Anti-oxidative response, photosynthetic pigments and water relation in *Hyptis saeveolense* against Sodic Stress Soil

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Abstract: *Vilayati tulsi (Hyptis suaveolens), is an antioxidant plant whose essential oil and derivatives have been shown to scavenge free radicals and decrease oxidative stress. Flavonoids, alkaloids, and tannin are among the phytochemical that are responsible for this vital role.The present study aims to grow Hyptis in sodic degraded land and screen out the tolerance limit soil sodicity as well as to develop. An experiment was conducted at different levels of soil sodicity ie., 4.37, 15.73, 35.6, and 54.54 Exchangeable Sodium Percentage (ESP) in a completely randomized design(CRD). The results indicated that vegetative growth and yield were significantly decreased on increasing the soil sodicity while essential oil content was increased on increasing the stress. Photosynthetic pigments i.e., chlorophyll 'a' , chlorophyll 'b', total chlorophyll and carotenoid were significantly decreased on increasing the soil sodicity. Chlorophyll carotenoid ratio was decreased on increasing the alkalinity stress while carotenoid chlorophyll ratio was increased which showed the tolerant mechanism against alkalinity stress. The anti-oxidative enzymes like CAT activities was significantly increased on increasing the sodicity stress while POX, SOD and GR activities were significantly decreased on increasing the sodicity stress while some non-enzymatic anti-oxidant like proline content, H2O2 content and total phenol contents were also determined. The relative water content (RWC) was significantly increased while water potential(ψ) significantly decreased on increasing the soil sodicity stress which indicates the change in osmoticum due to alkalinity stress. Thus, the Hyptis plant shows medium tolerance to soil sodicity and can easily grow up to 15-25 ESP.*

Keywords: **Anti-oxidant, Enzyme Activity, Chlorophyll, Water Content, Sodic soil.**

1. Introduction

Vilayati tulsi or bush mint (*Hyptis suaveolens* L.) an exotic weeds which is suffrutescent annual or perennial herbaceous weed of Lamiaceae family. Stem is quadrangular, velvety, thick, and covered with long hairs and small erect glandular dots [1]. It is widely distributed near road side as well as agricultural and open land in rainy season in India. Although, it is a rich source of medicinally important phytochemicals like essential oils, tannins, saponins, phenol, flavonoids, terpenoids, alkaloids, and sterols and have one or many of these compounds have anti-oxidative, anti-inflammatory, antispasmodic, anti-septic, anticancer, anti-ulcer, antimicrobial, antibacterial, antiviral, anti-fungal, anti-diabetic, anti-fertility, diaphoretics, anticutaneous, anticatarrhal, antirheumatic, gastro protective, immunomodulatory, analgesic, and antiviral activity [2]. But due to overgrowth during season which affect the local variety of plant and crop yields. So, there are task to sustainable management of this exotic weeds.

One of the main issues causing land degradation is the prevalence of soil salinity and sodicity in all parts of the world. The issue of soil salinity and sodicity is very common in arid and semi-arid regions of the world [3]. Roughly 932 million hectares (Mha) or 7% of the world's land is covered by salt-affected soils [4], with 316 million hectares located in developing nations. In India, the damaged area by salt was estimated to be between 6.1 and 23.3 million Ha [5,6]. Several researchers have already noted the negative effects of alkalinity and salinity on the growth, dry matter production, and grain yield of several crops [7, 8, 9, 10].

Singh et al. [11] evaluated the tolerance status and mechanism of plants' tolerance against sodic stress in mango ginger plants at various levels of soil sodicity. They found that

plants showed tolerance against sodic stress up to medium levels of ESP without lowering rhizome yield and quality. By preserving electrolytic equilibrium, raising water potential, activating antioxidant enzymes, and increasing metabolite content, the plant resisted stress. Therefore, the primary threat to cultivable land is salinization, which might result in the loss o f 30% of land within the next 25 years and possibly 50% by 2050[12]. Salt deposition in the soil affects plants through alternative the water potential (Ψ) and causing ion-specific toxicity by upsetting the tissue ions homeostasis [13]. Cellular and physiological alterations are brought on by an inadequate water balance and a shift in the osmotic pressure in tissues. In addition to osmotic stress and ion toxicity, plants also exhibit other negative consequences related to the generation of toxic molecules and the integrity of cellular membranes, which start a slow disruption of the cellular process [14]. Although plants implement a variety of strategies to minimize salt damage, these strategies are based on adjusting the water potential and either excluding or sequestering excess salt from sites of metabolism by cell organelles like chloroplasts and mitochondria in cells that are highly susceptible to oxidative damage by inducing a high oxidative burst/exposure, which reduces the molecular oxygen and converts it into some over oxidized moieties commonly referred to as ROS, such as superoxide (O^{2-}), peroxide (H_2O_2), and free radicals (OH^-), which cause rapid cell damage by starting a series of reactions [14,15]. Cytosolic and organelle-specific ROS have a greater effect on cellular membranes and other macro molecules such as proteins, nucleic acids, lipids, glycosides, etc. ROS slows down cellular function by accelerating cellular damage in terms of various metabolic processes. Plants are also partially or completely sensitive to these oxidative risks, depending on the levels of ROS accumulation in the tissues and the over-expression of both enzymatic and non-enzymatic mechanisms against salinity stress [16]. A number of anti-oxidative enzymes, including SOD, POX, and CAT, work in a sequential cascade to lower ROS [17,18, 19].

So, the present study aims to explore sodic wasteland for growing of *Hyptis* plant and screening of tolerance limit and mechanism of tolerance by which degraded land can be utilized in sustainable manner.

2. Material and Methods

Plant material: *Hyptis suaveolens* (L.) Poit., popularly known as vilayati tulsi, bush mint, bush tea in India, is suffrutescent annual or perennial herbaceous weed growing along road sides. The stem is quadrangular, velvety, thick, and covered with long hairs and small erect glandular dots [1].

Experimentation:

Hyptis plants were grown at diffèrent exchangeable sodium percement (ESP) levels *i.e.* control (4.37 ESP), low (15.7 ESP), medium (35.6 ESP), and high (54.5 ESP) in four replicates using a complete randomized design (CRD) in naturally collected sodic soils from different locations of Banthara village near the Lucknow-Kanpur Highway, Lucknow, U.P., India. ESP is a common diagnostic metric for a soil's sodicity risk. In order to determine the tolerance limit of plants at various ESP levels at which they can readily grow and reproduce, the investigation was carried out in earthen pots. Only surface soils, up to a depth of 0-15 cm, were collected and stored for drying. The soils were appropriately blended and crushed after drying. The soil samples analysis was done (Table 1) followed by standard methods [20]. The earthen clay pots were lined on the inner side with alkathene sheets to check for leaching and contamination from the clay. Each pot contained 15 kg of soil. Four plantlates was transplanted in each pot. A basal dose of nutrients was given in two split doses *i.e.*, 200 $mgNkg^{-1}$ soil as $Ca(NO_3)_2$, 100 $mgPkg^{-1}$ soil as KH_2PO_4, and 100 $mgkg^{-1}$ soil as $MgSO_4$.

Table 1. Soil chemical properties used in experiment.

Soil Sodicity levels	Soil Chemical Properties				
	Soil pH	EC (dSm^{-1})	Org. C (%)	CEC ($cmolkg^{-1}$)	ESP (%)
Control	7.98±0.17	0.28±0.036	0.43±0.01	18.00±0.43	**4.37±0.086**
Low ESP	8.50±0.03	0.43±0.007	0.36±0.02	15.00±0.34	**15.73±0.771**
Medium ESP	9.54±0.09	0.44±0.007	0.14±0.01	14.20±0.26	**35.66±3.54**
High ESP	10.13±0.04	0.69±0.03	0.09±0.01	13.60±0.36	**54.50±3.49**

Measurement of vegetative growth and yield

Growth measurement was taken as plant height and biomass yield. Plant height was measured from soil surface to young leaf. When plants were about 75days old, different parameters of waters relation were measured. Biomass yield was taken at the time of harvest. Uprooted plants were washed and thoroughly separated into root and shoot and dried in an oven at 70°C for 24 hours.

Essential oil content

Essential oil content was determined by hydro-distillation method [21] through Clevenger type apparatus[22]in the dried leaves of *Hyptis* plant for 4 hours. Essential oil content was calculated with following formula:

$$Essential\ oil\ content(\%) = \left(\frac{volume\ of\ essential\ oil}{Weight\ of\ material}\right) \text{ x } 100$$

Plant Water Relation

The water potential was measured hydrometrically in the leaves using a Wescor micro-voltmeter and C-52 leaf chambers when plant growing at field capacity. Standard methods were used to calculate the Specific Water Content (SWC) and Water Saturation Deficit (WSD) [21].

Photosynthetic Pigments

Photosynthetic pigments, such as chlorophyll and carotenoid were assessed in the extract (80% acetone) of the young, completely expanded fourth leaf [23]. The residue was removed by centrifuging the homogenate at 4000xg for 10 minutes. The color of the supernatants was assessed at 663.2, 646.8, and 470 nm for chlorophyll a, chlorophyll b and carotenoid respectively which have been expressed as mg of chlorophyll or carotenoid g^{-1} fresh weight.

Enzyme Assey

2.5g of fresh leaf tissue was homogenized using a cooled pestle and mortar kept in an ice bath with 10.0 mL of chilled 50mM potassium phosphate buffer (pH 7.0) containing 1.0% insoluble Polyvinyl Pyrrolidone (PVP). The homogenate was centrifuged at 20000xg for 10 minutes in a chilled centrifuge at 2°C after being filtered through two layers of muslin cloth. Within four hours, the supernatants were used for enzyme assay after being kept at 2°C.

Assays of anti-oxidative enzymes

Catalase [CAT (EC, 1.11.1.6)] enzyme activity was measured in 10 ml of reaction mixture, standardized against 0.1M $KMnO_4$, containing 0.5 mM H_2O_2 and 1 mM phosphate buffer (pH 7.0) in test tube stabilized at 25°C (as described by Bisht [24] a variant method of Euler and Josephson). One milliliter of tissue extract was added to start the reaction, and everything was carefully mixed. Two milliliters of 4M H_2SO_4 were added to terminate the reaction after five minutes. Titrating the reaction mixture with 0.1 N $KMnO_4$ allowed for the assay of H_2O_2 decomposition after a 5-minute reaction. The unit of measurement for CAT activity is mg protein^{-1} (µ mole H_2O_2 decomposed min^{-1}).

Peroxidase [POD (EC. 1.11.1.7)] activity was assayed by modified method of Luck [25]. In a centrifuge tube 5 mL of 0.1M phosphate buffer (pH 6.0), 1 mL of 0.01% H_2O_2, and 1 mL of 0.05% (w/v) p-phenylene diamine were taken and stabilized at 25°C. After adding 1 mL of diluted tissue extract, the reaction was allowed to continue for 5 minutes before being stopped the reaction by adding 2 mL of 4N H_2SO_4. The contents were centrifuged for 15 minutes at room temperature at 4000 x g. A spectrophotometer (Spectro-chem MK II Manufactures AIML) was used to measure the supernatant's color intensity at 485 nm. The unit (mg protein)$^{-1}$ has been used to express the enzyme's activity.

Superoxide dismutase [SOD (EC 1.15.1.1)] activity was determined by measuring the ability to inhibit the photochemical reduction of Nitro-Blue Tetrazolium (NBT) in 3mL of reaction mixture containing 50mM phosphate buffer pH 7.8, 13 mM methionine, 75 µM NBT, 2 µM riboflavin, 0.1 mM EDTA, and 0 to 50µL enzyme extract. The change in absorbance was measured at 560 nm (Beauchamp and Fridovich) [26]. After adding riboflavin, the tubes were exposed to light for ten minutes. The reaction combination above without the enzyme extract reached its maximum color at 560 nm, and blanks were not lighted.

Glutathione Reductase [GR (EC 1.6.4.2)] assay was performed in a 3 mL reaction mixture containing 100 mM phosphate buffer pH 7.0, 1 mM GSSG, 1 mM EDTA, 0.1 mM NADPH, and 25–50 µL of the enzyme extract. The oxidation of NADPH was followed by monitoring the decrease in absorbance per min at 340nm. The amount of NADPH oxidized was calculated using the extinction coefficient [27].

Protein levels in tissue extracts were measured in order to identify the specific activity of an enzyme. In tissue extracts of 20% cooled Trichloro Acetic Acid (TCA), protein was measured using the folin Ciolcalteu reagent of Lowry et al [28] and left to stand at 4°C for four hours. Following centrifugation, the residue was dissolved in 0.1M NaOH at 80°C for 10 minutes in a water bath, and the amount of protein was calculated. The spectronic colorimeter was used to test the color intensity at 660 nm. The results have been expressed on percent fresh weight basis.

Estimation of non-enzymatic antioxidant

The amount of H_2O_2 was measured in a supernatant made from freshly chopped leaves in 100% cooled acetone and centrifuged at 10,000g for five minutes was measured by Brennan and Frenkel [29]. At 415 nm, the color intensity was measured. µmol H_2O_2 g^{-1} fresh weight has been used to express the results. The Folin Ciolcalteu (FC) reagent was used to calorimetrically determine the total phenol content of the leaf extract, which was prepared up to 10mL using distilled water and 1.5mL of 20% sodium carbonate after it had been properly shaken [30].

The absorbance of the final solution was measured at 765 nm using a UV spectrophotometer. The Bates et al[31]. method was used to estimate the ~~prolien~~ protein content. After grinding freshly chopped leaves in sulphosalicylic acid and filtering them with Whatman No. 1, an appropriate aliquot was obtained, mixed with glacial acetic acid and ninhydrin reagent, and heated for one hour. The color was measured at 520 nm after being extracted in toluene.

Statistics

A one-way ANOVA was used to identify significant differences between the treatments by using Sigma Stat 3.2. The data represent means plus/minus SE, with a significant difference at $P<0.05$.

3. Results and Discussion

Vegetative growth and yield

The results of vegetative growth and yields of *Hyptis* plant indicated that plant height was significantly decreased on increasing the soil sodicity stress (Table 2). Number of branches per plant was also significantly decreased. Biomass was significantly decreased on increasing the soil alkalinity stress in *Hyptis* plants. Steep declination was observed from medium and high sodicity stress while at low sodicity level non-significant decrement was observed (Table 2).

Table 2: Effect of soil sodicity on the growth, biomass yields and photosynthetic pigments of Vilayati tulsi (Hyptis suaveolens (L.) plants.

Parameters	Soil Sodicity (ESP)			
	Control	Low ESP	Medium ESP	High ESP
Vegetative growth and yields				
Plant height (cm)	91.33±2.52	71.67±5.13	51.00±4.36	37.67±2.52
Number of branches / plants	12±0.82	11.25±1.26	9.5±0.58	6.5±1.29
Stover weight (g/plant)	218.33±15.28	201.67±17.56	86.67±7.64	62.67±5.03
Root weight (g/plant)	36.67±1.53	31.67±2.89	21.67±2.89	16.33±1.53
Total Biomass (g/plant)	255±16.09	233.3±20.21	108.3±7.64	79±6.56
Essential oil yield (%)	0.20	0.25	0.31	0.38
Photosynthetic pigments				
Chlorophyll ‘a’	0.9095±0.0115	0.7285±0.0421	0.6835±0.0735	0.572±0.0368
Chlorophyll ‘b’	0.45±0.01	0.35±0.023	0.4205±0.0542	0.4005±0.0205
Total Chlorophyll	1.3595±0.0215	1.0785±0.0651	1.104±0.128	0.9725±0.0573
Carotenoids	0.738±0.011	0.6095±0.0135	0.664±0.023	0.6215±0.027
Chlorophyll /Carotenoid ratio	1.84	1.77	1.66	1.56
Carotenoid /chlorophyll ratio	0.543	0.565	0.601	0.639

Similar finding was also observed by other medicinal crops by previous workers like mango ginger's growth, survivability, and metabolic processes were all affected by rising soil ESP. Singh et al. [8] examined several turmeric accessions cultivated in sodic soil and found that during different stages of crop growth, accessions differed greatly in terms of plant height, number of leaves, and shoots per plant. Similar results were found in Osmium species by Singh et al. [7] and in fennel plants by Singh et al. [8] & Garg et al. [9], who reported no negative effects up to 25 ESP. Similar findings were also noted by another employee [32,33]. *Hyptis* plants may therefore withstand low ESP without suffering negative consequences. As a result, it appears that medicinal plants can thrive in low to medium sodic soils, making them more cost-effective than other common crops and perhaps an option to bio-reclamation of sodic soil. The essential content was significantly increased on increasing the alkalinity stress in Hyptis plant (Table 2).

Photosynthetic pigments

Photosynthetic pigments *i.e.*, chlorophyll 'a', chlorophyll 'b', total chlorophyll and carotenoid were significantly decreased on increasing the soil sodicity in *Hyptis* plant (Table 2). Chlorophyll carotenoid ratio was decreased on increasing the alkalinity stress while carotenoid chlorophyll ratio was increased which showed the tolerant mechanism in *Hyptis* plant against alkalinity stress. The effectiveness of photosynthesis, which is closely linked to the photosynthetic pigments of plants cultivated at high ESP and decreased in the current study, determines the decrease in dry matter production. Other workers have also noticed similar finding [6, 7, 34, 35]. The increased activity of chlorophyllase is linked to these decreases in chlorophyll concentration [36]. High salinity has damaged the chlorophyll's fine structure, causing the pigment protein complex to become unstable and lowering the amount of chlorophyll [37]. Free radicals produced by excess excitation energy from chlorophyll during photosynthesis are eliminated by carotenoid [38]. There was a notable reduction in the carotenoid concentration. In comparison to the control, the carotenoid/chlorophyll ratio was marginally higher in medium ESP but much lower in low and high ESP.

Anti-oxidative enzymatic activities

An antioxidant is a substance that can prevent or repair damage caused to the body's cells by oxygen through delaying or suppressing the oxidation of lipids or other molecules by preventing the start or spread of oxidative chain reactions [39]. A number of anti-oxidative enzymes, including SOD, POX, and CAT, work in a sequential cascade to lower ROS [17,18, 19].

In present study, various antioxidative enzymes activities and some non-enzymatic antioxidants were assayed. Results indicated that CAT activities were significantly increased on increasing the sodicity stress while POX, SOD and GR activities were significantly decreased on increasing the sodicity stress (Fig.1) while some non-enzymatic anti-oxidant like proline content, H_2O_2 content and Total Phenol contents were also determined. Results indicated that proline and total phenol contents was significantly increased on increasing the sodicity stress while generation of H_2O_2 content decreased (Fig.2). Oxygen free radicals were generated during plant metabolism and must be eliminated in order to maintain normal growth. Numerous plant systems provide evidence of environmental stresses, particularly salt stress, which modifies the quantity and activity of enzymes involved in scavenging oxygen free radicals. The main scavenger is the enzyme that converts SOD to hydrogen peroxide, which is removed by ascorbate peroxidase at the cost of oxidizing ascorbate to monodehydroascorbate. These two enzymes are present in a number of isozymes that are active in the cytosol and chloroplast [40]. POX inactivation may have resulted from an increase in H_2O_2 levels. Other workers similarly found that SOD activity was uncontrolled in various plants as a result of H_2O_2 generation following salinity treatment [41–44].

In *Crithmum maritimum* and mangroves, total CAT activity declined as salinity increased, demonstrating that CAT reacts similarly in glycophytes and halophytes under salt stress conditions. Additionally, the POX contributes to the defense mechanisms of plants, including as reactions to insects and a coordinated reaction called the oxidative burst [45-48]. As a result, SOD demonstrated the first line of defense against oxidative stress in plants and produces H_2O_2 by dismutating superoxide radicals at nearly diffusion-limited rates [49]. It is crucial to the defense mechanism against the toxicity of free radicals because it influences the concentration of O_2 and H_2O_2 in plants [50]. Many workers also seen a similar tendency [51–55]. Therefore, Hyptis plant's physiological specialization and robust antioxidative response mechanism support its ability to withstand sodicity stress.

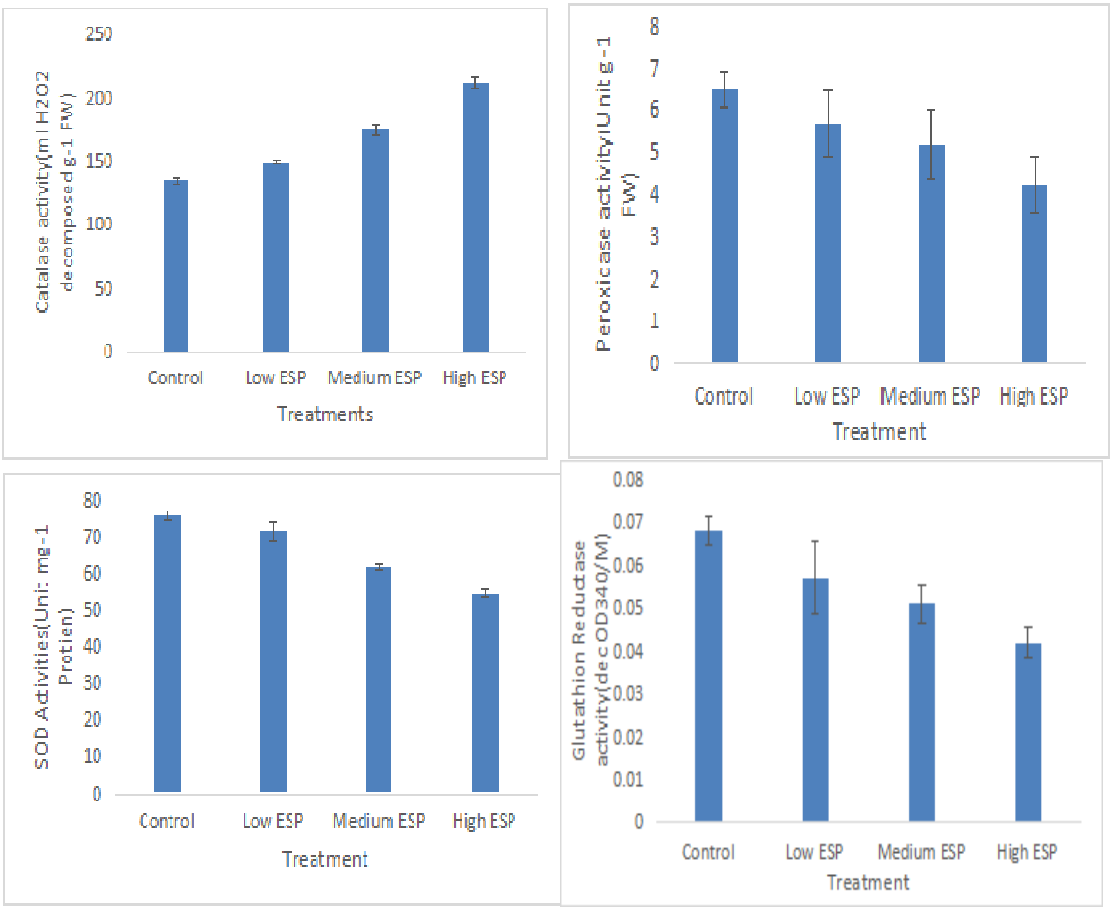

Figure-1: Soil sodicity induced changes in anti-oxidative enzyme activity against stress condition.

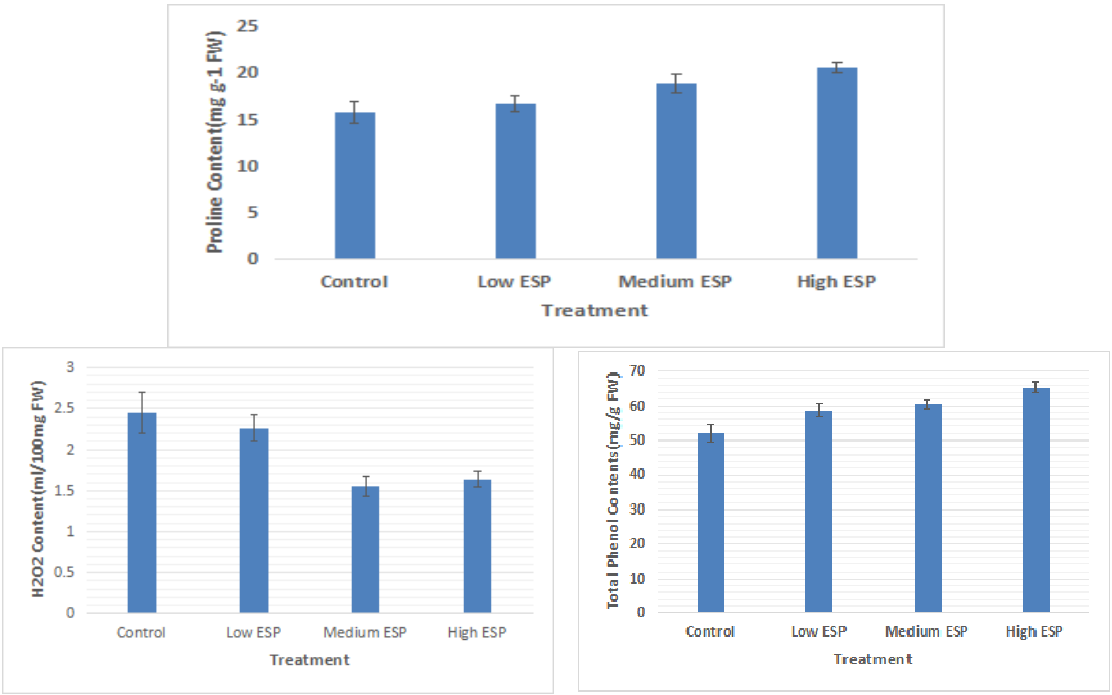

Figure-2: Soil sodicity induced changes in some non-enzymatic anti-oxidant against sodic stress condition.

Prolien is a stress protein that accumulated in plants and indicated stress tolerance. According to the current study, the accumulation of proline content increased when the sodicity stress plant increased. The plant cultivated in high ESP had the highest prolien content, whereas the control had the lowest (Fig.2). Similar findings were also observed by other workers, who obseve that suitable solutes play a role in protecting against free radicals in stressful situations [53]. Recent research, however, points to a connection between halophytes' higher osmolyte content (such as prolien) and lower ROS generation, which demonstrated improved resistance to salt stress. Increased proline content has been linked to better resistance to abiotic stress. Prolien is an excellent osmoticum and aids in membrane stability [55. 56].

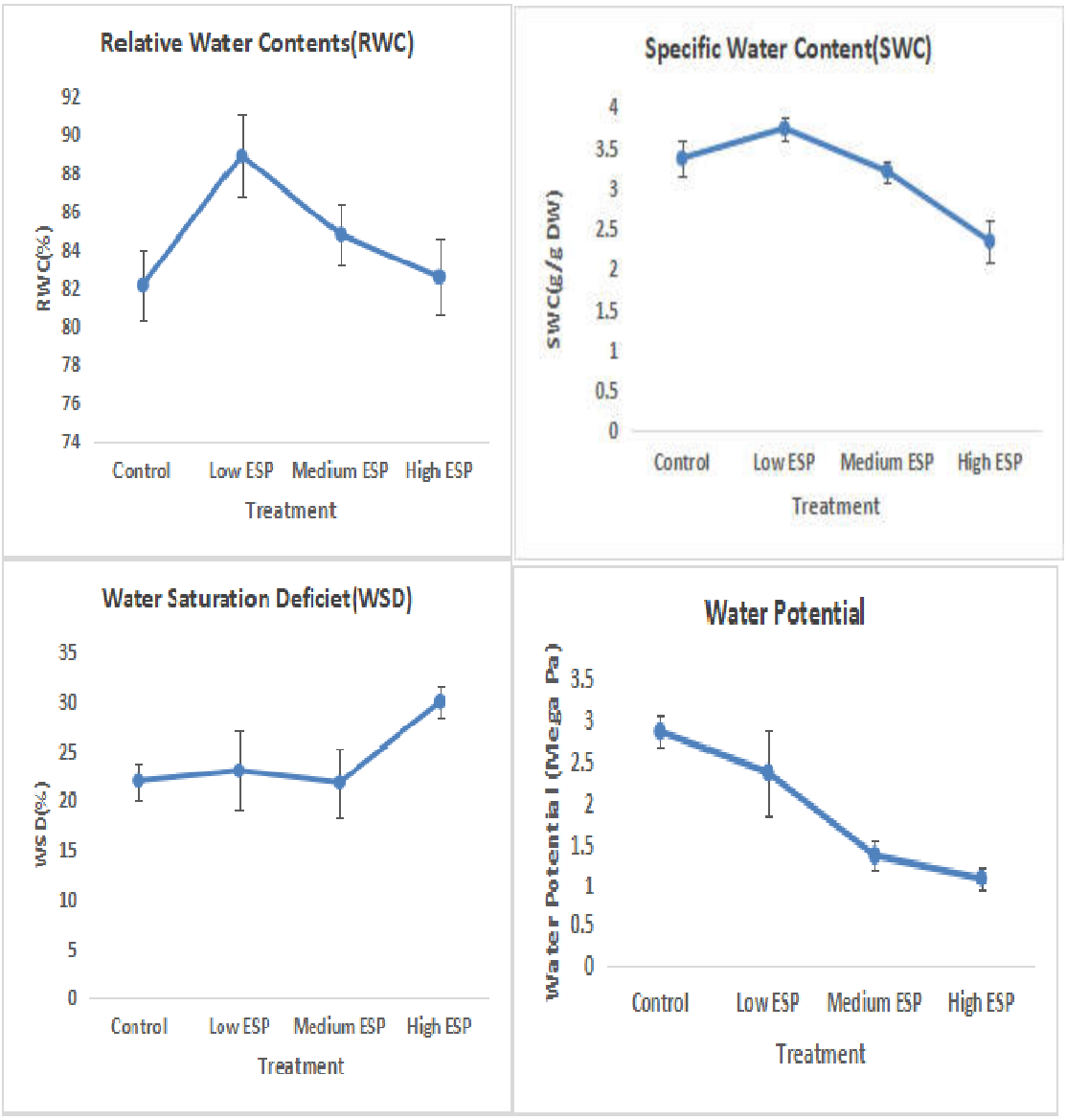

Figure-3: Soil sodicity induced changes in water relation parameters like RWC, SWC, WSD and Water potential in the leaf of Vilayati tulsi (Hyptis suaveolens (L.) plants.

Water relation and water contents

Plant suffer from physiological water stress due to soil sodicity and salinity. Reduced growth on increasing soil ESP levels is caused by the sodicity of the soil, which increases the osmotic potential of the circulating soil solution and causes an ionic imbalance. Plants under sodic stress need greater energy to make osmotic adjustments by building up organic and inorganic solutes, which counteract by lowering the osmotic potential inside their cells relative to the soil solution outside. Growth is inhibited by the energy expended during osmotic adjustment [57].

In present study, results indicated that relative water content (RWC) and specific water content (SWC) at lower ESP levels elevated while further these declined sharply (Fig.3) but water saturation deficit (WSD) slightly decreases up to medium ESP levels while at high ESP levels it was sharply increased (Fig.3). Results of water potential(ψ) indicated that it was significantly decreased on increasing the soil sodicity (Fig.3). The plant underwent water stress, which can accelerate water intake, as evidenced by the decreased water potential, RWC, and SWC but increased WSD at the high ESP level. Another worker made similar

observations [58, 59]. Increased proline content in the leaf tissue is caused by elevated WSD at high ESP. When there is water stress, proline builds up [60,61].

Conclusion

Phytochemicals, primarily phenol, proline, and anti-oxidative enzymes (CAT, POX, SOD and GR) were observed in the leaf extract of *Hyptis* which play important role for preventing oxidative damage in sodic stress environment. Although plant height and biomass yield were adversely affected at medium and high ESP levels but at low ESP levels (15-25 ESP) obtained optimal yield. Therefore, it was strongly advised to cultivate *Hyptis* plants on moderately sodic degraded land in order to restore sodic degraded land.

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