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The Morphological and Physiological Traits of Periwinkle (*Catharanthus roseus* L.) as an Ornamental-Medicinal Plant Species in Response to Salinity Stress and Biochar

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Abstract

Biochar is a sort type organic fertilizer derived from the pyrolysis of plant residuals and crop waste and is recommended for improving soil fertility and modifying saline soils. In this regard, the effect of biochar on moderating the effects of salinity stress on growth and some physiological traits *Catharanthus roseus* L. was explored in a factorial experiment based on a randomized complete block design with two treatments including salinity stress (0, 1000, 2000, and 3000 mg/kg NaCl) and biochar (0, 2, and 4%) in 3 replications, 36 plots, and 6 plants per plot. The study was conducted in pots in the spring of 2022. The results showed that salinity negatively influenced all studied morphological traits and relative water content (RWC) whereas biochar, especially at the rate of 2%, helped their preservation and improvement. With increasing the salinity level, the proline content and total soluble solids (TSS) increased versus the control. The highest level of proline (346.48 mmol/kg FW) and TSS (1.42°Brix) were recorded for $NaCl_{3000\ mg/kg}$ × $biochar_{0\%}$. A decline was recorded in malondialdehyde (MDA) with the application of 2% biochar at all four NaCl levels, but biochar at the rate of 4% failed to alleviate salinity effects at the highest level of NaCl (3000 mg/kg) and this treatment exhibited the highest MDA level. The highest activities of peroxidase (POD) (1.56 IU/g FW/min) and ascorbate peroxidase (APX) (9.40 IU/g FW/min) were observed in the treatment of $NaCl_{1000\ mg/kg}$ × $biochar_{2\%}$. With increasing the NaCl level (2000 and 3000 mg/kg), POD and APX activities decreased, which was accompanied by an increased accumulation of MDA. Based on the results, it can be concluded that by applying 2% biochar at the salinity level of 1000 or 2000 mg/kg, periwinkle plants with acceptable morphophysiological traits can be produced.

Keywords: Active carbon, Antioxidant enzymes, Environmental stress, Proline, Soil fertility.

INTRODUCTION

Soil fertility, water and nutrient availability, and the efficiency of the photosynthesis system are some factors influencing the ideal growth of plants. Salinity is the most important plant growth-limiting stress that adversely impacts soil fertility, water and nutrient uptake, chloroplast health, and photosynthesis rate, thereby disrupting the natural growth of the plant (Jimenez-Mejia *et al*., 2022; Safdar *et al*., 2019). Data shows that 50 percent of irrigated lands and 20 percent of total arable lands of the world are suffering from salinity and that there is a global deficiency of fresh water and non-saline soils for crop production. So, it is necessary to use huge saline water and soil resources for agricultural sustainability and continuation. The identification and cultivation of salinity-resistant or tolerant plants and the application of moderators of the adverse NaCl effects on soils and plants, e.g., active carbon or biochar, are effective and available solutions for preserving agricultural sustainability in saline regions (de Almeida Cartaxo *et al*., 2022; Rizwan *et al*., 2015; Luo *et al*., 2017).

Biochar is a carbon-based compound produced by the pyrolysis or burning of organic matter (plant residuals, agricultural and food waste, substrates and residuals of animal and poultry farming, and so on) at high temperatures (300-1000 °C) and in oxygen-deficient or oxygen-free conditions. Biochar has drawn the attention of many researchers and experts, especially in the agricultural and environmental sector, as an ideal method of agricultural waste recycling, soil modification, carbon sequestration, and the reduction of greenhouse gases (Wijitkosum, 2022; El Nahhas *et al*., 2021). Biochar application reportedly contributes to improving soil fertility by reducing pH, improving water retention capacity, increasing microbial diversity, and increasing nutrients in the root zone (Enders *et al*., 2012; Carter *et al*., 2013; Song *et al*., 2022; El Nahhas *et al*., 2021). In biochar-applied soils, stable particulates form, soil permeability, water retention capacity, and microflora increase, water availability to plants and nutrient cycles are regulated, and consequently, the uptake of nutrients required for plant growth increases (Song *et al*., 2022; Martinez-Gomez *et al*., 2022). Biochar has a high specific area and cation exchange capacity and can take up soluble nutrients and salts, thereby maintaining soil nutrients (Ali *et al*., 2017; Kookana *et al*., 2011). With the application of biochar in saline soils, Na and Cl uptake by plants increases. Therefore, the application of biochar in saline soils can be effective in partially mitigating the adverse effects of NaCl by Na uptake and mobilization to plant tissues. Biochar application in saline soils has been reported to improve cation exchange capacity, increase water and mineral uptake and retention, regulate stomatal opening and closure, and preserve and increase plant growth and development (Ekinci *et al*., 2022; Kanwal *et al*., 2018; Ali *et al*., 2017).

Yang *et al*. (2020) found that salinity stress (400 mM NaCl) reduced the growth of quinoa, but the application of 5% biochar at this salinity level reduced Na uptake and increased vegetative growth, plant height, fresh weight, and yield of this plant. Hussein Ibrahim *et al*. (2020) reported the significant interactive effect of biochar (0, 2.5, 5, and 10%) and salinity (0.26, 5.8, and 12.6 dS/m) on sorghum and found that the application of 5% biochar at the highest level of salinity increased plant height, leaf area, and plant fresh and dry weight. In Kanwal *et al*.'s (2018) study, the application of 1% and 2% biochar in saline and non-saline conditions increased the vegetative growth and physiological processes of wheat. By improving the soil condition, biochar caused an increase in fresh and dry weight of roots and shoots, leaf relative water content, chlorophyll content, and decreased the activity of superoxide dismutase and catalase enzyames of feverfew plants under water stress (Naeemi Golzard *et al*., 2023).

Ali and Majeed (2017) in a research investigated the effect of 0, 1, 2 and 3% biochar and 0, 140 and 280 mg/kg of nitrogen fertilizer in growing chrysanthemum and reported that with the application of 3% biochar, plant height, plant fresh weight, The number of leaves and flowers, stem diameter and flower diameter increased. Also, the efficiency of biochar improved in combination with nitrogen fertilizers.

Madagascar periwinkle (*Catharanthus roseus* L.) is an herbaceous perennial from the family of Apocynaceae that is used in the pharmaceutical industry for the anticancer alkaloids of its roots and shoots (vinblastine and vincristine) in addition to its ornamental applications. These compounds are, however, found in very small amounts in the leaves and roots of this plant, so its cultivation area should be extended to produce plenty of leaves and roots for extracting significant amounts of them. Nonetheless, owing to the increase in saline water and soil area in the world and the allocation of non-saline soils to the production of staple and strategic crops, there is a shortage of high-quality land and water for the production of medicinal and non-strategic plant species, like Madagascar periwinkle. So, we need to consider their cultivation in regions with saline water and soil (Kalanaki *et al*., 2022; Vu *et al*., 2022). In this regard, the present research aimed to shed light on the effect of different levels of NaCl on the morphological and physiological traits of Madagascar periwinkle and the effect of biochar on moderating the impact of salinity stress on this plant.

MATERIALS AND METHODS

The study was conducted in March-February, 2022 to study the effect of biochar and salinity stress on some morphophysiological traits of Madagascar periwinkle in a factorial experiment based on a randomized complete block design with two treatments in three replications, amounting to a total of 36 plots with 6 plants/plot. The treatments included biochar at three levels (0, 2, and 4% of soil weight) and salinity stress at four levels (0, 1000, 2000, and 3000 mg/kg NaCl). The soil used in the experiment was the basic soil composed of 25% garden soil and 75% perlite. The seeds used had vigor of 99% supplied from Parmis Institute, Mahallat, pure NaCl was procured from Merck, Germany, and rice husk biochar was bought from a reputed company of biofertilizers in Guilan province, Iran. The experiment commenced by sowing the seeds on March 24. Before sowing, they were disinfected with 1.5% sodium hypochlorite and 2 mg/L Benomyl for 5 seconds. They were, then, sown in transplanting trays containing the basic soil and watered every day until reaching the 4-leaf stage. Thirty-five days after sowing, the four-leaf seedlings were transplanted in plastic pots with a mouth diameter of 12 cm and a height of 10 cm containing 0.5 kg of basic soil (25 % garden soil and 75 % perlite) plus different levels of biochar and NaCl. After transplanting, the plants were watered every day for seven days after which the irrigation was reduced to once every three days. At each irrigation step, the plants were watered with 100 mL of water/plant. It should be noted that the seeds were sown in a commercial greenhouse in Isfahan province, Iran and after transplanting, the pots were kept in open space outside the greenhouse until the end of the experiment (4 months).

The physical and chemical characteristics of the substrates used the water and soil laboratory of Isfahan Azad University were measured and the obtained data are shown in table 1.

Table 1. The physical and chemical characteristics of the substrates used in the research.

Media	Soil texture	pH	EC (ds/m)	Organic matter (%)	HCO_3 (meq/l)	N (%)	P (mg/kg)	K (%)	Cl (meq/l)	Na (%)
$NaCl_0 \times Biochar_{0\%}$	Clay Loam	7.01	2.8	1.8	11	0.52	115.5	0.33	10	0.20
$NaCl_0 \times Biochar_{2\%}$	Clay Loam	7.16	3.0	3.1	10	0.63	135.2	0.88	15	0.21
$NaCl_0 \times Biochar_{4\%}$	Sandy Clay Loam	7.30	3.1	3.3	15	0.52	129.4	0.103	15	0.21
$NaCl_{1000\ mg/kg} \times Biochar_{0\%}$	Clay Loam	7.34	12.3	1.01	14	0.41	119.5	0.29	85	0.26
$NaCl_{1000\ mg/kg} \times Biochar_{2\%}$	Clay Loam	7.44	13.8	2.8	10	0.63	127.9	0.42	90	0.32
$NaCl_{1000\ mg/kg} \times Biochar_{4\%}$	Sandy Clay Loam	7.55	15.4	1.4	15	0.52	130.17	0.69	95	0.37
$NaCl_{2000\ mg/kg} \times Biochar_{0\%}$	Clay Loam	7.38	15.2	0.93	20	0.31	109.6	0.29	100	0.40
$NaCl_{2000\ mg/kg} \times Biochar_{2\%}$	Clay Loam	7.49	16.1	2.0	15	0.47	129.06	0.39	115	0.44
$NaCl_{2000\ mg/kg} \times Biochar_{4\%}$	Sandy Clay Loam	7.65	20	1.1	17	0.40	120.8	0.44	170	0.53
$NaCl_{3000\ mg/kg} \times Biochar_{0}$	Clay Loam	7.55	15.7	0.53	29	0.27	107.3	0.22	180	0.53
$NaCl_{3000\ mg/kg} \times Biochar_{2\%}$	Clay Loam	7.70	20.5	1.7	20	0.49	120.9	0.35	110	0.64
$NaCl_{3000\ mg/kg} \times Biochar_{4\%}$	Sandy Clay Loam	7.78	39.7	0.9	25	0.40	117.7	0.78	380	0.79

Assessment of traits

Morphological traits

The morphological traits were assessed on three randomly selected periwinkle plants. The number of leaves was counted during the experiment and the number of flowers was counted during the flowering period, and their averages were reported. The duration of the flowering period was determined as the number of days from the emergence of the first flower until the end of the flowering period. At the end of the experiment, the roots and shoots of the plants were dissected from the crown. The roots were washed with water, and then, the length of the longest root was measured with a ruler. After the fresh weights were recorded, the roots and shoots were oven-dried at 75 °C for 48 hours. The fresh and dry weights of the roots and shoots were measured with a 0.001g-precision digital scale. The specific weight of the leaves was determined as the leaf fresh weight divided by its area.

Physiological traits

Leaf relative water content (RWC) was measured by the weight measurement in distilled water. In the end, the following equation was applied to estimate leaf RWC (Ritchie and Nguyen, 1990):

$$RWC = [(FW - DW)/(TW - DW)]/100$$

in which RWC represents leaf relative water content (%), FW represents leaf fresh weight (g), DW represents leaf dry weight (g), and TW represents leaf turgid weight (g).

The proline content was determined by Bates *et al*.'s (1973) method using the standard curve of pure proline. So, 0.5 g of fresh leaf was extracted using 5 mL of sulfosalicylic acid 3%. Then, 2 mL of the plant extract was mixed with 2 mL of the ninhydrin acid reagent and 2 mL

of pure acetic acid. It was then put in a water bath at 100 °C and was immediately transferred to an ice bath. Then, 6 mL of toluene was added to the sample, and they were mixed. In the end, the absorbance of the solution was read at 520 nm with spectrophotometry (Shimadzu UV-120-02, Japan) at the level of plant samples and standard samples, and the proline content was calculated by the standard curve.

Total soluble solids (TSS) were measured by Irigoyen *et al*.'s (1992) method for which an alcoholic extract was first prepared. Then, the supernatant of the samples was separated after three steps of centrifuging at 3500 rpm. Then, 0.1 mL of the resulting supernatant was mixed with 3 mL of the anthrone solution and kept in a hot bath at 100 °C until the emergence of a colorful material. In the next step, the absorbance of the solution was read at 665 nm with spectrophotometry (Shimadzu UV-120-02, Japan), and the standard glucose curve was used to calculate TSS.

The malondialdehyde (MDA) content was determined by Heath and Parker's (1968) method for which 0.5 g of the leaf extract was prepared and added with 1 mL of potassium phosphate buffer 50 mM (pH = 7) and 0.5 M of ethylenediaminetetraacetic acid (EDTA). The resulting extract was centrifuged at 14000 rpm at 4 °C for 20 minutes, and the supernatant was extracted with a sampler. This procedure was repeated three times. Then, 200 µL of the resulting sample was mixed with 1000 µL of trichloroacetic acid (TCA) 20% containing 0.5% of thiobarbituric acid (TBA). The samples were put in a hot bath at 95°C for 30 minutes and were immediately cooled down in ice. After 10 minutes of centrifugal at 10500 rpm, the red material of the supernatant (MDA-TBA) was separated and its absorbance was read at 532 and 600 nm with spectrophotometry (Shimadzu UV-120-02, Japan). Finally, the following equation was applied to calculate the MDA content of the periwinkle leaf texture:

$$\text{MDA (nmol/g FW)} = A_{532\ nm} - A_{600\ nm}$$

Antioxidant enzymes activity

To prepare the enzymatic extract, 0.5 g leaf disks were extracted with liquid nitrogen one week after the emergence of the first flower. Then, 5 mL of 100 mM potassium phosphate buffer (pH = 7), 0.5 mM EDTA, and 1 mM polyvinylpyrrolidone (PVP) 1% were added to the extract. The samples were centrifuged at 15000 rpm at 4°C for 30 minutes. The supernatant was separated and used as the enzymatic extract (Sairam and Srivastava, 2002).

Peroxidase (POD) activity was measured by MacAdam *et al.* (1992) method for which 100 µL of the enzymatic extract was mixed with 450 µL of guaiacol and 450 µL of H_2O_2. The absorbance of the resulting solution was read at 470 nm by spectrophotometry (Shimadzu UV-120-02, Japan).

Ascorbate peroxidase (APX) activity was determined by Nakano and Asada's (1987) method. First, the reaction mixture that was composed of 100 µL of the enzymatic extract, 1 mM of hydrogen peroxide, 50 mM of potassium phosphate buffer (pH = 7), and 0.25 mM ascorbate 10 mM was prepared. The absorbance was then read at 290 nm by spectrophotometry (Shimadzu UV-120-02, Japan).

Statistical analysis

All data were analyzed by the statistical software suite of SPSS 19, and the means were compared by Duncan's multiple range test.

RESULTS

Morphological traits

According to the analysis of variance (ANOVA), the interactive effect of "salinity × biochar" was significant ($P < 0.01$) on leaf number, specific leaf weight, root length, and root and shoot fresh and dry weight. Also, the interactions of the experimental treatments were significantly ($P < 0.05$) different for flower number and flowering period duration (Table 2).

Table 2. Analysis of variance for the effect of salinity and biochar on some morphological traits *Catharanthus roseus* L.

S.o.V	df	MS								
		Leaf no.	SLW	Flower no.	FPL	Root length	Root FW	Root DW	Shoot DW	Shoot FW
NaCl (S)	3	16.02**	1.91**	4.44**	43.44**	46.93**	1.37**	0.033**	0.004**	3.97**
Biochar (B)	2	75.08**	6.47**	16.71**	82.63**	89.32**	1.35**	0.040**	0.063**	2.19**
S × B	6	4.93**	1.35**	2.31*	6.43*	22.50**	3.50**	0.099**	0.040**	2.18**
Error	22	0.51	0.15	0.64	2.00	0.65	0.02	0.002	0.0008	0.05
CV (%)		4.40	14.50	24.84	18.84	5.90	4.48	10.77	5.33	5.57

* and **: Significant at the $P < 0.05$ and $P < 0.01$ levels based on Duncan's multiple range tests, respectively. SLW: Specific leaf weight; FPL: Flowering period length; FW: Fresh weight; DW: Dry weight.

Leaf number and specific leaf weight

As the comparison of means revealed, these two traits were lower at higher NaCl levels. But, the application of biochar (especially at the rate of 2%) significantly alleviated the adverse effects of NaCl on these traits. At the highest salinity level (3000 mg/kg NaCl), biochar at the rate of 4% did not differ from the control ($NaCl_{3000\ mg/kg} \times biochar_{0\%}$) significantly and had the lowest leaf number (12.40 leaves) and specific leaf weight (1.34 mg/cm^2) among all treatments (Table 3).

Table 3. The comparison of the mean effect of different treatments on the morphological traits of *Catharanthus roseus* L.

	Leaf no.	SLW (mg/cm^2)	Flower no.	FPL (day)	Root length (cm)	Root FW (g)	Root DW (g)	Shoot FW (g)	Shoot DW (g)
$NaCl_0 \times Biochar_{0\%}$	18.63^{b}	3.59^{ab}	4.44^{bc}	10.35^{c}	15.60^{c}	3.70^{bc}	0.54^{b}	4.57^{bc}	0.56^{cd}
$NaCl_0 \times Biochar_{2\%}$	20.02^{a}	4.11^{a}	5.89^{a}	13.88^{a}	12.67^{def}	4.04^{a}	0.62^{a}	5.29^{a}	0.73^{a}
$NaCl_0 \times Biochar_{4\%}$	19.07^{ab}	3.61^{ab}	5.32^{ab}	9.35^{d}	13.57^{d}	3.79^{bc}	0.55^{ab}	4.71^{b}	0.60^{c}
$NaCl_{1000\ mg/kg} \times Biochar_{0\%}$	15.9^{c}	2.67^{c}	2.39^{def}	7.57^{e}	12.47^{def}	3.30^{d}	0.38^{c}	4.13^{d}	0.53^{d}
$NaCl_{1000\ mg/kg} \times Biochar_{2\%}$	18.27^{b}	3.39^{b}	3.69^{cd}	11.79^{b}	16.50^{c}	3.90^{ab}	0.55^{ab}	5.18^{a}	0.66^{b}
$NaCl_{1000\ mg/kg} \times Biochar_{4\%}$	18.24^{b}	2.72^{c}	3.36^{cde}	6.39^{f}	13.33^{de}	3.60^{c}	0.39^{c}	4.25^{cd}	0.58^{cd}
$NaCl_{2000\ mg/kg} \times Biochar_{0\%}$	14.23^{d}	2.19^{cd}	2.32^{def}	6.08^{f}	12.00^{ef}	2.55^{f}	0.27^{d}	3.30^{fg}	0.50^{de}
$NaCl_{2000\ mg/kg} \times Biochar_{2\%}$	16.42^{c}	2.40^{c}	2.98^{cf}	6.37^{f}	20.83^{a}	3.10^{de}	0.31^{d}	3.74^{e}	0.53^{d}
$NaCl_{2000\ mg/kg} \times Biochar_{4\%}$	14.64^{d}	2.22^{cd}	2.33^{def}	6.19^{f}	17.93^{b}	2.95^{e}	0.30^{d}	3.64^{ef}	0.51^{de}
$NaCl_{3000\ mg/kg} \times Biochar_{0}$	12.60^{e}	1.35^{e}	1.77^{f}	3.72^{j}	6.80^{h}	1.33^{i}	0.12^{e}	2.46^{h}	0.33^{g}
$NaCl_{3000\ mg/kg} \times Biochar_{2\%}$	13.97^{d}	1.91^{de}	2.20^{def}	5.78^{g}	11.67^{f}	2.02^{g}	0.28^{d}	3.11^{g}	0.40^{f}
$NaCl_{3000\ mg/kg} \times Biochar_{4\%}$	12.40^{e}	1.34^{e}	2.10^{ef}	4.54^{h}	9.97^{g}	1.64^{h}	0.25^{d}	2.56^{h}	0.39^{f}

*In each column, averages that have at least one common letter are in statistically similar groups with Duncan's multiple range tests at the 5% possibility. SLW: Specific leaf weight; FPL: Flowering period length; FW: Fresh weight; DW: Dry weight.

Flower number and flowering period duration

As the NaCl level was increased, the number of flowers decreased. The lowest number (1.77 flowers) was related to $NaCl_{3000\ mg/kg}$ × $biochar_{0\%}$). The highest number of flowers (5.89 flowers) was observed in non-saline conditions treated with 2% biochar. In saline conditions, the application of biochar, especially at the rate of 2%, increased flower number versus its non-application (Table 3). The negative effect of salinity was recorded on the duration of the flowering period, but biochar at the rate of 2% mitigated the effects of salinity at all four levels on this trait. The longest and shortest flowering periods were 13.88 and 3.72 days recorded for the treatments of $NaCl_{0\ mg/kg}$ × $biochar_{2\%}$ and $NaCl_{3000\ mg/kg}$ × $biochar_{0\%}$, respectively (Table 3).

Root length

Based on the comparison of means, the root length decreased with increasing the salinity level and the application of biochar in non-saline conditions. The application of biochar increased this trait at the NaCl levels of 1000, 2000, and 3000 mg/kg. The plants treated with biochar 2% exhibited the longest roots at all three levels of NaCl. The maximum root length (20.83 cm) was for the treatment of $NaCl_{2000\ mg/kg}$ × $biochar_{2\%}$ and the minimum (6.80 cm) to the highest salinity level and no biochar application (Table 3).

Root and shoot fresh and dry weights

The comparison of means for these traits showed that the increase in the NaCl level decreased these traits and that the application of biochar alleviated this reducing effect. As is observed in table 3, the application of biochar at the rate of 2% was related to the highest root and shoot fresh and dry weights at all four levels of NaCl, so it is a good treatment to alleviate the negative effects of salinity on root and shoot growth (Table 3).

Physiological traits

According to the results of ANOVA, the interaction of "salinity × biochar" was significant ($P < 0.01$) for RWC, TSS, proline, MDA, and POD and APX activities (Table 4).

Table 4. Analysis of variance for the effect of salinity and biochar on some physiological traits of *Catharanthus roseus* L.

S.o.V	df	MS					
		RWC	TSS	Proline	MDA	POD	APX
NaCl (S)	3	166.7**	0.18**	8546**	0.41**	0.27**	53.24**
Biochar (B)	2	203.1**	0.07*	7950**	0.32**	0.24**	6.66**
S × B	6	350.3**	0.08**	33411**	0.31**	0.16**	18.57**
Error	22	1.10	0.02	58.52	0.002	0.0006	0.006
CV (%)		1.85	13.02	5.35	3.97	2.49	1.63

*, ** and ^{ns}: significant at $P < 0.05$, $P < 0.01$ and insignificant based on Duncan's multiple range tests, respectively. RWC: Relative water content; TSS: Total soluble solids; MDA: Malondialdehyde; POD: Peroxidases; APX: Ascorbate peroxidase.

Leaf relative water content (RWC)

The comparison of means revealed that RWC decreased with increasing the salinity level, but the application of biochar alleviated the negative effects of NaCl on RWC at its all four levels. The biochar rate of 2% outperformed its 4% rate in RWC retention. The maximum and minimum RWC among all treatments was obtained from $NaCl_{0\ mg/kg}$ × $biochar_{2\%}$ (70.54%) and $NaCl_{3000\ mg/kg}$ × $biochar_{0\%}$ (36.98%), respectively (Table 5).

Table 5. The comparison of the mean effect of different treatments on some physiological traits of *Catharanthus roseus* L.

	RWC (%)	TSS (°Brix)	Proline (mmol kg^{-1} F.W.)
$NaCl_0$ × $Biochar_{0\%}$	65.09^{b}	0.87^{de}	88.74^{h}
$NaCl_0$ × $Biochar_{2\%}$	70.54^{a}	0.84^{de}	55.04^{i}
$NaCl_0$ × $Biochar_{4\%}$	66.85^{b}	0.78^{e}	56.99^{i}
$NaCl_{1000\ mg/kg}$ × $Biochar_{0\%}$	54.49^{d}	0.96^{bcd}	105.43^{g}
$NaCl_{1000\ mg/kg}$ × $Biochar_{2\%}$	66.78^{b}	0.92^{bcd}	89.71^{h}
$NaCl_{1000\ mg/kg}$ × $Biochar_{4\%}$	58.83^{c}	0.95^{bcd}	103.32^{g}
$NaCl_{2000\ mg/kg}$ × $Biochar_{0\%}$	53.39^{de}	1.23^{ab}	168.93^{d}
$NaCl_{2000\ mg/kg}$ × $Biochar_{2\%}$	54.77^{d}	1.07^{bcd}	119.68^{f}
$NaCl_{2000\ mg/kg}$ × $Biochar_{4\%}$	54.32^{d}	1.06^{bcd}	143.82^{e}
$NaCl_{3000\ mg/kg}$ × $Biochar_0$	36.98^{g}	1.42^{a}	346.48^{a}
$NaCl_{3000\ mg/kg}$ × $Biochar_{2\%}$	52.36^{e}	1.14^{bc}	182.21^{c}
$NaCl_{3000\ mg/kg}$ × $Biochar_{4\%}$	46.76^{f}	1.17^{bc}	255.28^{b}

*In each column, averages that have at least one common letter are in statistically similar groups with Duncan's multiple range tests at 1% possibility. RWC: Relative water content; TSS: Total soluble solids.

Total soluble solids (TSS)

Based on the comparison of means, TSS in the periwinkle leaves increased with increasing the NaCl level, whereas the application of biochar at all four levels of NaCl reduced TSS. The highest TSS was obtained from $NaCl_{3000\ mg/kg}$ × $biochar_{0\%}$ (1.42 °Brix) and $NaCl_{2000\ mg/kg}$ × $biochar_{0\%}$ (1.23°Brix), not differing from one another significantly. The lowest was 0.78°Brix recorded by $NaCl_{0\ mg/kg}$ × $biochar_{4\%}$ (Table 5).

Proline

Table 5 demonstrates that the proline content was the lowest in non-saline conditions, but it started to increase with the increase in the NaCl level and culminated in the treatment of $NaCl_{3000\ mg/kg}$ × $biochar_{0\%}$ (346.48 mmol/kg FW). The application of biochar at the rates of 2% and 4% reduced the proline content at all four levels of NaCl versus its non-application. However, its 2% rate was more effective than its 4% rate in reducing the proline content in saline conditions (Table 5).

Malondialdehyde (MDA)

Fig. 1 displays that MDA increased with increasing the NaCl level. The application of 2% and 4% biochar at the NaCl levels of 0, 1000, and 2000 mg/kg reduced the MDA accumulation versus its non-application. The 2% rate of biochar outperformed its 4% rate at all three NaCl levels so that the lowest MDA was related to $NaCl_{0\ mg/kg}$ × $biochar_{2\%}$ (0.36 mmol/kg FW) and $NaCl_{1000\ mg/kg}$ × $biochar_{2\%}$ (0.84 mmol/kg FW), respectively. At the highest level of NaCl, biochar 2% reduced MDA significantly versus its non-application, but when biochar was applied at the rate of 4%, MDA increased significantly and reached its maximum level (1.60 mmol/kg FW) among all treatments (Fig. 1).

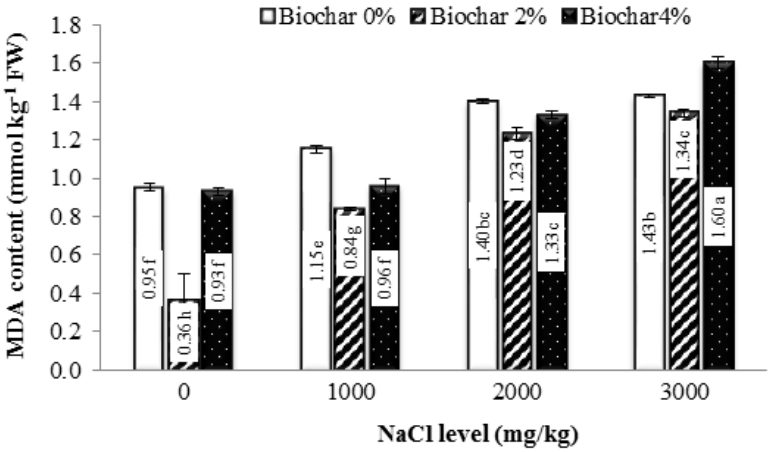

Fig. 1. The interactive effects of "NaCl × biochar" on the accumulation of malondialdehyde (MDA) content in the periwinkle leaves.

Peroxidase (POD) activity

Based on the comparison of means for the interactive effects of the experimental treatments on the POD activity, the activity of this enzyme significantly declined with increasing the salinity level. The application of biochar at the rates of 2% and 4% significantly increased the POD activity at the salinity levels of 0, 1000, and 3000 mg/kg NaCl. The maximum POD activity was 1.45 IU/g FW/min recorded by the treatment of $NaCl_{1000\ mg/kg}$ × $biochar_{2\%}$. There was not a significant difference among the biochar rates of 0%, 2%, and 4% at the salinity level of 2000 mg/kg. In general, 2% biochar was more successful in increasing the POD activity at all four NaCl levels. The efficiency of 4% biochar decreased with increasing the NaCl level and reached its lowest level at the salinity level of 3000 mg/kg. This treatment had the lowest POD activity (0.58 IU/g FW/min) among all treatments (Fig. 2).

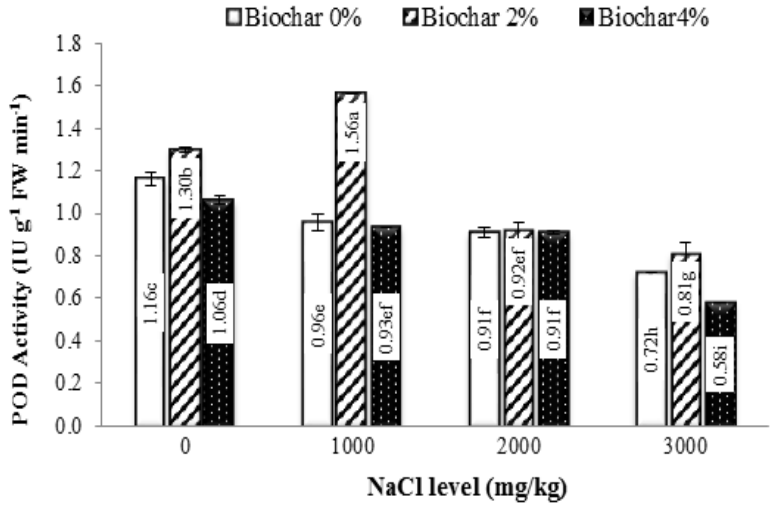

Fig. 2. The interactive effects of "NaCl × biochar" on peroxidase (POD) activity in the periwinkle leaves.

Ascorbate peroxidase (APX) activity

Based on Fig. 3, the application of biochar at the rates of 2% and 4% in non-saline conditions decreased and increased the APX activity versus the control, respectively; but the reverse happened at the salinity level of 1000 mg/kg so that 2% biochar increased this trait, but 4% biochar decreased it. The application of biochar at the salinity level of 2000 mg/kg significantly increased the APX activity versus the control ($NaCl_{2000\ mg/kg}$ × $biochar_{0\%}$). At the salinity level of 3000 mg/kg, no significant differences were observed among the treatments. In total, the highest APX activity among all treatments was related to $NaCl_{1000\ mg/kg}$ × $biochar_{2\%}$ (9.40 IU/g FW/min). The treatments of $NaCl_{3000\ mg/kg}$ × $biocahr_{0,2,\ and\ 4\%}$, as well as $NaCl_{2000\ mg/kg}$ × $biochar_{0\%}$, had the lowest APX activities respectively, but they did not differ from one another significantly (Fig. 3).

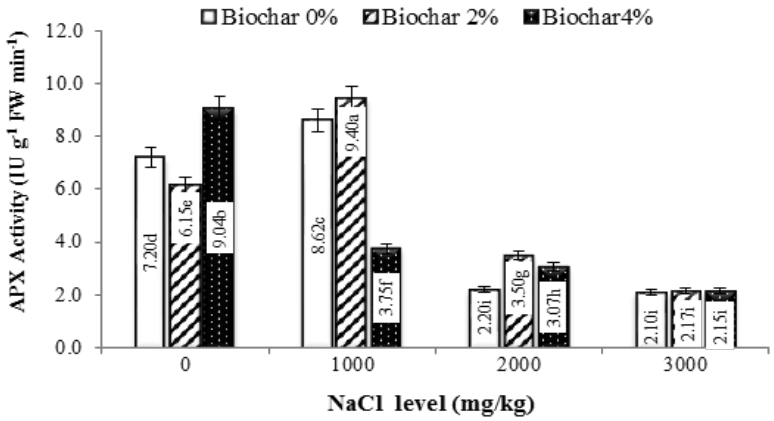

Fig. 3. The interactive effects of "NaCl × biochar" on ascorbate peroxidase (APX) activity in the periwinkle leaves.

DISCUSSION

Salinity is one of the most important environmental stresses and a factor limiting plant growth and development. The ion imbalance in saline soils disrupts the natural performance of the plant's metabolic system. The over-existence of Na^+ and Cl^- ions in saline soils is a source of disturbance in the uptake of essential elements and water. The increase in ROS, the damage to membranes and macromolecules, the increase in membrane permeability and electrolyte leakage, the damage to the performance of the photosynthesis system, and the disruption in the plant's hormone metabolism are some mechanisms by which salinity stress disrupts the natural growth and development of the plant, accelerates the shedding of the plant organs, and causes early senescence and plant death (Farhangi Abriz and Torabian, 2017; El Nahhas *et al*., 2021; Al Tabbal *et al*., 2023). Previous research has also reported the negative effect of salinity on the morphological, physiological, and biochemical traits of various plant species (Ali *et al*., 2017; Farhangi Abriz and Torabian, 2017; Al Tabbal *et al*., 2023). Likewise, we recorded the destructive effect of NaCl on leaf number and area, flower number, flowering period, root and shoot fresh and dry weight, and physiological attributes of the Madagascar periwinkle, which were not unexpected considering substrate analysis and the negative effects of salinity on substrate characteristics. Unlike salinity, the application of biochar to the soil improved these characteristics and partially alleviated the negative effect of salt on the substrates (Table 2). As was mentioned in the Results section, biochar mitigated the adverse impact of NaCl on the

periwinkles, which can mainly be attributed to its effect on improving substrate characteristics (Ali *et al*., 2017).

The loss of water potential in the soil, osmotic stress, and the loss of plant access to water are some mechanisms by which salinity disrupts or reduces plant growth and development rate. Owing to its high specific area and cation exchange, biochar can absorb salt from the root rhizosphere, dilute salt in the soil solution by improving the physical and chemical properties of the soil, such as its water retention capacity, and prevent osmotic stress by preserving root access to water, thereby creating tolerable conditions for plant growth in saline soil (Ali *et al*., 2017; Al Tabbal *et al*., 2023). El Nahhas *et al*. (2021) reported that salinity contributed to reducing water uptake by the plant, reducing water mobilization from the roots to the leaves, reducing cell division, and consequently, reducing vegetative and reproductive growth. Biochar increases the water retention capacity of the soil and water absorption and mobilization from the root to the shoot and helps preserve cell turgor and photosynthesis performance, resulting in the increased growth of the plant. Ali *et al*. (2017) ascribed the positive effect of biochar on the vegetative growth and yield of the plant to its effect on soil attributes, increasing water productivity, and reducing root sensitivity to oxidative stress. All these factors together reduce ABA synthesis by the roots and maintain stomatal conductance, which is accompanied by an increase in photosynthesis and vegetative growth. This is consistent with Ekinci *et al*. (2022) who reported that biochar suppressed the negative effect of salinity on cabbage by reducing NaCl concentration, increasing nutrient availability, preventing ROS over-production, adjusting ABA synthesis, preserving stomatal conductance, and regulating the activity of antioxidant enzymes. There are reports as to the increased growth and yield of Kochia (Al Tabbal *et al*., 2023) and the increased vegetative and reproductive growth of faba beans (Farhangi Abriz and Torabian, 2017) and cabbage (Ekinci *et al*., 2022) with the application of biochar in saline conditions, which agrees with our findings. Also, in the Naeemi Golzard *et al*. (2023) research, the application of 5% biochar to the soil under water stress conditions increased the fresh and dry weight of the shoots and roots of rose plants. Ali and Majeed (2017) reported that the application of 3% biochar in chrysanthemum cultivation medium improves the vegetative and reproductive growth of this plant.

The leaf RWC of the periwinkles decreased in salinity stress and increased with biochar application. In El Nahhas *et al*.'s (2021) study too, RWC decreased in plants grown in salinity stress significantly, whereas the treatment with biochar significantly increased this trait in saline conditions. It seems that RWC preservation and increase with the application of biochar is related to the effect of this compound on increasing water retention capacity and water productivity. Ekinci *et al*. (2022) reported that the cabbage plants grown in saline conditions had lower RWC than the control and that the application of biochar in saline conditions increased it significantly. These researchers argue that salinity reduces cell turgor and RWC in plants by inducing osmotic stress and that the application of biochar improves leaf RWC by modifying the soil and increasing water availability and retention capacity. An increase has been reported in RWC with biochar application in corn (Haider *et al*., 2015), feverfew plants (Naeemi Golzard *et al*., 2023), and tomato (Akhtar *et al*., 2014) under environmental stresses.

The accumulation of compatible osmolytes in stressful conditions contributes to keeping cell health and structure by stabilizing and preserving the structure of macromolecules and membranes and reducing the osmotic potential of the cell. The adjustment and accumulation of osmolytes, like prolines, total soluble sugars, and glycine betaine, is one of the crucial

defensive mechanisms of plants against environmental stresses. Proline is the most important non-enzymatic antioxidant in living organisms that plays a fundamental role in suppressing the detrimental effects of ROS. Prolines and total soluble sugars help the preservation of cell turgor and the natural activity of the cell in stressful conditions by establishing osmotic balance and adjusting water flow (Farhangi Abriz and Torabian, 2017). In the present work, salinity increased the concentrations of proline and soluble sugars, but the application of biochar in both saline and non-saline environments reduced them, which marks the mitigation of the stress imposed on the plants. El Nahhas *et al*. (2021) reported that in saline conditions, the proline content of faba beans significantly increased versus the control (non-saline conditions) and maximized at the highest salinity level, whereas the application of biochar reduced the proline concentration in the plant tissues significantly. Ekinci *et al*. (2022) reported a decrease in ROS, soluble sugars, and MDA with the application of 5% biochar in cabbage plants grown in saline conditions.

In saline conditions, more solutes accumulate in plant cells, entailing oxidative stress and the increased synthesis of ROS and subsequently, the increased permeability of membranes. ROS accumulation and over-activity result in the degradation of membrane lipids as a critical component of cell membrane structure. MDA is a major factor to assess the extent of damage to membrane lipids or lipid peroxidation, which increases in stressful conditions (Al Tabbal *et al*., 2023). In the present research, MDA significantly increased with the increase in the salinity level, and the application of biochar, especially at the rate of 2%, reduced lipid peroxidation at all four NaCl levels. A similar result was reported by El Nahhas *et al*. (2021) for faba beans. Researchers argue that biochar reduces peroxidation and increases plant growth in both saline and non-saline conditions by absorbing salts and reducing their translocation to plant tissues, and also by preventing oxidative stress and adjusting the activity of antioxidant enzymes (Ali *et al*., 2017; El Nahhas *et al*., 2021).

The activity of antioxidant enzymes is necessary for keeping osmotic balance and inhibiting ROS activity. In stressful conditions, plants activate their enzymatic and non-enzymatic defensive systems to scavenge excessive ROS, thereby reducing oxidative stress and preserving the health of membranes and macromolecules (El Nahhas *et al*., 2021). APX and POD are antioxidant, defensive, and ROS-scavenger enzymes. APX plays a key role in ROS suppression. On the other hand, POD decomposes ROS into water and oxygen in apoplastic space and vacuoles. The activity of these enzymes in stressful conditions protects the plants against the fatal effects of salt by osmotic adjustment and the preservation of membrane structure (Farhangi Abriz and Torabian, 2017). It was revealed in the present research that the increased activity of POD and APX contributes to protecting cell structure and reducing lipid peroxidation (MDA). Farhangi Abriz and Torabian (2017) suggest that biochar reduces ROS levels by reducing the toxic effects of Na^+ and Cl^-, so the application of biochar at some salinity levels reduces the activity of antioxidant enzymes compared to their activity in stressful conditions. There are reports about the increase in MDA in salinity and the decrease in its accumulation with the application of biochar to cabbage (Ekinci *et al*., 2022) and the decrease in ROS, MDA, and the activity of antioxidant enzymes in faba beans with the application of biochar in saline conditions (Farhangi Abriz and Torabian, 2017). Ali *et al*. (2017) argue that biochar helps scavenge ROS, protect cell structure, reduce MDA, and adjust the activity of antioxidants by alleviating the stress imposed on the plant and preventing oxidative stress. In the present study, the application of biochar at the rate of 4% at the NaCl levels of 0, 1000, and

3000 mg/kg was related to lower POD activity than that in the control (no biochar application). However, the comparison of the activity of antioxidant enzymes (POD and APX) with MDA shows that even biochar could not adjust enzymatic activity and scavenge ROS at high salinity levels (2000 and 3000 mg/kg NaCl), and these factors increase MDA accumulation. The elevated activity of POD and APX at lower salinity levels can be ascribed to the higher sensitivity of these enzymes and the activation of cells to cope with oxidative stress.

CONCLUSION

In summary, it can be concluded that biochar increased periwinkle tolerance to saline conditions and increased its vegetative and reproductive growth, as well as plant fresh and dry weight, through improving substrate features. From the perspective of physiological traits, the application of biochar improved RWC and reduced TSS, prolines, and lipid peroxidation in periwinkle leaves, reflecting the decrease in the stress imposed on the plant. However, a decrease was observed in APX and POD activity with the increase in salinity (2000 and 3000 mg/kg NaCl). Given the higher activity of SOD and GPX in these treatments (data are not shown), it can be said that APX and POD activities were lower. In total, in regions where NaCl concentration is at the level of 3000 mg/kg, Madagascar periwinkle plants with optimal vegetative and reproductive growth can be produced by applying 2% biochar, but to produce plants in good ornamental conditions (with over 3.5 flowers, 18.27 leaves, and 11.79 days of the flowering period), the application of 2% biochar at the salinity of 1000 mg/kg NaCl is a more appropriate option.

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