properties of the investigated soil**.**

Soil particle size distribution	Total N	organic matter	CaCO3	Available P	K^+	Ca^{2+}	Mg^{2+}	ECe	pH
	%	%	%	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}	dS m^{-1}	-
47% silt, 32% clay, 21% sand	0.09	1.15	19.8	6.8	278	0.43	98.4	2.03	7.48

K^+, Ca^{2+}, Mg^{2+}; Soluble cations concentrations, EC_e; electrical conductivity of soil saturated paste extract.

2.3. Preparation of amendments

The cattle manure was passed through a 2-mm sieve and pyrolyzed at 450-650 °C. The biochar (with a size smaller than 2 mm) was mixed with distilled water at a ratio of 1:5 (biochar to water) and left for 24 hours. Afterward, the pH was measured in saturated paste extract by a pH meter (Model METROHM632), EC by an EC meter (Model JENWAY4310), and cation exchange capacity (CEC) was measured at pH=7 using 1 mol L^{-1} ammonium acetate, respectively (Moncada et al., 2014). The Walkley-Black method was utilized to measure the organic matter content (Prasetya et al., 2021)., Moreover, the concentrations of total nitrogen N, phosphorus P, and potassium K were determined through the Kjeldahl method (Page et al., 1982). The bulk density (BD), particle density (PD), and total porosity of the soil mixture were measured using the cutting ring method with a volume of 100 cm3 at the central laboratory of Lorestan University (Fan et al., 2015). The ASTM D-285 methods with minor adjustments [ASTM, ASTM Book of Standards, 2007] were used to measure the apparent density of biochar. To achieve this, samples were poured into a pre-weighed100-mL graduated glass cylinder (with an inner diameter of 25.4 mm) through a funnel (with an outlet diameter of 20 mm) using an electric vibratory spatula until the cylinder was filled to over 50% of its capacity. The spatula was held at a constant vertical distance from the cylinder throughout the feeding process. The recorded the bulk volume of the sample inside the cylinder, and subsequently reweighed the cylinder to determine the mass of the sample. Bulk density (g cm^{-3}) was estimated as:

$$Bulk\ density = \frac{m}{v} \quad (1)$$

The oven-dry mass of biochar (m) in grams, and volume of biochar it occupies in the cylinder (v) in cm^{-3}.

The super absorbent used was the hydrogel Stockosorb® 660 Agro manufactured by (Degussa-Hüls Ibérica, S.A., Barcelona, Spain. The biochar and superabsorbent were mixed with soil at a depth of 0-20 and 0-15 cm, respectively. Table 2 displays specific physicochemical properties of both biochar and the superabsorbent.

Table 2. Physicochemical properties of biochar and super absorbent used in this experiment

Amendment	pH	EC	CEC	C	N	P	K	BD	PD	WHC	PS
	-	(ds m^{-1})	(cmolc kg^{-1})	(%)	(%)	g Kg^{-1}	meq L^{-1}	g cm^{-3}	g cm^{-3}	(%)	mm
Biochar	8.8	12.8	129	21.86	1.79	2.39	25.8	0.65	-	139	< 2.0
Super absorbent	7.70	13.21	400	-	-	-	4048.7	0.85	1.36	9×10^4	0.2

EC, electrical conductivity; CEC, cation exchange capacity; BD, bulk density; PD, particle density; WHC, Water-holding capacity; PS, Particle size

Each experimental plot consisted of 4 rows 3 m in length. The distance between plants and planting rows was 30 and 60 cm, respectively. The distance between the main plots in each block was 1 m, and the distance between the blocks was 2 m. At the beginning of May 2018, when the height of the savory plants was between 5 and 7 cm, it was transferred to the farm with a density of 4 plants m^{-2}.

2.4. Water holding capacity (WHC)

To determine the WHC, the unground biochar (15 gr dry matter) was soaked in deionized water for 24 h. Afterward, the soaked mixture was placed in a Kontes flex glass column (2.5cm × 10 cm, Kontes Glass Company, Inc., Vineland, NJ). A vacuum at 0.6 bar was applied to the column for 10 minutes to eliminate any trapped air in the biochar pores. Following a settling period of 24 h, the column underwent a vacuum suction of 0.1 bar until no more gravitational water was drained out. Finally, the amount of water retained by the biochar in the column was measured by weighing (Song & Guo, 2012). Finally, the WHC was calculated using the following equation:

$$WHC\ (\%) = \frac{water\ retained(g)}{biochar\ dry\ mass\ (g)} \times 100 \quad (2)$$

2.5. Application of drought stress treatments

As previously mentioned, the application of water treatments was determined based on field capacity (FC). To determine the percentage of soil moisture at the point of field capacity a 3×3 plot of land was selected on the farm. This plot was then filled with water and immediately covered with plastic to prevent evaporation from its surface. At intervals of 12 hours, a sample was extracted from the central area of the plot and measured to determine the percentage of moisture it contained. The process continued until two consecutive measurements showed the same soil moisture level. At this point it was determined that the soil had reached its field

capacity. The obtained number from these measurements was then used as the soil moisture percentage of at the field capacity point for all subsequent calculations.

The Theta Probe device (Spectrum Technologies Inc., IL, USA). Was utilized to measure soil moisture. To accomplish this the field was irrigated and allowed to drain naturally. Soil samples were collected from the field every 24 hours for two weeks. Simultaneously the reading on the Theta Probe device was recorded. These soil samples were transported to the laboratory for volumetric moisture measurement, which provided the volumetric moisture content. The intact sample of field soil was weighed to determine its wet weight. Subsequently, the soil sample was placed in an oven set at 105 ℃ for 24 h, allowing for the determination of its dry weight. The weight percentage of soil moisture was calculated by subtracting the dry weight from the wet weight. Additionally, the volumetric percentage of soil moisture was obtained by multiplying the weight percentage of moisture by the apparent specific gravity of the soil.

The Theta Probe device was calibrated using data obtained from water content measurements using a weight method. Following the start of irrigation treatments in both years of the experiment, the volumetric soil moisture content up to a depth of 30 cm was measured daily using the Theta Probe device. Whenever the moisture content of the treatments reached its lowest level, the water deficiency for each treatment was calculated using specific formulas. The required amount of water for each treatment was then accurately measured using the following formula and added to the respective plots. The soil moisture deficiency for irrigation per meter of soil depth (θ_{defT}) can be determined by comparing the current soil moisture (θ_w) with the soil moisture at field capacity (θ_{Fc}), (Askari and Ehsanzadeh, 2015).

$$\%\theta_{defT} = \%\theta_{Fc} - \%\theta_w \quad (3)$$

If the root depth of the plant is d_z (in meters), the deficiency of soil moisture for the field (θ_{def}) during irrigation can be expressed as:

$$\theta_{def} = \theta_{defT} \times d_z \quad (4)$$

Since drip irrigation was used, the obtained value should be multiplied by the coefficient of percentage of wet surface (Pw). This coefficient represents the ratio of the width of water droplets (w) to the distance between rows (D_r). In this study, the width of water droplets was measured to be 30 cm, while the distance between planting rows was 60 cm. Therefore, P_w was calculated as follows:

$$P_w = \frac{w}{D_r} = \frac{30}{60} = 0.5 \quad (5)$$

Thus, the irrigation depth (d_I) in millimeters was derived using the following equation:

$$d_I = 10 \times \theta_{def} \times P_w = 10 \times \theta_{def} \times 0.5 = 5\theta_{def}\ (mm) \quad (6)$$

To calculate the volume of water required for irrigation (V_I) in liters for each plot, we need to multiply the irrigation depth (mm) by the area of the plot (S) in m^2. This will give us the following equation:

$$V_I = d_I \times S \quad (7)$$

In the case of treatments with 80% and 60% FC, the volume mentioned above was multiplied by 8.10 and 6.10 respectively to obtain the water volume for each plot.

2.6. Measurement of morpho-physiological plant parameters

During two years (2017-2019), after the 50-60% flowering stage, six plants from each plot were randomly selected to measure the morphophysiological traits. The plant parameters included above-ground dry mass (DM), leaf relative water content (RWC), photosynthetic pigments (Chl *a* and Chl *b,* carotenoids (Car), flavonoid, electrolyte leakage (EL), and proline. To measure leaf relative water content, ten leaves were harvested from the second fully developed upper leaves. The fresh weight of the leaves was determined immediately after harvesting. Afterward, the leaves were placed in test tubes filled with distilled water and left for 4 hours at room temperature (25°C) in the low-light laboratory environment. Turgid masses were then estimated. Leaf dry matter (DM) was measured by drying the leaf samples in an oven at 72°C for 48 hours. Finally, the relative water content (RWC) was calculated using the provided equation (8) (Ritchie & Hanson, 1990):

$$\mathrm{RWC}(\%) = \left(\frac{\text{Fresh Weight} - \text{Dry Weight}}{\text{Turgid Weight} - \text{Dry Weight}} \times 100\right) \quad (8)$$

Determination of chlorophyll a (Chl *a*), chlorophyll b (Chl *b*) and carotenoid in fresh leaves was done using method Arnon (1967).

$$\mathrm{Chl}\ a = (19 \cdot 3\mathrm{A}^{663\cdot2} - 0 \cdot 86\mathrm{A}^{645\cdot8})\ \mathrm{V}/100\mathrm{W} \quad (9)$$

$$\mathrm{Chl}\ b = (19 \cdot 3\mathrm{A}^{645\cdot8} - 3 \cdot 6\mathrm{A}^{663\cdot2})\ \mathrm{V}/100\mathrm{W} \quad (10)$$

$$\mathrm{Total\ Chl} = (\mathrm{Chl}\ a + b) \quad (11)$$

$$\mathrm{Car\ (mg/\ g)} = 100(\mathrm{A}^{470}) - 3 \cdot 27(\mathrm{mg\ Chl}\ a) - 104(\mathrm{mg\ Chl}\ b)/227 \quad (12)$$

Where A(663.2 and 645.8) is the light absorption at wavelengths 663, 645 and 470 nm, V is the volume of filtered solution and W is the sample weight. utterly crushed,

The amount of free proline in the leaves was measured using the method of Bates et al. (1973). 0.5 g of fresh leaves sample was wholly crushed by liquid nitrogen inside the mortar. For each sample, 10 ml of aqueous sulfosalicylic acid (3%) was added, and it was purified using Whatman filter paper. Afterward, the Falcon tube containing 2 ml of ninhydrin reagent and 2 ml of acetic acid was supplemented with 2 ml of the extract. The resulting mixture was then subjected to heating in a hot water bath at 100°C for 60 minutes. Once the heating process was complete, the mixture was allowed to cool on ice. Following that, 4 ml of toluene was introduced to each sample. The samples were then vigorously mixed using a vortex for approximately 20-30 seconds until two distinguishable phases emerged. In the last step, the toluene phase was separated, and its absorbance was measured at a wavelength of 520 nm using a spectrophotometer (Hitachi U1800, Japan) against blank toluene.

$$\mathrm{Proline\ (\mu mol\ g\ FW^{-1})} = \left[\left(\mu\mathrm{g}\frac{\mathrm{Prolin}}{\mathrm{ml}} \times \mathrm{ml}\frac{\mathrm{toluene}}{115.5}\right)\right]/\left[\mathrm{g}\frac{\mathrm{Samples}}{5}\right] \quad (13)$$

The flavonoid content was determined using the colorimetric method of aluminum chloride (Chang et al., 2002), and rotinoside was used as a standard. 0.5 ml of the standard solution was combined with 2.8 ml of distilled water with 5% Sodium nitrite (150 µL). After 5 minutes, the

mixer was combined with 10% AlCl3 (150 µL) and 4% NaOH (2 mL). Distilled water was poured into a 5 ml volume. After giving the samples a 30-minute incubation at 25°C, the absorbance of the samples was measured at a wavelength of 300 nm, comparing it against the blank. Then, the total amount of flavonoids was expressed as rutinoside equivalents in mg 100 g^{-1} of dry extract. Intire flavonoid content was calculated as follows:

$$C = {}^{cV}/_{m} \quad (14)$$

"C represents the total flavonoid content in mg of rutinoside equivalents per g-1 dry extract. 'c' denotes the concentration of quercetin obtained from the calibration curve, expressed in mg per ml. 'V' refers to the volume of the extract in ml, and 'm' represents the mass of the extract in gr."

To measure polyphenols in savory, the following steps were followed:

1 g of savory was powdered using a mill and mixed with a magnetic stirrer. The powdered savory was then extracted in 100 ml of 95% ethanol at 22°C for one hours. After extraction, the resulting extract was filtered using Whatman filter paper to remove plant tissue particles. The polyphenolic content of the extract was determined using the Folin-Ciocalteu reagent, following the procedure described by Meda et al. (2005). The extracted sample was mixed with 1.0 ml of Folin-Ciocalteu reagent, diluted 10-fold, and 0.8 ml of a 7.5% sodium carbonate solution. The mixture was left to reach room temperature and was allowed to stand for at least one h.The concentration of polyphenols was measured using a spectrophotometer, which read the absorbance at a wavelength of 765 nm.The results were expressed as mg of gallic acid equivalents per gram of fresh weight (mg galik g-1 FW).

To measure electrolyte leakage (EL%), first, 15 leaves were separated from each plant and placed in 10 ml of double distilled water. After 24 hours, the electrical conductivity of each sample was calculated using an EC meter (model. Jenway) (EC_1). To measure the total rate of EL, the samples were put in a freezer at -80°C for 24 hours, and the electrical conductivity of the samples was measured again (EC_2). EL % was calculated as follows (Marcum, 1998):

$$EL(\%) = [EC_1/EC_2] * 100 \quad (15)$$

2.7. Data analyses

The experiment was a factorial split-plot randomized complete block with three replications. Homogeneity of variances was done using Bartlett's test. The year was considered a random variable. Cattle manure biochar, Stockosorb superabsorbent and drought stress were considered as fixed variables. A two-way ANOVA test was conducted to analyse data from the two-year experiment using the PROC GLM of SAS version 9.1, as a statistical test, to appraise differences between variables. Means' comparison was done using the test (LSD at $P < 0.05$.

3. Results

3.1. Effects of year, drought stress, biochar, and superabsorbent on the examined traits

The variance analysis showed that drought stress and superabsorbent levels had a significant effect on all investigated traits in savory ($P<0.01$). In contrast, biochar had an important ($P<0.01$) effect on Chl *a* and Chl *b*, total chlorophyll, flavonoids, polyphenol, leaf relative water content (RWC), and above-ground dry mass (DM) (Supplemental Table S3). An interaction between drought stress and biochar influenced the chlorophylls a (Chl *a*), total chlorophyll (Total Chl), carotenoids, flavonoid, leaf polyphenol, proline, and RWC. The drought stress treatment × superabsorbent interaction affected chlorophyll, proline concentrations, leaf carotenoids and RWC ($P<0.01$) (SupplementalTable S3). And in addition, the year affected leaf carotenoids (Caro), electrolyte leakage (EL), and above-ground dry mass (DM). There was an apparent increase in leaf carotenoids (13.14%), electrolyte leakage (9.80%), and above-ground dry mass (50.4%) for the second-year crop than the first-year (Table 4).

Table 3. Variance analysis of photosynthetic traits and non-enzymatic antioxidants of *savory* in different experimental treatments.

SOV	df	Chl a (mg g^{-1}FW)	Chl b (mg g^{-1}FW)	Total chl (mg g^{-1} FW)	Caro (mg g^{-1} FW)	Flav (mg QE g^{-1}Shoot DW	Polyph (mg galik g^{-1} FW)	Proe (µmol g^{-1}FW)	El (%)	RWC (%)	DM (g $plant^{-1}$)
Y	1	0.116ns	0.042ns	0.281ns	0.085**	15.0ns	5.28ns	0.068ns	397*	39.7ns	2641**
Y (R)	4	0.455	0.48	0.771	0.003	50.3	2.61	1.84	47.6	8.51	41.3
DS	2	9.43**	0.487**	14.1**	0.972**	5576**	688**	85.0**	282**	3987**	1141**
DS×Y	2	0.040ns	0.006ns	0.065ns	0.003ns	5.71ns	3.65ns	0.570ns	9.75ns	7.23ns	153**
Error A	8	0.086	0.010	0.152	0.008	29.2	11.2	1.30	47.6	90.7	11.5
B	1	0.469**	0.143**	1.15**	0.100**	132**	58.1**	0.475ns	5.17ns	337**	103**
SA	1	13.1**	1.26**	22.1**	1.14**	104**	46.9**	43.5**	1156**	1298**	98.2**
B×SA	1	0.008ns	0.0005ns	0.011ns	0.00001ns	4.55ns	1.36ns	0.009ns	26.0ns	17.7ns	50.8**
DS ×B	2	0.810**	0.005ns	0.825**	0.015*	61.0**	29.2**	5.91**	12.5ns	150**	7.76ns
DS ×SA	2	2.52**	0.163**	4.09**	0.160**	26.7ns	6.36ns	3.83*	32.4ns	255**	8.39ns
DS ×SA×B	2	0.036ns	0.001ns	0.025ns	0.004ns	2.52ns	1.56ns	1.87ns	0.747ns	5.92ns	1.51ns
Y×B	1	0.019ns	0.0003ns	0.023ns	0.005ns	0.011ns	4.85ns	0.360ns	11.6ns	7.54ns	27.5*
Y×SA	1	0.004ns	0.001ns	0.003ns	0.00001ns	7.28ns	0.516ns	2.31ns	48.8ns	20.6ns	5.17ns
Y×B×SA	1	0.00003ns	0.002ns	0.0001ns	0.001ns	1.71ns	0.087ns	1.79ns	7.54ns	14.5ns	12.2ns
Y× DS ×B	2	0.002ns	0.001ns	0.004ns	0.003ns	1.04ns	0.223ns	0.012ns	41.5ns	1.19ns	10.0ns
Y× DS ×SA	2	0.035ns	0.005ns	0.067ns	0.002ns	2.02ns	0.633ns	0.655ns	20.5ns	0.915ns	5.05ns
Y× DS ×SA×	2	0.010ns	0.0002ns	0.010ns	0.0001ns	2.70ns	0.807ns	1.17ns	0.364ns	0.210ns	3.73
ErrorB	36	0.049	0.004	0.057	0.004	8.86	2.44	1.02	25.5	12.1	4.97
CV	-	13.5	14.2	11.0	11.6	13.3	12.7	15.6	10.3	4.91	12.2

** and * stand for significant effects at $p < 0.01$ and $p < 0.05$, respectively; and ns stands for non-significant; SOV, source of variation**;** DF, degree of freedom; Y, years; DS, drough stress; B, biochar; SA, superabsorbent; Chl, chlorophyll; Caro, carotenoids; Flav, flavonoid; Polyph, polyphenol; Pro, proline; EL, electrolyte leakage; RWC, relative water content; FW,fresh weight; DM, above-ground dry mass.

3.2. Effect of experimental treatments on photosynthetic pigments

The highest content of Chl a, Chl b, Total Chl, and leaf carotenoids were observed in non-drought stress (DS_0). By increasing DS_0 to DS_1, a decreasing trend was observed for the Chl a (25) and Chl b (14.2), carotenoids (27.2), and total chlorophyll (23%) contents. Likewise, a decreasing trend was observed with increasing DS_0 to DS_2 (Table 4). The biochar and SA alone increased Chl a (10.2 and 71.1 %), Chlb (22.2 and 84.6 %), carotenoids (14.1 and 58%), and total Chlorophyll (13.3 and 72.5%) than the control treatment (without - biochar and SA) in non-drought stress conditions (DS_0) (Table 4). The interaction between DS and B had a significant impact on photosynthetic pigments. In the non-stress treatment (DS0), the use of biochar resulted in a 7.9% decrease in the content of Chlorophyll a (Chl a). In contrast, there was an increase of 14% for the DS1 treatment and a substantial increase of 65.3% for the DS2 treatment, as compared to the control(without biochar) (Table 5).

Biochar treatment decreased total chlorophyll content by 17.3% in treatment DS_0, whereas increased by 12.2 and 63% in treatments DS_1 and DS_2 compared to the control (Soil without B) (Table 5). Carotenoid content increased by 14.6, 2.99 and 33.2% for DS_0B_1, DS_1B_1 and DS_2B_1 treatments compared to the control (non- using biochar) in different levels of drought stress, respectively (Table 5). There was no notable distinctions observed in the treatment of 80% Fc (DS1) when comparing the utilization and non-utilization of biochar (Table 5). The SA had different behavior compared to drought stress (Table 6). The SA_1 increased Chl a by 9.3, 145, 155%, Chl b by 25.1, 196, 124%, carotenoids by 16.7, 129, 80.8%, proline by11.2, 164, and 144%, respectively, for DS_0, DS_1 and DS_2 compared to the control (without SA) (Table 6).

3.3. Effect of experimental treatments on flavonoids and Polyphenols

Drought stress reduced all examined traits except flavonoids and Polyphenols. The highest and lowest concentrations of flavonoids and total phenol were observed as 35.9 and 5.81 mg QE g^{-1} shoot DW and 17.3 and 7.69 mg galik g^{-1} FW in DS_2 and DS_0, respectively (Table 4). The concentration of flavonoids and total phenol in the DS_2 treatment increased by 4.35% and 125% compared to the DS_0 treatment, respectively (Table 4). The using of biochar alone increased the concentration of flavonoids by 12.8% than the control (non- using biochar). The using of SA did not affect the flavonoids. However, the using of SA resulted in a 4.5% decrease in total phenol than the control (non- SA) in non-drought stress conditions (Table 4). There was a significant positive interaction of DS ×B treatment on the concentration of flavonoids, total phenol and proline depending on the degree of drought stress (Table 5). The highest concentrations of flavonoid and total phenol were 36.5 mg QE g^{-1} shoot DW and 17.6 mg galik g^{-1} FW for the combined drought stress and biochar (DS_2B_1 treatment). There was no significant difference between the DS_2B_0 treatment (60% FC + soil without biochar) and the DS_0B_0 treatment (100% FC + soil without biochar) (Table 5).

3.4. Effect of experimental treatments on proline

The variance analysis showed that the proline concentration was significantly ($p<0.01$) affected by the water stress (Table 3). Proline concentrations increased significantly with increasing levels of drought stress. The concentration of proline in the DS_2 treatment (8.85 µmol g^{-1} FW) increased by 73.3% compared to the DS_0 (4.95 µmol g^{-1} FW) treatment (Table 4). Biochar led to an increase in proline by 6.55 µmol g^{-1} FW, but this increase was not significant compared to the control without biochar (B_0) (Table 4). The highest concentration of proline (8.82 µmol g^{-1} FW) was recorded for the DS_2B_1 treatment. The biochar application resulted in a 70.0% decrease in proline concentration compared to the control (soil without B) in non-drought stress (Table 5).

Superabsorbent dramatically decreased proline concentration by 21.4% compared to the control (SA_0), (Table 4). The interaction between drought stress and SA on proline content was significantly different. The SA decreased proline by 36.1%, 22.8% and 24.3% in DS_0, DS_1 and DS_2, respectively than the control (soil without SA) (Table 6).

3.5. *Effect of experimental treatments on electrolyte leakage* (EL %)

The electrolyte leakage (EL %) concentrations were significantly influenced by drought stress and SA application ($P<0.01$) (Table 3). EL concentrations in savory leaves increased with enhancing drought stress (46.6 to 52.8% in DS_0 and DS_2 treatments) (Table 4). The SA_1 treatment led to a reduction (15.1%) in electrolyte leakage than the control treatment (non –using SA), While the use of biochar led to a 1.2% increase in electrolyte leakage compared to the control treatment without the use of biochar, it was observed no significant difference between the B_1 and B_0 treatments (Table 4).

Table 4. Comparison of the average photosynthetic traits and non-enzymatic antioxidants of Sovary in different experimental treatments

Traits / Treatment	Chl **a**	Chl **b**	Total Chl	Carotenoids	Flavonoids	Polyphenol	Proline	El	RWC	DM
	(mg g^{-1} FW)				(mg QEg^{-1} Shoot DW)	(mg $galikg^{-1}$ FW)	(µmol g^{-1} FW)	%		(g $plant^{-1}$)
Years										
Y(1)	1.60a	0.42a	2.03a	0.52b	22.8a	12.6a	6.50a	46.5b	70.0a	11.9b
Y(2)	1.67a	0.47a	2.15a	0.59a	21.9a	12.0a	6.44a	51.2a	71.5a	24.0a
Drought stress										
DS_0	2.24a	0.56a	2.82a	0.76a	5.81c	7.69c	4.95c	46.6b	84.2a	24.8a
DS_1	1.68b	0.48b	2.17b	0.55b	25.3b	12.9b	5.88b	47.1b	69.6b	18.1b
DS_2	0.99c	0.29c	1.29c	0.36c	35.9a	17.3a	8.58a	52.8a	58.5c	11.0c
Biochar										
B_0	1.56b	0.40b	1.96b	0.52b	21.0b	11.4b	6.39a	48.5a	68.6b	15.7b
B1	1.72a	0.49a	2.22a	0.59a	23.7a	13.2a	6.55a	49.1a	72.9a	18.3a
Superabsorbent										
SA_0	1.21b	0.31b	1.53b	0.43b	21.1a	13.1a	7.24a	52.8a	66.5b	16.8b
SA_1	2.07a	0.58a	2.64a	0.69a	21.5a	12.5b	5.69b	44.8b	74.9a	19.1a

Y, years; DS_0, DS_1 and DS_2: drought stress at 100, 80 and 60% FC, respectively; B_0 (Non- biochar), B_1(10 ton/ ha); SA_0 (Non- Superabsorbent) and SA_1(6g/ plant), respectively; Chl: chlorophyll; Caro: carotenoids; Flav: flavonoid; Polyph: polyphenol; Pro: proline; EL: electrolyte leakage; RWC: relative water content; FW,fresh weight; DM: above-ground dry mass. Different letters in each column indicate significant differences among the treatments ($p < 0.05$, LSD test).

Table 5. The interaction of drought stress and biochar on the concentration of photosynthetic pigments and non-enzymatic antioxidants.

Traits	Chl a	Carotenoids	Total Chl	Flavonoids	Polyphenol	Proline
Treatment		(mg g^{-1} FW)		(mg QE g^{-1} Shoot DW)	(mg galik g^{-1} FW)	(µmol g^{-1} FW)
DS_0B_0	2.36a	0.71b	2.88a	5.61d	6.37d	5.61b
DS_0B_1	2.13b	0.82a	2.67b	6.03d	7.03d	3.30c
DS_1B_0	1.57d	0.55c	2.04d	22.1c	10.8c	6.06b
DS_1B_1	1.79c	0.56c	2.29c	28.5b	15.1b	5.71b
DS_2B_0	0.75f	0.31e	0.98f	35.2a	17.2a	8.34a
DS_2B_1	1.24e	0.41d	1.60e	36.5a	17.6a	8.82a

DS_0, DS_1 and DS_2: drought stress at 100, 80, and 60% FC, respectively; B_0 (Non- biochar), B_1(10 ton/ ha); Chl, Chlorophyll; respectively. Different letters in each column indicate significant differences among the treatments ($p < 0.05$, LSD test).

Table 6. The interaction of drought stress and superabsorbent on the concentration of Chl a, chl b, total chl, carotenoids and proline.

Traits	Chl a	Chl b	Total Chl	Carotenoids	Proline
Treatment			(mg/g FW)		(µmol/g FW)
DS_0SA_0	2.14b	0.50c	2.67b	0.70b	4.56c
DS_0SA_1	2.34a	0.63b	2.97a	0.82a	3.35d
DS_1SA_0	0.99d	0.26e	1.19d	0.34d	6.64d
DS_1SA_1	2.43a	0.70a	3.14a	0.18a	5.13c
DS_2SA_0	0.56e	0.18f	0.75e	0.26c	9.77a
DS_2SA_1	1.43c	0.39d	1.83c	0.46e	7.39b

DS0, DS1 and DS2: 100, 80 and 60% FC; SA0 and SA1: 0 and 6g plant-1 superabsorbent; Chl, chlorophyll; respectively. Different letters in each column indicate significant differences among the treatments ($p < 0.05$, LSD test).

3. 6. *Effect of experimental treatments on relative water content*

The DS, B, SA and the interaction of DS × B, and DS × SA significantly affected the relative water content (RWC) (Table 3). The RWC decreased with increasing drought stress (a 30.5% reduction in DS_2 treatment compared to DS_0) (Table 4). Biochar and SA treatments increased the RWC by 0.6% and 12.6% than the control treatments (non- using biochar and SA), respectively, in non-drought stress conditions (Table 4). The effect of SA × RWC was different in water stress treatments. There was no significant difference between the use of SA_1 and SA_0 at the non-drought stress treatment (DS_0). The SA_1DS_1 and SA_1DS_2 treatments increased RWC by 19.4 and 23.1% in comparison to the control (i.e., SA_0), respectively (Supplemental Table S3;Fig. 1). The effect of biochar application depended on the drought stress. The RWC declined simultaneously with the drought stress in the B_1 and B_0. There was no significant difference between B_1 and B_0 at the non-drought stress treatment (DS_0), (Supplemental Table S3;Fig. 2). However, biochar application at DS_1 and DS_2 resulted in a 15.6% and 11.9% increase in RWC compared to the control (soil without biochar), respectively (Fig. 2). In the non-drought stress treatment (DS_0), the application of biochar and SA had no impact on the relative water content, however, it led to an increase in RWC during moderate (DS_2) and severe (DS_3) stress conditions as shown in Figures 1 and 2.

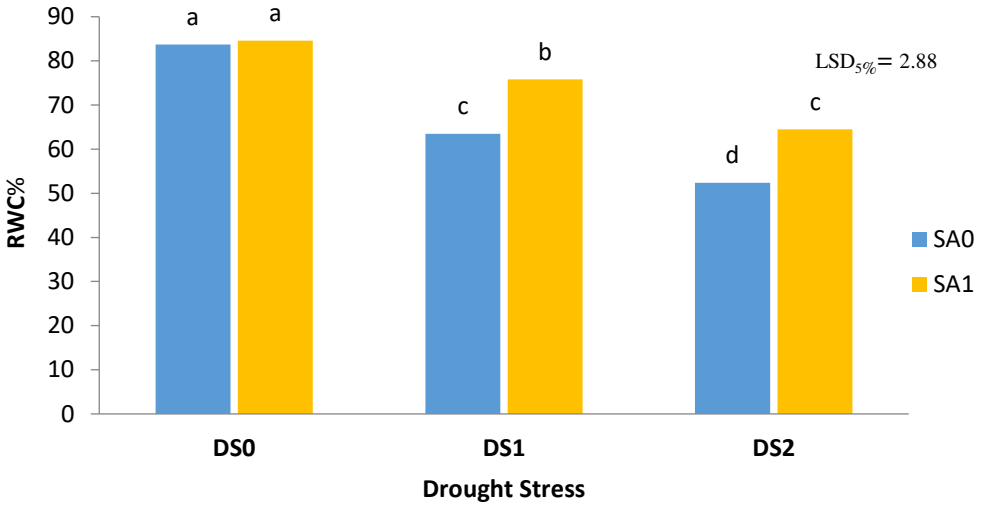

Fig. 1. Mean comparisons for the interaction of drought stress and superabsorbent on the relative water content (RWC) of savory.

DS_0, DS_1 and DS_2: 100, 80 and 60% field capacity; SA_0 (Non- Superabsorbent) and SA_1(6 g. $plant^{-1}$), sequentially. Different letters in each column indicate significant differences among the treatments ($p < 0.05$, LSD test).

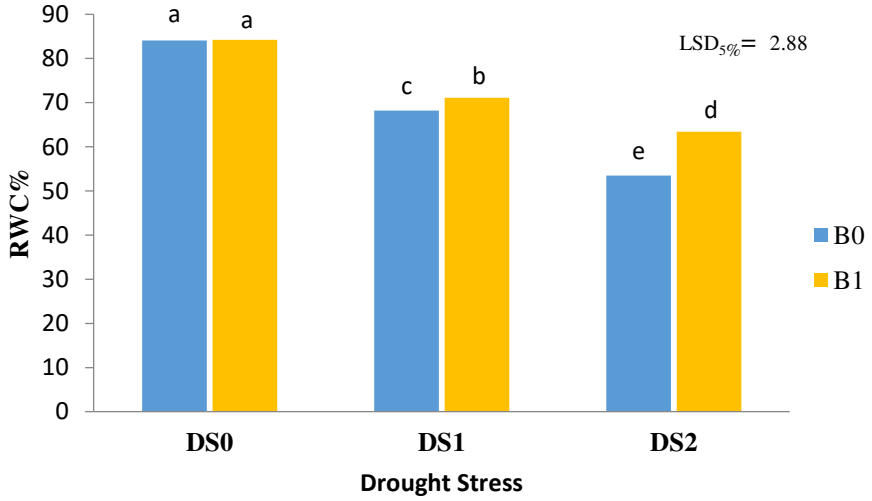

Fig. 2. Mean comparisons for the interaction of drought stress and biochar on the relative water content (RWC) of savory.

DS_0, $DS_1$and DS_2: 100, 80 and 60% field capacity; B_0 (Non- biochar), B_1(10 ton/ ha), sequentially. Different letters in each column indicate significant differences among the treatments ($p < 0.05$, LSD test).

4. DISCUSSION

Physiological indicators serve as measures to determine the physiological condition of plants, offering valuable insights into their growth, development, health, and overall performance. Physiological indicators are closely related to plant water status. When plants are exposed to drought stress, their physiological functions are affected, leading to reduced growth and yield. In this study, the use of cow manure biochar and SA significantly improved the physiological index of *S. rechingeri* under drought stress conditions. Our results show that the use of biochar increased the content of Chl *a,* Chl *b*, total Chl, carotenoid, antioxidants and RWC compared to treatments without biochar (B_0), (Table 4). A higher RWC under stress conditions indicates the plant's ability to tolerate water shortage. A decrease in RWC and leaf Chlorophyll content and an increase in the activity of non-enzymatic antioxidants (flavonoids, total phenol, proline) in the plant during drought conditions (Table 4), agree with other studies (Wang, 2014; Jaafar et al., 2012; Bettaieb et al., 2009). The presence of nitrogen, phosphorus, and a significant quantity of potassium in cattle manure biochar can increase the concentration of soil nutrients. The porous structure, negative charge, and cohesion forces present in biochar particles led to an increase in the concentration of nitrogen, phosphorus, and potassium in the soil (Hass et al., 2012). Biochar has been shown to increase the availability of phosphorus in soil by adsorbing it onto its surface and releasing it slowly over time (Glaser et al., 2002; Kookana et al., 2011). This can improve soil structure and water-holding capacity, as well as provide a habitat for beneficial microorganisms (Fan et al., 2015). Biochar has the potential to improve soil fertility and crop yields by affecting the uptake of nutrients by plants. The reduction of photostatic pigments can be explained by metabolic changes. The production of reactive oxygen species is one of the factors that reduce chlorophyll content during drought stress, which is followed by lipid peroxidation and chlorophyll degradation (Silva et al., 2007). The main causes of the decrease in carotenoid and chlorophyll synthesis under drought stress have been attributed to the decrease in the efficiency of carbon use and the increase in ethanol and lactate production (Ahanger et al., 2021). Our findings are similar to those of Ullah et al. (2023) who reported that by reducing nitrogen loss and increasing nitrogen absorption, biochar enhances the concentration of photosynthetic pigments. Drought stress decreased the amount of nitrogen in aerial parts (data not shown). The use of biochar and superabsorbent increased nitrogen by 31.8 and 30%, respectively, compared to the control (without biochar and SA) in conditions without drought stress (DS_0), (n= 3). The possible reason for the reduction of photosynthetic pigments during water stress can be attributed to the instability of protein complexes and the destruction of chlorophyll and chlorophyllase.The utilization of both nutrients and water can be enhanced through the use of biochar (Nanda et al., 2015). Additionally, Lima et al. (2018) have reported that the effectiveness of utilizing both nutrients and water can be improved by incorporating biochar. High nitrogen availability of biochar (1.79%) can increase the content of photosynthetic pigments (Supplemental Table S2). A decrease in total Chl content has been reported as a typical symptom of drought stress in various crops like milk thistle (*Silybum Marianum*) (Keshavarz Afshar et al., 2016) and sunflower (*Helianthus annuus*) (Gobadi et al., 2013), which limits photosynthesis. The limitation of photosynthesis caused by drought stress, especially in the treatment of DS_2, led to a decrease in biomass (Table 4). The reduction of photosynthetic pigments during drought stress may be caused by the instability of protein complexes and the breakdown of chlorophyll and chlorophyllase (Moller et al., 2007). Similarly, exposure of plants (*S. rechingeri*) to drought stress reduced fresh and dry weight (Beiranvandi et al., 2022), which was attributed to a decrease in chlorophyll content and photosynthetic rate. In addition, Misra & Srivastava (2000) reported that drought stress in mint (*Mentha spp*) plants reduces carbon dioxide exchange, nutrient uptake and chlorophyll levels, which in turn reduces dry matter synthesis. In agreement with other studies, including those by Novak et al. (2010), McCormack et al. (2013), and Beiranvandi et al. (2022), biochar has

been found to have a positive effect on leaf water potential and soil water retention. This, in turn, has been linked to an increase in various physiological processes such as photosynthesis. Additionally, these studies suggest that biochar can help mitigate the negative impacts of drought stress by stimulating Rubisco activity and pigment concentrations, which are believed to contribute to increased photosynthetic rates. The osmolyte index is a measure of the ability of a molecule to stabilize protein structures under conditions of stress, such as high temperatures or extreme pH. The relationship between osmolytes and physiological index is complex and multifaceted. Osmolytes can play a protective role in maintaining cellular function and preventing damage under stressful conditions. The findings of this study support the earlier research (Sigita et al., 2023) that the proline content remains consistent between the first and second year of the crop.

The accumulation of proline in drought-stressed plants indicates a functional mechanism for osmotic regulation, cell structure maintenance, and a drought stress adaptation strategy (Gunes et al., 2008). The beneficial effects of proline on enhancing drought resistance in various crops, such as fennel (*Foeniculum vulgare* Mill.) (Gholami Zali & Ehsanzadeh, 2018) and milk thistle (Keshavarz Afshar et al., 2016), have been previously documented. These findings align well with the results of the present study. The inhibition of oxygen free radicals is a crucial protective function of flavonoids against reactive oxygen radicals. Plants increase phenolic compounds in stress conditions due to the increase of free radicals so that they can initiate appropriate defense reactions. Some studies have reported an increase in flavonoids due to the use of biochar in plants (Różyło et al., 2017; Hashemi & Shahani, 2019). The increase in flavonoids can be explained by the relative humidity and the amount of nitrogen in the leaves (Yang et al., 2018; Shiwakoti., et al., 2016), and the biochar improves those properties. There is some evidence suggesting that the application of SA can improve plant growth and yield under water-limited conditions by increasing soil moisture availability and reducing water stress. Likewise, the application of SA regulated the drought stress conditions by wettening the root zone in the soil and reducing the quantity of proline (Su et al., 2017). Proline accumulation in plants has been linked to improved tolerance to various abiotic stresses by regulating osmotic balance and protecting cellular structures from damage (Gholami Zali & Ehsanzadeh, 2018). Therefore, SA application may induce proline accumulation in plants as a response to improved water availability and reduced stress. Drought stress causes peroxidation of fatty acids in the cell membrane by inducing oxidative stress and creating oxygen-free radicals (Cruz de Carvalho, 2008). This leads to increased membrane permeability and electrolyte leakage from plant cells. As a result, ions such as K^+ and Ca^{2+} leak out of the cells, disrupting cellular homeostasis (Fan et al., 2015). The concentrations of EL% were found to increase under drought stress. However, strengthening the antioxidant defense system through biochar modification led to the reduction of the harmful effects of drought stress. Furthermore, The SA can decrease EL by reducing humidity fluctuations. Changes in humidity levels can lead to the expansion and contraction of soil, resulting in the disintegration of soil aggregates. Additionally, research studies by Fan et al. (2015) and Fouladidorhani et al. (2020) have suggested that high concentrations of cations such as Ca, Mg, K, and other organic amendments can enhance soil fertility and structure. Liu et al. (2012) reported that the addition of SA resulted in increased absorption of nutrients, specifically N and K, as well as enhanced accumulation of dry matter. Additionally, the study found that water retention was positively affected by the use of SAP. Overall, reducing humidity fluctuations and adding organic amendments with high amounts of available cations can lead to improved SA, fertility, and soil structure.

5. Conclusion

Drought stress had a significant impact on the physiological characteristics of S. rechingeri in both years of the experiment. The decreased leaf RWC resulted in reduced photosynthesis, dry weight, and photosynthetic pigment content, as well as stomatal closure and reduced gas exchange with the environment. Drought stress also caused a notable increase in non-enzymatic antioxidants in the plant. Although there is limited knowledge about the amendment of cow manure biochar as a substrate, the study indicates that it could have a positive effect on certain physiological indexs of S. rechingeri when subjected to drought stress. This suggests that using cow manure biochar as a sustainable solution might enhance drought resistance. The combined use of biochar and superabsorbent proved more effective in mitigating the effects of drought stress on most traits. This can be attributed to their mutual effect on soil properties, which play a significant role in determining plant performance and nutrient absorption.

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