International Journal of Scientific & Engineering Research, Volume 7, Issue 10, October-2016 54
ISSN 2229-5518

Effect of salicylic acid and salt stress on the growth and some biochemical parameters of *Mentha suaveolens*

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Abstract— salinity presents a limiting factor for plant growth in the word, in this vision we have tried in this work tostudy the effect of salicylic acid at concentrations of 10 mM, 20 mM and 30 mM, on growth and biochemical response of *Mentha suaveolens* grown under salt stress at 150 mMNaCl.

In result salt stress causes a reduction of the growth parameters (height, number and lengths of internodes) and changes in biochemical parameters: a significant decrease in chlorophyll pigments, strong accumulation of total phenolics, condensed tannins, soluble sugars, proline and hydrogen peroxide.

However application of salicylic acid increasing concentrations in case of salt stress aims to lessen the negative effects of salt. These levels decline gradually especially with the application of AS 30 mM.

Index Terms— salt stress, salicylic acid, *Mentha suaveolens*, hydrogen peroxide, total phenolics compounds.

1 Introduction

Salinity can be defined as an excessive accumulation of salts in soils or waters with a threshold that may impact on normal plant growth. Ecosystems are characterized by high variability of rainfall associated with significant evaporation promoting the accumulation of salts in the soil, which explains the poor quality of available water resources in these areas [1] .In Morocco, soil salinization becoming alarming reducing arable land [2].

Tolerance to the presence of salts is then a quality largely sought in plants of agronomic interest to broaden their culture in those regions.

The exogenous application of salicylic acid has an effect on many physiological processes in unfavorable conditions. Several studies have shown that the salicylic acid participates the on regulation of metabolic and physiological pathways of the plant [3].

The correlation between the concentration of salicylic acid and plant resistance suggests that the AS acts as a molecule signals in plants, which induces tolerance to certain biotic and abiotic stress [4]. For this purpose we tried to study the combined effects of salt stress and salicylic acid on morphology and biochemical response of an aromatic and medicinal plant widely used in Morocco *Mentha suaveolens*

2-Material and methods

2-1Plant material and growth conditions

The plant material used is constituted by cuttings of Menthasuaveolens. Each cutting, 10 cm in length, comprises at least two nodes. Culturing is carried out in plastic pots (3kg) glasshouse Irrigation is provided by means of water containing 150 mMNaCl and at different concentrations of salicylic acid: 10 mM, 20 mM, 30mM according to the following device

- Treatment with NaCl (150 mM)
- Treatment with AS (10 mM, 20 mM, 30 mM)
- Treatment with NaCl (150 mM) + AS (10 mM, 20 mM, 30 mM)

Measurement Of the conductivity

The extraction of the electrolyte was produced by the technique described by addition of 20 ml distilled water to 4g of soil from each treatment followed by stirring for 30 min then the conductivity is measured by a conductivity meter [5] .

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2-2 Morphological parameters

The length of the stem of the plants and the number of lengths among nodes: Before sampling plant material we measured the height of the stem in centimeters (cm) using a ruler to 8 treatments.

2-3 Biochemical parameters

2-3-1 Dosage of chlorophylls

Fragments of leaves (1 g) were ground in a mortar previously placed in ice with a pinch of magnesium carbonate and 5 g of anhydrous sodium sulfate. Ensuite,10ml of 80% acetone are poured into the ground material, which is filtered on a Buchner, the residue is recovered in tubes essai.de further extractions are carried out with acetone to obtain a filtrate colorless (devoid of all traces of chlorophyll pigments) which the final volume is specified.

OD measurements were made with a spectrophotometer at wavelengths of around 663nm to 645nm for chlorophyll a and chlorophyll b.

McKinney [6] have established systems of equations that calculate the concentrations (g/l) chlorophyll from absorbance at 663 and 645 nm of an extract of 80% acetone are:

Chlorophyll a = (0.0127 D.O663) - (0.00269 OD 645)

Chlorophyll b = (0.0229 OD 645) - (663 0.00468 OD)

Total chlorophyll = (0.0202 OD 645) + (0.00802 OD 663)

2-3-2 Dosage of proline

Proline contents were determined by measuring the quantity of the coloured reaction product of proline with ninhydric acid[7]. The absorbance was read at 520 nm. The amount of proline was calculated using L-proline (Pancrea) for the standard curve and expressed in µg/g of fresh leaf matter

Extraction and determination of total phenols

Oxidation of phenols reduces this reactant in a mixture of the blue oxides of tungsten and molybdenum. The color intensity is proportional to the rate of oxidized phenolic compounds.

- Extraction of total phenolic compounds

Fragments of leaves and roots (0.5 g) were ground in a mortar containing a specific volume usually 5 ml, ethanol 50% (water-alcohol solution).then we Collect the extracts in tubes with lids and well numbered, then leave the tubes in the refrigerator overnight to allow time for ethanol to extract the maximum amount of phenol present in the extract.

In the tubes containing the leaf extracts there was a risk of the existence of chlorophylls we tried to eliminate it by adding in 3 ml of 0.5 ml of extract chloroforms, vortex tubes and tubes and centrifuged for 5 min at 5x1000 mtp, two phases were separated; a phase supernatant and pellet.

-Determination of total phenolic compounds

The assay of total phenols using the method based on the Folin-Ciocalteu method [8].

Prepare in test tubes the following mixture: 0.5 ml of water extrait,3ml, 0.5 ml of 20% Na2CO3, mix, wait 3 min then add reagent 0.5ml Folin-ciocalteu. Mix and place the tubes for 30 min at 40˚C. Reading absorbance at 760nm.

The amount of phenolic compounds was calculated using gallic acid for the standard curve and expressed in mg/g of fresh leaf matter.

2-3-3 Dosage of condensed tannins

Based on the property that tannins becomes anthocyanins by heating in acidic medium[8].In this assay a portion of the ethanol extract is used for the determination of condensed tannins. In two tubes, put 3 ml of the ethanolic extract which is added 3 ml of concentrated HCl. the tubes 1 is placed in the water bath at 100 ° C for 30 minutes, followed by rapid cooling, the tube 2 is maintained at room temperature. After 30 min, 0.5ml of ethanol is added to both tubes. The reading is taken at 550nm.The calculations are made to a relationship:

D.O (TRC) = (D.O1 - D.O2) × 19.33 for a dilution of 1/50

2-3-4 Dosage of soluble sugars

The total sugar content is determined by the phenol-sulfuric acid method [9]. The extraction of the carbohydrate is made by the 80% ethanol. 100mg of the samples were ground in 5ml ethanol 80% .the tube is heated to evaporate alcohol to each tube 10 ml of water was added distilled is the solution to be analyzed .1ml solution to be assayed which is added phenol solution of 1ml of 5% and 5 ml of sulfuric acid the tubes are shaken and placed in 5 0C for 45 min and 30 min in the dark reading is performed at 485 nm.

2-3-5 Dosage hydrogen peroxide

The extraction and the determination of hydrogen peroxide were carried out according to the method described below [10].

1 g of fresh leaf samples and root from each treatment are crushed in 15 ml of trichloroacetic acid, the homogenate is centrifuged for 15 min at 12,000 rpm. The assay is performed by adding 0.5 ml of supernatant 0.5 of pH 7 phosphate buffers and 1 ml of 1 mM potassium iodide and then the optical density of the tube is read at 390 nm

2-4 Statistical analysis

One-way analysis of variance was carried out for each parameter studied. Tukey's post hoc multiple mean comparison test was used to test for significant differences between treatments (at $p \leq 0.055\%$). Univariate analysis was used to test significant differences in treatments, accessions and their interaction for an

ISSN 2229-5518

individual parameter. All statistical analyses were performed with IBM.SPSS statistics, Version 19.The results of each experiment (biochemical essays) were repeated three times and (20 times for morphological essays).

3-Results

3-1 Effect of salicylic acid on the growth and biochemical parameters of *Mentha suaveolens* in non salt stress condition

In absence of salt stress, the addition of salicylic acid to the medium at the concentrations 10, 20 and 30 mM, causes an inhibition of plant growth (fig. 1). This inhibition is significant at low concentrations and decreases very slightly when the concentration of salicylic acid is increased to 30 mM. This growth inhibition affects the number of internodes and their length (fig. 2) since we found that these parameters fell strongly in the presence of AS at low concentrations. In these cases, increasing of the AS concentration does not seem to have a great influence, since there is no significant differences between the different concentrations.

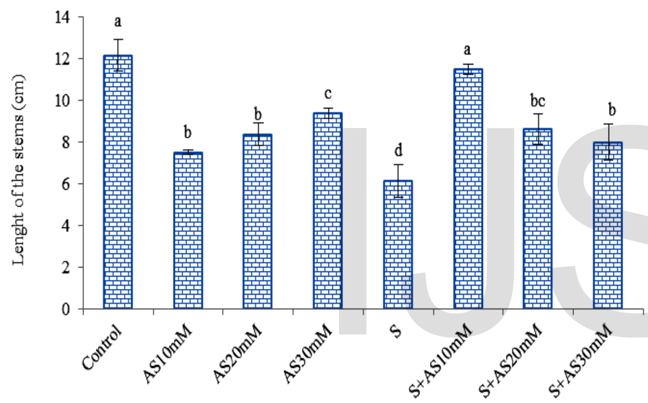

Figure 1: Changes of length of the stems in *Mentha suaveolens* after applications of different treatments. The values indicated by different letters are significantly different (P = 0.05)

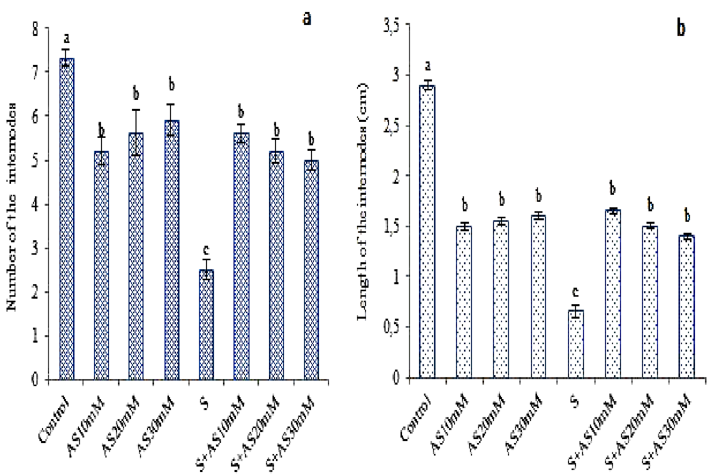

Figure 2: Changes of number (a) and length (b) of the internodes in *Mentha suaveolens* after applications of different treatments. The values indicated by different letters are significantly different (P = 0.05)

In addition, there is a significant decrease in total chlorophyll contents mainly when the concentration of AS increases to 30 mM (fig. 3). At low concentrations (10 and 20 mM), the Reduction of chlorophyll is not significant, which explains the inhibitory effect of AS at high concentrations.

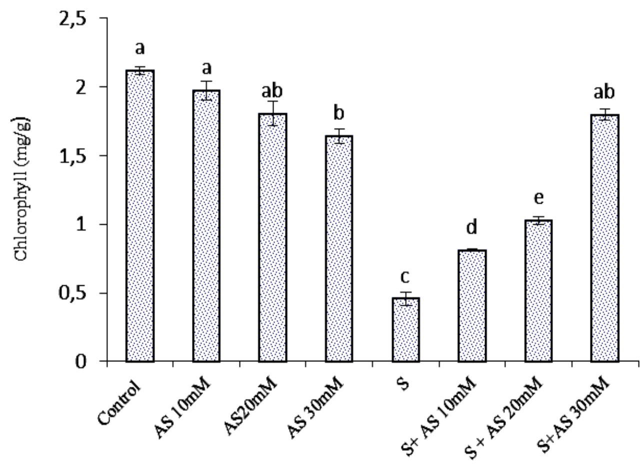

Figure 3: Changes of chlorophyll contents in *Mentha suaveolens* after application of different treatments. The values indicated by different letters are significantly different (P = 0.05)
AS: salicylic acid, S: salt stress

In leaves, proline contents do not seem affected by high concentrations of AS (30mM). these levels decrease significantly in the presence of low concentrations of AS. However, there has been no influence of AS on the levels of proline in roots (fig. 4a).

The accumulation of soluble sugars (Fig. 4b) of the leaves increases significantly in the presence of strong concentrations of salicylic acid (20 and 30Mm). The concentration of 10mM does not show any remarkable change. In the roots, the accumulation of soluble sugars is even more important that the concentration of AS is higher

Concentrations of phenolic compounds increase in the leaves and roots mainly in the presence of high concentrations of AS, 30 mM for the leaves and 20 mM for the roots (fig. 5a).

In Menthasuaveolens, phenolic compounds consist largely of condensed tannins (Figure 5b). The latter involve in the same manner as phenolics i.e. significantly increase in leaves and roots based on increasing concentrations of salicylic acid.

We also find that the contents of hydrogen peroxide enhance in the leaves and roots with the increase in the salicylic acid concentration (fig. 6).

3-2 Effect of salicylic acid on the growth and biochemical parameters of Menthasuaveolens in salt stress condition

Salt stress causes a significant inhibition of growth of Menthasuaveolens. This inhibition concerns the length of the stem, the number and length of internodes (fig. 1 and 2). There is also a very significant decrease of about 75% of total chlorophyll content (fig. 3). In addition, the salt stress has a very important influence on the accumulation of proline since their levels increase in a very significant manner in the leaves and roots (fig.

4a). The same observations can be made for soluble sugars (fig. 4b), total phenolic compounds (fig.5a) condensed tannins (fig.5b) and hydrogen peroxide (fig. 6).

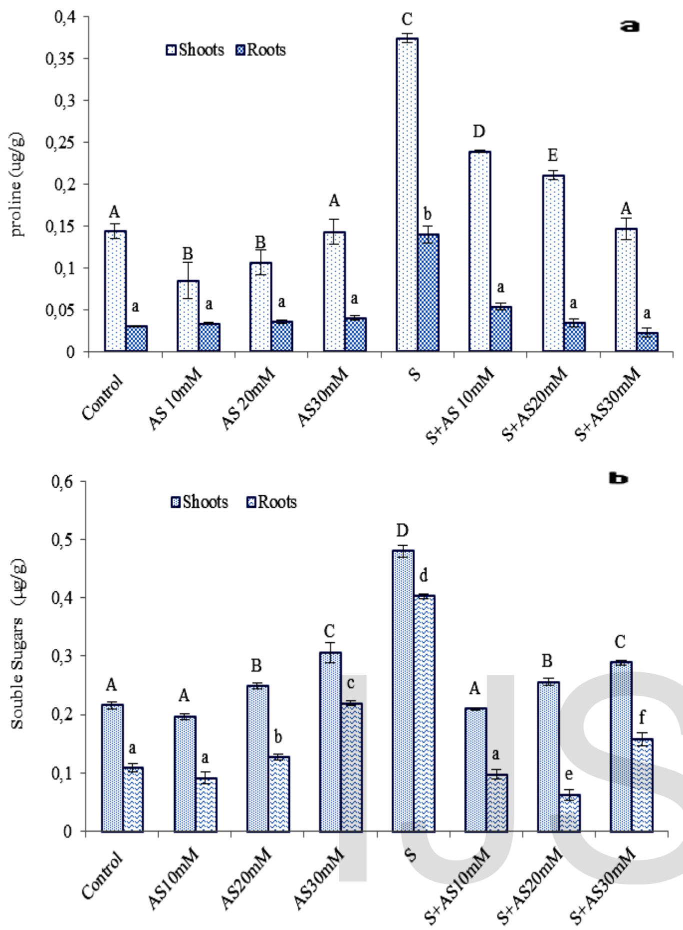

Figure 4: Changes of proline contents (a) and soluble sugars contents (b) in *Mentha suaveolens* after application of different treatments. The values indicated by different letters are significantly different (P = 0.05)
AS: salicylic acid, S: salt stress

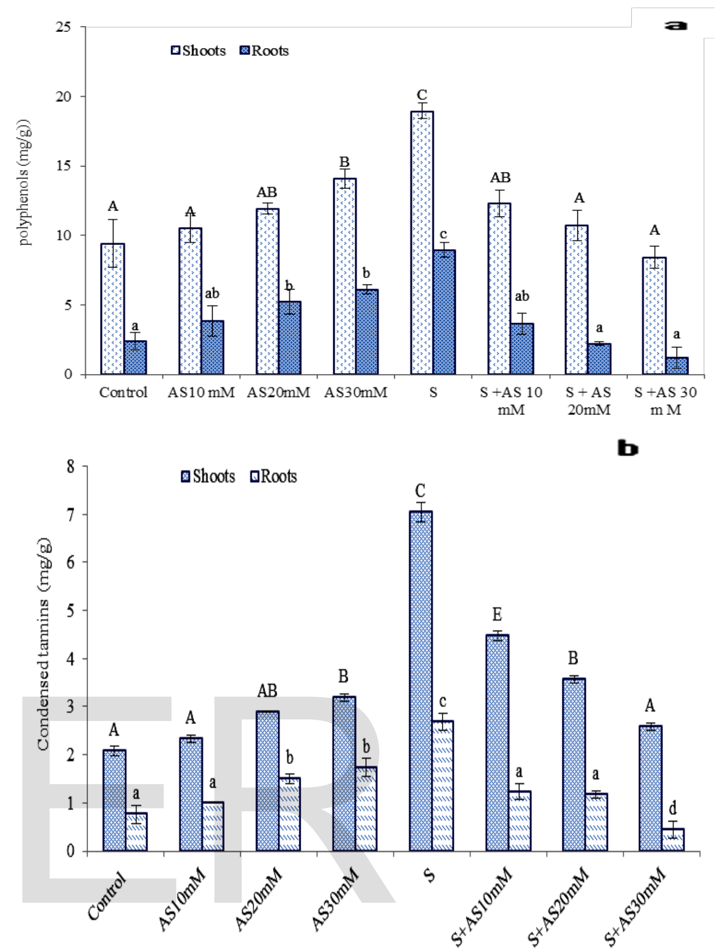

Figure 5: Changes of polyphenols contents (a) and condensed tannins (b) in *Mentha suaveolens* after application of different treatments. The values indicated by different letters are significantly different (P = 0.05)
AS: salicylic acid, S: salt stress

In addition, the harmful effects of salt stress may be reduced by application of salicylic acid. Indeed, concentrations of the AS that induce growth inhibition in absence of salt, cause contrariwise a growth stimulation in case of salt stress; the stems of length can return to normal by applying the AS at a concentration of 10 mM (fig. 1). At higher concentrations (20 and 30 mM), the increase in length is lower. The same observation can be made for the number and length of the internodes, which may increase when using the AS at 10 mM, however, increasing the concentration of AS has no significant influence (fig. 2). It should be noted in this case that the number and length of internodes remain in any case lower than those of the control. The contents of total chlorophyll may also increase in the presence of AS in salt stress (fig. 3). These levels are even higher than the concentration of AS increases; to 30 mM, the chlorophyll may reach about 90% compared to those of the control.

Proline levels are also influenced by the AS in salt stress (fig. 4a). There is a gradual decrease in proline contents with the application of increasing concentrations of salicylic acid. In leaves, proline levels almost return to the same level than the control at a concentration of 30 mM. In roots, proline contents reach those in the control at a lower concentration (20 mM).

The levels of soluble sugars in the leaves and roots also undergo a substantial decrease under the influence of AS in salt stress (fig. 4b). In this case, it is the low concentrations of AS (10 mM) that restore the normal level of soluble sugarsfound in the control. At high concentrations, we have a slight increase but is still lower than in the case of the stressed environment without AS. Identical results for the total polyphenol contents are obtained after application of the AS in the presence of NaCl (fig. 5a). There is a gradual decrease in the accumulation of these molecules as the concentration of AS increases. From 20 mM, concentrations of phenolic compounds are identical to those of control both in the leaves and in roots. Condensed tannins are following the same change as the CPT (Fig. 5b), since we noticed that the application of AS can reduce these levels as the concentration of AS increases. However, regardless of the concentration of AS applied, levels of condensed tannins are still higher than those of the control.

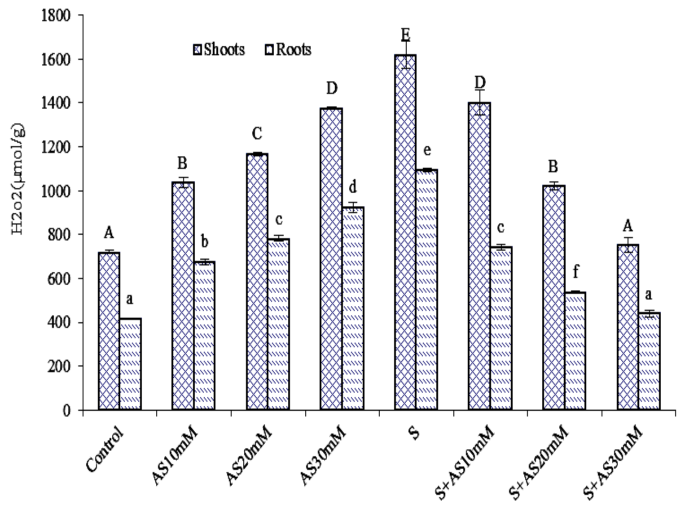

Figure 6: Changes of hydrogen peroxide contents in *Mentha suaveolens* after application of different treatments. The values indicated by different letters are significantly different (P = 0.05)
AS: salicylic acid, S: salt stress

The application of the AS in the presence of NaCl plays an important role in the levels of the plant H2O2 which strongly decreases with increasing concentration of AS (fig. 6). At 30 mM, the AS allows to bring these levels to those determined in control and this both in the leaves than in roots.

4-Discussion

Under normal irrigation conditions, addition of salicylic acid to the medium at concentrations of 10 mM causes inhibition of growth and number of internodes of *Mentha suaveolens*. A similar result was reported in the tobacco grown in vitro and treated with AS [11], and in the common bean plants whose total dry weight decreases after applying salicylic acid. Some scientist found a significant reduction in the growth of seedlings of Viciafaba treated with high concentrations (greater than 5 mM) of salicylic acid in the absence of stress [12].

Abiotic stress results in morphological, physiological and biochemical changes that negatively affect the growth and productivity of the plant [13, 14]. In the presence of salt stress, the results of this research showed that fresh weight of shoot and root of *Mentha suaveolens*was reduced. These results confirm those of many studies in this area : they showed that increasing the NaCl concentration decreases the growth of durum wheat (*Triticum durum* L.) Or even stop the growth of plant following a strong Na + accumulation in cells [15]. Some works showed that salinity caused a marked reduction in growth parameters of shoots and roots of sugar beet plant[16]. Growth reduction due to salinity is mainly attributed to water deficit due to lowered water potential in the root zone, nutritional imbalance and specific ion toxicity arising from higher concentration of Na+ and Cl- .

In this experiment we observed also that exogenous application of salicylic acid without stress, increased fresh weight of shoot and root. [17] reported increase in the growth of shoot and root of soybean and maize plants in response to salicylic acid treatment. Exogenous application of SA seems to inhibit the Cl- and Na+ absorption and helps for Mg, Fe, Mn, N and Cu absorption and decreases harmful effect of stress on growth

In the presence of salt stress, the application of salicylic acid overcomes the inhibitory effect of stress on the growth and this in function of the applied concentration. These results confirm those of [18] which showed that salicylic acid application overcomes the negative effect caused by salinity on growth and development of Atriplex and Spinacia plants. [19, 20] have shown that pretreating the tomatoes by salicylic acid increases the salinity tolerance

The changes in the growth of *Mentha suaveolens* affect the levels of chlorophyll, which vary depending on the environment. The salicylic acid or salt stress result in lower levels of total chlorophyll mainly when concentrations increase.

Effects of Exogenous SA on photosynthesis parameters vary among plant species and doses tested. In Menthasuaveolens, there is a significant decrease in total chlorophyll content mainly from the concentration of 20 mM. At a lower concentration (10 mM), the decrease in total chlorophyll content was not significant.[21] showed that the treatment of winter wheat plants with 500 µM for 24 hours causes a decrease in the rate of photosynthesis.[22] showed that high concentrations of AS (1-5 mM) result in a reduction of the rate of photosynthesis and RuBisco activity in barley plants.[23] also showed chlorophyll content reduction in cowpea, wheat and Arabidopsis. The decrease in RuBisco activity was attributed to a 50% reduction in protein levels compared to untreated plants [24]. Furthermore, exogenous AS induced alterations in leaf anatomy and these changes correlate with increased volume chloroplast thylakoid swelling and bleeding of the stroma [25]. Thus, reduction of photosynthetic activity is due to the effects of high concentrations of AS on the thylakoid membranes and reactions induced by light.

Furthermore, there is also a very significant decrease of about 75% of total chlorophyll contents of Menthasuaveolens. The reduction of photosynthetic activity in the presence of NaCl is explained by various authors as one of the causes of reduced growth and vegetative productivity [26]. These results are in agreement with those of [27] who noticed a decrease in pigment chlorophyll content after salt stress in wheat. [28] reported that salt stress causes a decrease in chlorophyll concentrations in mustard.

Under the conditions of salt stress, salicylic acid seems to play an important role in the accumulation of chlorophyll. These findings are in agreement with those of [29] which showed that the total chlorophyll levels increase in bean in the presence of salicylic acid. The same results were obtained by[30] in peas and [31] in sunflower.[32] also reported that salicylic acid can have stimulating effects on the photosynthetic capacity of the corn by inducing the activity of Rubisco.

Osmotic adjustment is the main part of the physiological mechanisms by which plants respond to salinity stress. The salinity stress often results in disturbance of plant water balance. The accumulation of certain osmotic adjustment solutes (e.g. proline, soluble sugars, and soluble protein) in the cytosol and organelles helps in the osmotic adjustment and improving growth and development of plants. The N. Tangutorum plants responded to NaCl treatments with the accumulation of proline, soluble sugars, and soluble protein.

Our study on *Mentha suaveolens* shows that salt stress causes a significant accumulation of proline in both leaves and roots. Our findings are consistent with those of [33] which showed the accumulation of proline in barley in response to salt stress and those of [34] who observed significant increase in proline content in rice leaves was subjected to salt stress. [35] found that explants of tomato a leaf was subjected to salt stress accumulate large amounts of proline. [36] was also a proline accumulation in wheat in case of salt stress.proline accumulation in plants exposed to salinity stress is due to low activity of antioxidant enzymes [37]. It is suggested that the salt leads to a greater activity of the pathway of synthesis of proline glutamate instead of chlorophyll synthesis; glutamate is a precursor of chlorophyll and proline biosynthesis [38].

The proline has been reported to exert multiple functions in stressful conditions, such as regulating osmotic pressure [39], protecting membrane integrity [40], scavenging ROS and stabilizing enzymes/proteins ratio [41].

In our study, in the absence of salt stress, the application of SA decreased proline content at low concentrations (10 and 20 mM). At a concentration of 30 mM, the level of proline contents is the same as that of the control. But under salt situation, low concentrations of 10 and 20 mm lead a gradual decrease in proline contents to carry these values to the same level as the control to the concentration of 30 mM. This is valid both for the leaves to the roots. Similar changes have been reported by [42] in wheat and barley plants.

The accumulation of soluble sugars is also one of the strategies used by plants to adjust their osmotic potential altered by Na^+ and Cl^- ions ([43, 44]. Our results show that NaCl induced a significant increase in total soluble sugar content in *Mentha suaveolens* but a much more significant in the leaves than in roots. These results confirm those obtained in Pistcia atlantica and in rice 'Taromazmom' [45] which indicate that the soluble sugar content increases significantly in the leaves and roots of plants exposed to NaCl.[32] observed that maize plants submitted to NaCl salinity treatment, showed a progressive increase in their soluble sugar content with increasing the salinity level. Saline conditions decrease the activity of ribulose 1, 5-bisphosphate carboxylase (Rubisco) which results in reduction in formation of carbohydrates [46]. In this situation, for escaping from plasmolysis performance and living during salt stress conditions the plant cell breaks down the sucrose to glucose and fructose, and by starch decomposition to glucose it increases its osmotic pressure cell[47].

On the contrary, without salt stress, a strong concentration of salicylic acid (20 and 30Mm) leads to an accumulation of soluble sugars in the leaves and roots. [48] also found that exogenous application of salicylic acid increases the amount of soluble solids (index brix). This can be attributed to the role of salicylic acid to improve membrane permeability, absorption and utilization of mineral nutrients.

Furthermore, our study shows that application of salicylic acid inhibits the effect of salt stress resulting in a significant decrease in soluble sugar content and these both in the leaves than in roots. These results are confirmed by those of [32] who observed that salicylic acid treatment caused a significant decrease in the content of soluble sugar in comparison with salinized plants. It seems that salicylic acid application can activate the metabolic consumption of soluble sugars to form new cell constituents to stimulate plant growth, and can balance the sugar levels under stress by inhibiting the polysaccharide-hydrolyzing enzyme system and / or accelerate the incorporation of soluble sugars of polysaccharides [32]. Soluble sugars were also found involved in adjusting osmotic pressure, ROS balance and responses to oxidative stress in plants [49]. Some research has indicated that salicylic acid causes an increase in the permeability of the membrane and facilitates the absorption and utilization of mineral nutrients and transport of assimilates. This would also contribute to improving the ability of plants processed for the production of biomass as shown by the increase of fresh and dry weight of plants [32]. [50] reported that application of salicylic acid is increased levels of soluble sugars and soluble proteins in rice.

Polyphenols are other compounds involved in the defense against salt stress. The accumulation of phenolic compounds and their role in the stimulation of plant resistance were provided in many species and in different abiotic and biotic stress situations [51].

Concerning these compounds, we note that the salt stress causes an increase in their accumulation both in the leaves and roots of *Mentha suaveolens*. Increasing polyphenol contents in tissue is a response to salinity and indicates the induction of synthesis of secondary metabolism to defend against salt stress [52]. This large accumulation is confirmed in Anethum graveolens L. [53] and Vetiveria zizanioides L. [44]. These molecules are involved in the process of defense against ROS which are produced during the photosynthetic metabolism established under environmental stress [54].

Without salt stress, salicylic acid causes an increase in the total polyphenol content in leaves and roots of *Mentha suaveolens* mainly in the presence of high concentrations of 20 mM. According to [54], in the presence of salicylic acid, PAL and TAL activities is maximum, respectively, at 75 and 100 of salicylic acid and the synthesis of phenolic compounds is simultaneous with the enzyme stimulation.

But under salt stress conditions, the application of salicylic acid causes a reduction of polyphenol contents. This decrease is proportional to the increase in the salicylic acid concentration, the concentration of 20 mM permits overcoming the harmful effect of NaCl and provides similar contents to those of the un-stressed control. According [55], lower polyphenol content is due to the effect of salicylic acid that can activate the polyphenol oxidase. [56] also reported that polyphenols levels increase after treatment of wheat plants by salicylic acid.

In *Mentha suaveolens*, these phenolic compounds consist largely of condensed tannins that undergo the same changes as the total polyphenol. Salt stress induces a significant increase of condensed tannins in the leaves and roots. These levels fell highly to identical values to those of the control when applying salicylic acid at doses of 20 mM and 30 mM. In the absence of salt stress, AS at these concentrations causes increased levels of condensed Tannins.

In the defense system of the plant, the production of active oxygen species (ROS) is one of the most rapidly observable events during biotic and abiotic stresses[11]. These forms consist essentially of hydrogen peroxide (H2O2).

We showed that in the presence of NaCl, the hydrogen peroxide levels increase significantly in the plant with a high accumulation in leaves. These results are in agreement with several studies that have shown that hydrogen peroxide accumulates during salt stress. [57] showed the role of H2O2 produced by oxidation of polyamines (PAs) in signaling during some stress responses.

Furthermore, we found that the AS results in an increase of the levels of hydrogen peroxide in in the whole plant. These levels enhance with increase in the salicylic acid concentration.. In previous study [58] observed that SA treatments enhanced H2O2 levels in the leaves of A. thaliana in a dose- and time-dependent manner. Plants treated with SA have previously been shown to accumulate H2O2 [59].

The application of salicylic acid on salt stress causes a significant decrease in concentrations of hydrogen peroxide in stressed plants. These results confirm those of [60] who showed that salt stress accelerates the production of H2O2 while the application of SA inhibits the production of H2O2 in the stressed plants. [61] have also shown that the application of salicylic acid on stressed plants decreases the levels of H2O2.

5- CONCLUSION

From this work, we can conclude that in *Mentha suaveolens*plants, salt stress is negatively affected several morphological and biochemical parameters. We recorded an increase in levels of proline, soluble sugars, polyphenols and hydrogen peroxide which means response of plants to salinity.

The data obtained from this study suggest that the AS application can enhance the adverse effects of salt stress, and this varies depending on the concentration applied.

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