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MICROMORPHOLOGICAL, PHYSIOLOGICAL, AND BIOCHEMICAL RESPONSES OF *OCIMUM BASILICUM* L. CV. „AROMAT DE BUZĂU" UNDER SALT STRESS

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Abstract: Excessive soil salinity severely limits crop productivity. This study evaluates the micromorphological, physiological, and biochemical responses of the Romanian basil variety ('Aromat de Buzău') to NaCl-induced salt stress (100–200 mM) against a control. Evaluated under both controlled laboratory and semi-open greenhouse conditions, the analyzed parameters included stomatal traits, photosystem II efficiency, assimilatory pigments, antioxidants, and oxidative stress enzymes. Results demonstrate an adaptive response dependent on both stress intensity and environment. Notably, the variety exhibited significant stomatal plasticity and shifted from enzymatic to non-enzymatic antioxidant defenses under high salinity, particularly in lab conditions. These findings characterize the genotype's salinity tolerance, offering practical guidelines for cultivating basil on salinised land in Romania.

Keywords: *Ocimum basilicum* L., salt stress, micromorphology, chlorophyll fluorescence, phenolic compounds.

INTRODUCTION

Abiotic stress induced by salinity currently poses a major threat to global agriculture, due to its adverse effects on the water and nutrient balance of plants. Excess salts in the substrate cause ionic toxicity, nutritional imbalances, and osmotic stress in plants, phenomena that inhibit root growth and leaf area expansion and ultimately affect the normal development and productivity of agricultural crops (da Silva et al., 2022; Balasubramaniam et al., 2023).

In this context, the cultivation of aromatic and medicinal plants is attracting increasing scientific and agronomic interest.

Among the species of recognized economic importance is basil (*Ocimum basilicum* L.), a taxon in the Lamiaceae family, valued for its high content of phenolic compounds and bioactive volatile oils with antioxidant, antibacterial, and antifungal properties, and widely used in the food, perfume, and cosmetics industries (Rewers and Jedrzejczyk, 2016; Ahmed et al., 2019; Ciriello et al., 2023).

The Romanian variety 'Aromat de Buzău', registered at the Buzău Research and Development Institute for Vegetable Crops in 2006, is the first indigenous semi-late-maturing variety of green basil, adapted to local soil and climate conditions, and recognized for its balanced, mint-like aromatic profile. The development of future agricultural strategies for cultivating this variety in areas of Romania prone to salinization requires systematic investigation of its mechanisms of response to salt stress, from the micromorphological level through to the physiological and biochemical levels (Vînătoru et al., 2019; Balanescu et al., 2020).

The aim of this study was to assess the salinity tolerance of plants of the 'Aromat de Buzău' variety through an integrated analysis of micromorphological changes in the stomatal apparatus, physiological parameters associated with the photosynthetic apparatus (chlorophyll fluorescence, content of assimilatory pigments) and the biochemical profile (phenolic compounds, total antioxidant activity and enzymatic activity), under two distinct experimental conditions: laboratory and semi-open greenhouse, in order to capture both the direct effect of salinity stress and the influence of microclimatic factors on the adaptive response of plants of this variety.

MATERIALS AND METHODS

Biological material

The biological material used was obtained from seeds of the indigenous basil variety (*Ocimum basilicum* L.) 'Aromat de Buzău' (batch no. 1BZ2011-1BZ01), sourced from the Buzău Research and Development Institute for Vegetable Growing (SCDL Buzău). The mature plant has the appearance of a globular, highly branched bush, ranging in height from 40 to 60 cm, with light green leaves and white flowers, and belongs to the Linalool or Linalool-Estragole chemotype (Burducea, 2018; Balanescu et al., 2020).

Cultivation conditions and saline treatment applied

The research was structured around two experimental approaches: Experiment I, conducted under controlled laboratory conditions (temperature 22–24°C, LED lighting, photoperiod of 17 hours light/7 hours dark), and Experiment II, conducted under field conditions, in a semi-open greenhouse (temperature 22-23°C during the day/18–20°C at night, natural light attenuated by the greenhouse film), located in the commune of Popricani, Iași County (47.278°N, 27.524°E).

Saline stress was experimentally induced by applying progressive concentrations of sodium chloride (NaCl) (100, 150, and 200 mM) administered in three successive treatments, at 14-day intervals, with the results reported relative to a control treatment (0 mM NaCl). The choice of NaCl as a stress agent is justified by its ability to induce a dual functional constraint on plants - initial osmotic stress and secondary ionic stress - through the accumulation of Na^+ and Cl^- ions to toxic levels in tissues (Tarchoune et al., 2012; Elhindi et al., 2016).

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Micromorphometric analyses

The density and surface area of the stomata on the lower epidermis of the test plants' leaves were assessed by scanning electron microscopy (SEM), using a Tescan Vega II - SBH microscope. The plant material was fixed in 2.5% glutaraldehyde (0.1 M phosphate buffer, pH 7.4), dehydrated in a series of acetone concentrations and dried using the critical point drying method with an EMS K850 apparatus, then sputter-coated by depositing a thin layer of gold (5–15 nm, EMS 550x) to ensure the conductivity required for scanning (Xu and Zhou, 2008; Pathan, Bond and Gaskin, 2010).

Physiological measurements

Chlorophyll fluorescence was analyzed in vivo using a portable fluorimeter (Hansatech FMS II), with the parameter φPSII (actual quantum yield of photosystem II) being monitored on mature, fully developed leaves located on the middle node of the plant stems (10-15 measurements per treatment).

The content of assimilatory pigments (chlorophyll a, chlorophyll b, total carotenoids) was determined spectrophotometrically from ethanolic extracts (96% ethyl alcohol) (Wellburn, 1994; Nayek et al., 2014; Norazah et al., 2020), by measuring absorbances at 664, 649, and 470 nm (Shimadzu 1240 UV-mini spectrophotometer) and applying the appropriate calculation formulas for each pigment fraction.

Biochemical analyses

The total polyphenol content was determined spectrophotometrically using the Folin-Ciocâlteu method (Herald, Gadgil and Tilley, 2012), based on a calibration curve with gallic acid, with the absorbance of the samples measured at 765 nm. The flavonoid content was assessed using the aluminum chloride colorimetric method (Herald, Gadgil and Tilley, 2012), with the absorbance of the samples measured at 510 nm and the results expressed as quercetin equivalents. Total antioxidant activity was determined using the DPPH (2,2-diphenyl-1-picrylhydrazyl) method, based on the ability of plant extracts to neutralize the DPPH free radical, quantified by the decolorization of the solution and the measurement of absorbance at 517 nm (Sharma and Bhat, 2009; Behrendorff et al., 2013).

For plants grown in the laboratory (Experiment I), the biochemical analyses were supplemented by the determination of the activity of the antioxidant enzymes superoxide dismutase (SOD, NBT method) and peroxidase (POD, method based on the oxidation of ortho-dianisidine), as well as total protein content, using the Bradford method (Bradford, 1976; Artenie, Ungureanu and Negură, 2008).

Statistical analysis

The experimental data were collated and expressed as mean ± standard deviation (SD) for each experimental treatment, using GraphPad Prism software. The statistical significance of the effect of salt stress was assessed by Analysis of Variance (ANOVA), followed by a post-hoc test; differences were considered significant at a threshold of $p < 0.05$.

RESULTS AND DISCUSSION

Micromorphometric responses of the stomatal apparatus

Under laboratory conditions (Experiment I), the number of stomata on the lower leaf epidermis decreased significantly at a concentration of 100 mM NaCl, reaching a minimum at 150 mM NaCl, before returning to values close to those of the control at 200 mM NaCl, - a phenomenon interpreted as a possible micromorphological compensatory response to environmental salinity in the leaf apparatus of the test plants. By contrast, the average stomatal area showed a notable increase at 100 mM NaCl, suggesting an adaptive mechanism of osmotic regulation of the leaf apparatus through stomatal opening/enlargement, with this parameter returning to values close to those of the control at 150 mM NaCl and decreasing significantly at 200 mM NaCl, as a result of the progressive activation of the abscisic acid (ABA)-mediated stomatal closure mechanism (Zhao et al., 2020).

Under semi-open greenhouse conditions (Experiment II), the number of stomata on the underside of the test plants' leaves increased progressively up to a concentration of 150 mM NaCl, followed by a slight decrease at 200 mM NaCl, whilst remaining at a level significantly higher than that of the control. The stomatal area showed a non-linear trend, with a significant increase at 100 mM NaCl and a drastic decrease to the minimum value in the experiment at 150 mM NaCl (Robinson, 1997; Barbieri et al., 2012; Yu and Assmann, 2016; Eisenach and De Angeli, 2017).

By correlating the two types of micromorphological response in the test plants (Fig. 1), the clearest adaptive strategy of the plants belonging to the 'Aromat de Buzău' variety emerges at a concentration of 150 mM NaCl, at which they develop the highest number of stomata, but with a minimal surface area – a classic strategy for fine and rapid control of transpiration, characteristic of plants subjected to osmotic stress (Bertolino, Caine and Gray, 2019; Hasanuzzaman, Zhou and Shabala, 2023). Overall, we can therefore conclude that plants of the 'Aromat de Buzău' variety demonstrate significant micromorphological plasticity of the stomata, indicating a moderate degree of salinity tolerance.

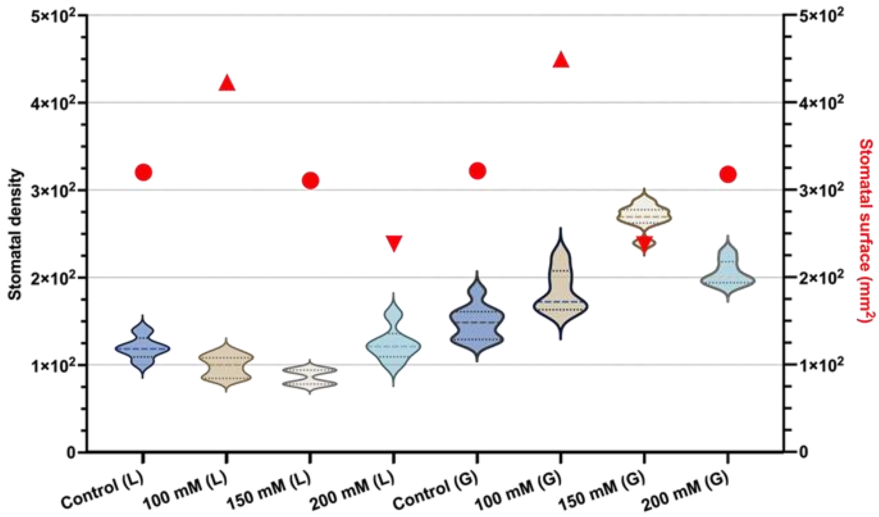

Fig. 1. Stomatal density of *Ocimum basilicum* L. 'Aromat de Buzău' plants grown under experimental conditions. Experimental variants: Control, 100 mM NaCl, 150 mM NaCl, 200 mM NaCl; (L) – Laboratory conditions, (G) – Semi-open greenhouse conditions. On the secondary axis (in red) is marked the stomatal surface area (●- non-significant difference related to Control, ▲- significant increase of stomatal surface area, ▼- significant decrease of stomatal surface area).

Chlorophyll fluorescence

Under laboratory conditions, only a concentration of 200 mM NaCl resulted in a statistically significant reduction in the effective quantum efficiency of photosystem II (φPSII) compared with the control, whilst concentrations of 100 and 150 mM were not associated with significant differences in this functional parameter. In the semi-open greenhouse, all three concentrations tested resulted in significant reductions in φPSII, with the most pronounced decrease recorded at 150 mM NaCl, suggesting greater photosynthetic sensitivity of the test plants under variable environmental conditions, where the interaction between salinity and uncontrolled microclimatic factors accelerates the decline in photosystem II efficiency.

The critical threshold for photosynthetic tolerance of the 'Aromat de Buzău' variety is thus reduced from 200 mM NaCl under laboratory conditions to 150 mM NaCl in a semi-open greenhouse, highlighting the importance of environmental conditions in determining the taxon's photosynthetic response to salinity stress (Khaliq, Tita and Zafar, 2017; Santos et al., 2020) (Fig. 2).

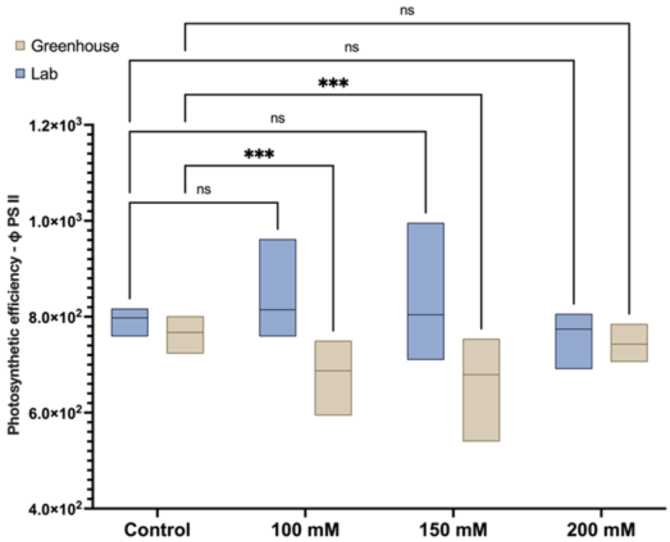

Fig. 2. Photosynthetic efficiency of *Ocimum basilicum* L. 'Aromat de Buzău' plants grown under experimental conditions. Experimental variants: Control, 100 mM NaCl, 150 mM NaCl, 200 mM NaCl; Statistically significant differences are marked with *.

Assimilatory pigment content

Chlorophyll a content remained relatively stable across the entire salinity range tested in both experiments, suggesting an intrinsic tolerance of the photosynthetic apparatus of plants belonging to the 'Aromat de Buzău' variety to NaCl-induced ionic disturbances (Ashraf and Harris, 2013). Chlorophyll b, by contrast, exhibited the most dynamic response: in the laboratory, its concentration increased at 100 mM NaCl and peaked at 200 mM NaCl, with a notable decrease at the intermediate concentration of 150 mM; in the greenhouse, a sharp increase in the concentration of this chlorophyll fraction was recorded at 150 mM NaCl, reaching a level comparable to that of chlorophyll a, and remained at a high level even at 200 mM NaCl.

In both experiments, the carotenoid pigment content showed a downward trend at concentrations of 150 and 200 mM NaCl, indicating a high sensitivity of this pigment fraction to intense salt stress, possibly through inhibition of the isoprenoid biosynthetic pathway or accelerated oxidative degradation, resulting in a significant reduction in the plants' photoprotective capacity, which is more pronounced under variable environmental growing conditions (Hashimoto, Uragami and Cogdell, 2016) (Fig. 3).

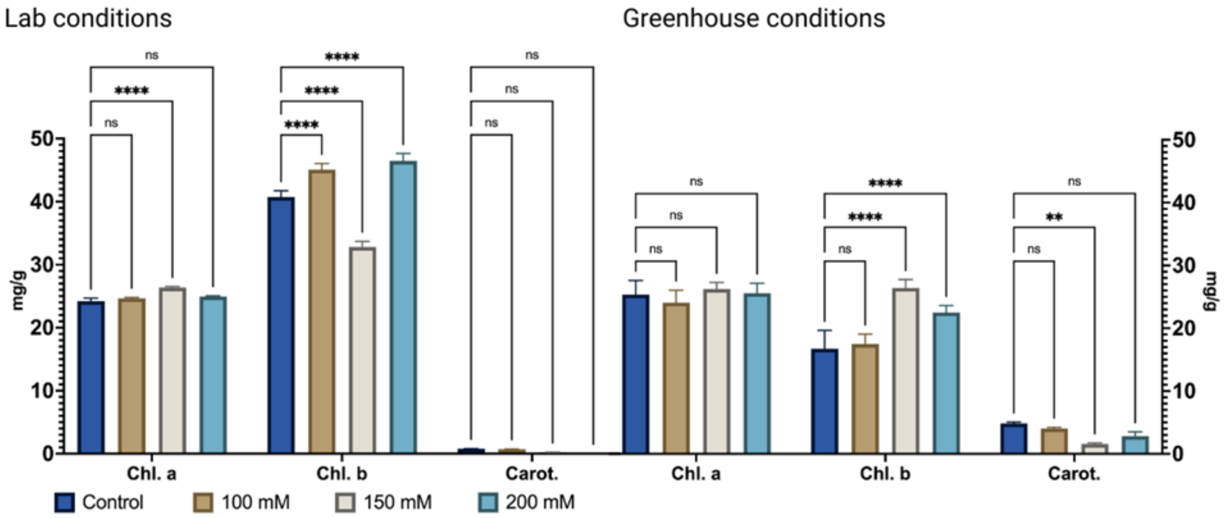

Fig. 3. Assimilatory pigments content of *Ocimum basilicum* L. 'Aromat de Buzău' plants grown under experimental conditions. Experimental variants: Control, 100 mM NaCl, 150 mM NaCl, 200 mM NaCl; Statistically significant differences are marked with *.

Total polyphenol content

Under laboratory conditions, the total polyphenol content showed a non-linear response: a slight increase at 100 mM NaCl (possibly a hormesis-type response), a slight decrease below the control level at 150 mM NaCl, and an increase -almost double that of the control - at 200 mM NaCl, representing an intense defense response by the test plants to severe salinity stress in the growth medium, through the neutralization of reactive oxygen species (ROS) (Valifard et al., 2014; Sharma et al., 2019).

In the semi-open greenhouse, the biological variability in polyphenol content was more pronounced, with no statistically significant differences compared to the control at any of the tested concentrations, suggesting that, under semi-open greenhouse conditions, plants of the 'Aromat de Buzău' variety do not significantly activate their polyphenol biosynthesis pathways at the salinity levels tested, possibly due to interaction with other environmental factors that mask the effect of salinity (Fig. 4).

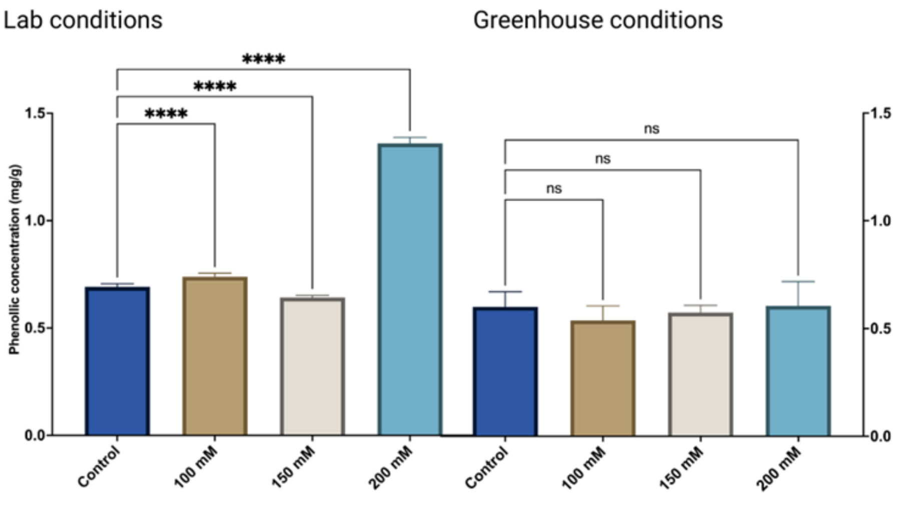

Fig. 4. Polyphenol content of *Ocimum basilicum* L. 'Aromat de Buzău' plants grown under experimental conditions. Experimental variants: Control, 100 mM NaCl, 150 mM NaCl, 200 mM NaCl; Statistically significant differences are marked with *.

Flavonoid content

In the laboratory, flavonoid content followed a non-linear pattern, increasing at 100 mM NaCl, a significant decrease below the control level at 150 mM NaCl, and an absolute maximum - statistically highly significant - at 200 mM NaCl. In the semi-open greenhouse, the dynamics were different, progressive and orderly, with flavonoid content increasing in proportion to the intensity of salinity stress, from values not significantly different from the control at 100 mM NaCl, to a significant value at 150 mM NaCl and a maximum at 200 mM NaCl (Wang et al., 2023) (Fig. 5).

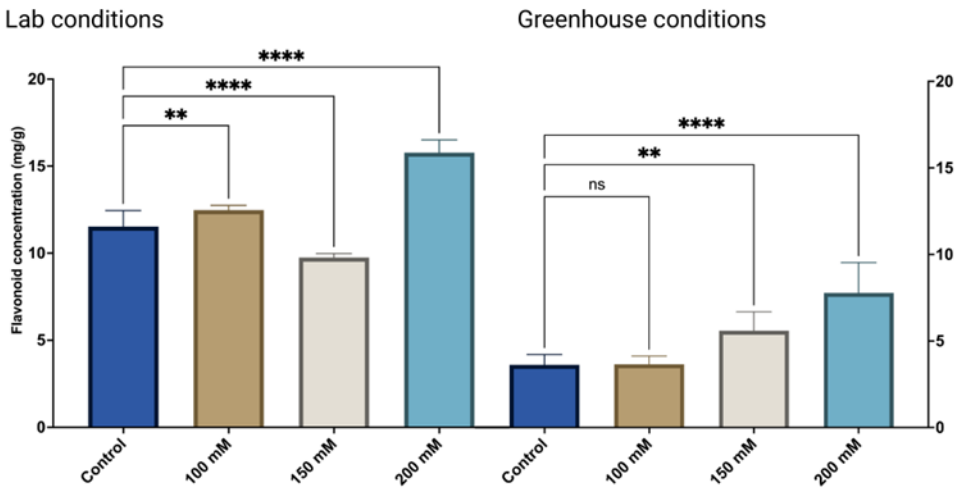

Fig. 5. Flavonoid content of *Ocimum basilicum* L. 'Aromat de Buzău' plants grown under experimental conditions. Experimental variants: Control, 100 mM NaCl, 150 mM NaCl, 200 mM NaCl; Statistically significant differences are marked with *.

Total antioxidant activity (DPPH)

Under laboratory conditions, DPPH antioxidant activity was slightly lower than that of the control at 100 and 150 mM NaCl, with a notable increase - almost double that of the control - at 200 mM NaCl, which can be interpreted as a hormetic-type adaptive response, in which plants intensively mobilize endogenous phenolic compounds and flavonoids to neutralize reactive oxygen species generated by the severe osmotic and ionic stress of the cultivation environment (Ciriello et al. 2023; Kandil et al. 2023).

In the semi-open greenhouse, the observed variations showed a slight decrease at 100 and 150 mM NaCl, followed by a recovery at 200 mM NaCl; these changes did not reach the threshold for statistical significance, but were attributed rather to the biological variability of the plant material under study in fluctuating environmental conditions than to the direct effect of the saline treatment (Fig. 6).

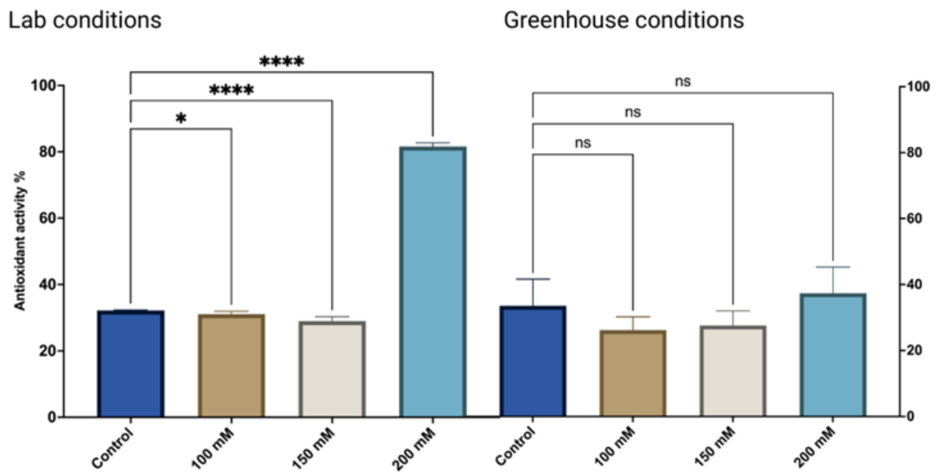

Fig. 6. Total antioxidant activity of *Ocimum basilicum* L. 'Aromat de Buzău' plants grown under experimental conditions. Experimental variants: Control, 100 mM NaCl, 150 mM NaCl, 200 mM NaCl; Statistically significant differences are marked with *.

Antioxidant enzyme activity (SOD, POD)

Enzymatic analyses (Fig. 7), carried out exclusively on plants grown in the laboratory, showed that superoxide dismutase (SOD) activity was highest in the control group, with all three applied salt concentrations causing a significant decrease in activity, followed by a slight recovery at 150 and 200 mM NaCl. This seemingly paradoxical decrease can be explained by the direct inhibition of the enzyme in question by Na^+ and Cl^- ions, by the enzymatic capacity being overwhelmed by excessive ROS production, or by the test plants switching their defense strategy to non-enzymatic defense mechanisms, correlated with increased DPPH antioxidant activity at the 200 mM NaCl treatment (Tarchoune et al., 2010).

Peroxidase (POD) activity decreased significantly at 100 mM NaCl and reached a minimum at 150 mM NaCl (one-third of the control value), only for its activity to recover almost completely at 200 mM NaCl, returning to values not significantly different from the control, a pattern suggesting a possible temporary accumulation of H_2O_2 in the mesophyll cells of the plants at the intermediate concentration, followed by the activation of genes involved in the synthesis of new antioxidant enzymes and phenolic compounds (Tarchoune et al., 2012; Hasanuzzaman et al., 2021).

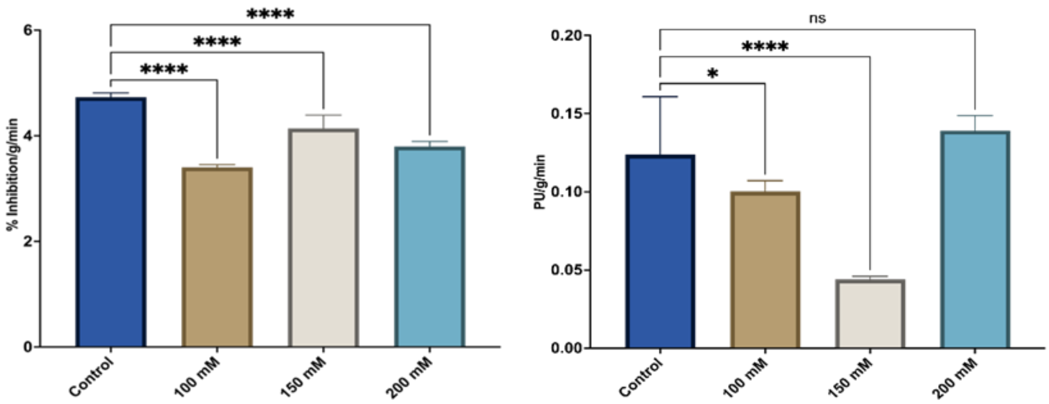

Fig. 7. SOD activity (Left) and POD activity (Right) of *Ocimum basilicum* L. 'Aromat de Buzău' plants grown under experimental conditions. Experimental variants: Control, 100 mM NaCl, 150 mM NaCl, 200 mM NaCl; Statistically significant differences are marked with *.

Total protein content

The total protein content (Fig. 8) of basil plants grown in the laboratory responded to salt stress with a biphasic pattern: an initial decrease at 100 mM NaCl, followed by a progressive and significant increase at 150 and 200 mM NaCl. Although the absolute variations were of small magnitude, the high statistical significance and the correlation with the other biochemical parameters support the interpretation of these changes as part of a coordinated adaptive functional response, in which the plants synthesize protective proteins in proportion to the intensity of the applied salt stress (Taiz et al., 2015; Athar et al., 2022).

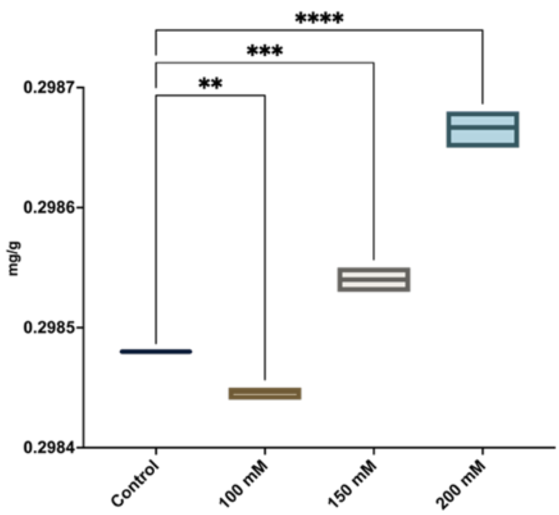

Fig. 8. Total protein content of *Ocimum basilicum* L. 'Aromat de Buzău' plants grown under experimental conditions. Experimental variants: Control, 100 mM NaCl, 150 mM NaCl, 200 mM NaCl; Statistically significant differences are marked with *.

Studia Universitatis "Vasile Goldiş", Seria Ştiinţele Vieţii
Vol. 36, issue 3, 2026, pp. 149 - 157

CONCLUSIONS

The indigenous basil variety 'Aromat de Buzău' exhibits a complex adaptive response to experimentally induced salt stress with NaCl, which is dependent on the growing environment. At the micromorphological level, the plants demonstrate significant stomatal plasticity, with an adaptive optimum at a concentration of 150 mM NaCl, at which high stomatal density, combined with a reduced stomatal area, ensures fine-tuned control of transpiration and limits water loss.

At the physiological level, the photosynthetic apparatus of plants of the 'Aromat de Buzău' variety proves to be relatively resistant to moderate salt stress induced under controlled laboratory conditions; however, its tolerance is significantly reduced under variable environmental conditions, where the critical threshold for photosynthetic resistance decreases from 200 mM to 150 mM NaCl. The plants' pigment strategy is based on maintaining a stable chlorophyll a content and increasing chlorophyll b content, whilst the progressive degradation of carotenoid pigments highlights a limitation in the photoprotective capacity of the basil plants, which is more pronounced in the solar chamber.

At the biochemical level, plants of the 'Aromat de Buzău' variety demonstrate two distinct adaptive strategies, depending on the growing environment. In the laboratory, at high salt concentrations, the plants activate a robust antioxidant response, based on the massive accumulation of polyphenols, flavonoids, and protective proteins, with a gradual shift from enzymatic defense (SOD, POD) - whose activity decreases as salinity increases - towards a non-enzymatic, phenolic defense, suggesting hormesis-type biochemical plasticity. In the semi-open greenhouse, these defense mechanisms are not significantly activated, as the plants' biochemical response is attenuated and masked by the variability of external abiotic factors, suggesting that the combined stress specific to the field environment may deplete the plants' metabolic resources before they can mobilize an effective antioxidant defense.

Overall, the results obtained highlight a moderate degree of salinity tolerance in the 'Aromat de Buzău' variety, with effective plant adaptive mechanisms under controlled cultivation conditions, but more limited under field conditions, which are variable, and may inform future cultivation strategies for this indigenous genotype on land prone to salinization.

AUTHORS CONTRIBUTIONS

Conceptualization, E.-I.O. and M.-M.Z.; methodology, E.-I.O.; data collection, E.-I.O.; data validation, E.-I.O. and M.-M.Z.; data processing, E.-I.O.; drafting – initial version, E.-I.O.; revision and editing, E.-I.O. and M.-M.Z.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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