TATIANE SANTOS CORREIA

MORPHO-PHYSIOLOGICAL, NUTRITIONAL, AND YIELD RESPONSES OF TOMATO ETHYLENE-INSENSITIVE MUTANT *NEVER RIPE* TO MANGANESE ABSENCE

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Prof. Dr. Vitor de Laia Nascimento
Orientador

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RESPOSTAS MORFO-FISIOLÓGICAS, NUTRICIONAIS E DE PRODUÇÃO MUTANTE ETILENO-INSENSITIVO EM TOMATEIRO *NEVER RIPE* À DEFICIÊNCIA DO MANGANÊS

Dissertação apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Agronomia/Fisiologia Vegetal, área de concentração em Fisiologia Vegetal, para a obtenção do título de Mestre.

APROVADA em 09/05/2025
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ABSTRACT

Manganese (Mn) is a micronutrient for plants, fundamental for several metabolic process and its deficiency can drastically reduce crop productivity. Ethylene is a phytohormone that can act in responses to nutritional stress that limits growth and development. However, little is known about the role of the ethylene perception and its impact in physiological responses to Mn absence. The tomato ethylene-insensitive mutant *Never ripe* (*Nr*) is more tolerant to some nutritional stress. Therefore, the aim of this work was to evaluate the connections of the ethylene perception with Mn absence on the level of plant growth and metabolism. The experiment was conducted in a completely randomized design, comparing two genotypes, the wild-type (WT) and the ethylene-insensitive mutant *Nr*, in two nutritional levels (control and Mn absence), totaling four treatments. Tomato plants were sown in trays with sand and irrigated daily until cotyledon protrusion. Afterward, the seedlings were transferred to 1L pots containing sand. Control plants received Hoagland's nutrient solution, initially at half strength and then at full strength after one week, while other plants received the Mn-absence treatment. At the end of the vegetative stage, analyses of chlorophyll fluorescence, gas exchange, and sampling of leaves for biochemical analysis were performed. Photosynthetic pigments, carbohydrates, nitrogen compounds, stress-related metabolites and enzymatic activity were quantified. At the end of the experiment, growth and production analyses were performed. For biomass quantification, the plant material was dried and weighed to quantify the dry biomass, which was also used for the determination of nutrient content in the root, stem and leaves. The total nutrient content, accumulation, and manganese use efficiency were determined. In total, 94 parameters of plants subjected to Mn absence were analyzed. Our results show that WT plants presented more stress-related responses than *Nr* during the Mn absence with several changes in its physiology, here observed for instance by the increase in root size and partition and affected chlorophyll fluorescence. Overall, *Nr* plans performed better than WT in the Mn absence. We can infer that this was due to the low sensitivity to ethylene, which allowed these plants to maintain their balanced metabolism even under stress due to the Mn absence, observed here by the nutrient use efficiency and gas exchange parameters.

Keywords: *Solanum lycopersicum*; Micro-Tom; Never Ripe; Nutritional Stress.

RESUMO

O manganês (Mn) é um micronutriente essencial para plantas, fundamental para diversos processos metabólicos, e sua deficiência pode reduzir drasticamente a produtividade das culturas. Etileno é um fitormônio que pode atuar em respostas ao estresse nutricional que limita o crescimento e o desenvolvimento. No entanto, pouco se sabe sobre o papel da percepção do etileno e seu impacto nas respostas fisiológicas à deficiência de Mn. O mutante de tomate insensível ao etileno *Never ripe* (*Nr*) é mais tolerante a alguns estresses nutricionais. Portanto, o objetivo deste trabalho foi avaliar as conexões da percepção do etileno com a deficiência de Mn no nível de crescimento e metabolismo das plantas. O experimento foi conduzido em um delineamento inteiramente casualizado, comparando dois genótipos, o selvagem (WT) e o mutante insensível ao etileno *Nr*, em dois níveis nutricionais (controle e deficiência de Mn), totalizando quatro tratamentos. As plantas de tomate foram semeadas em bandejas com areia e irrigadas diariamente até a protrusão dos cotilédones. Em seguida, as plântulas foram transferidas para vasos de 1L contendo areia. As plantas controle receberam a solução nutritiva de Hoagland, inicialmente em meia concentração e, após uma semana, em concentração total, enquanto as demais plantas receberam o tratamento com ausência de Mn. Ao final do estádio vegetativo, foram realizadas análises de fluorescência da clorofila, trocas gasosas e coleta de folhas para análise bioquímica. Foram quantificados pigmentos fotossintéticos, carboidratos, compostos nitrogenados, metabólitos relacionados ao estresse e atividade enzimática. Ao final do experimento, foram realizadas análises de crescimento e produção. Para a quantificação da biomassa, o material vegetal foi seco e pesado para quantificar a biomassa seca, que também foi utilizada para a determinação do teor de nutrientes na raiz, caule e folhas. Foram determinados o teor total de nutrientes, o acúmulo e a eficiência do uso de manganês. No total, foram analisados 94 parâmetros de plantas submetidas à deficiência de Mn. Nossos resultados mostram que as plantas WT apresentaram mais respostas relacionadas ao estresse do que as *Nr* durante a deficiência de Mn, com diversas alterações em sua fisiologia, aqui observadas, por exemplo, pelo aumento no tamanho e partição da raiz e pela fluorescência da clorofila afetada. De modo geral, as plantas *Nr* tiveram um desempenho melhor do que as WT na deficiência de Mn. Podemos inferir que isso ocorreu devido à insensibilidade ao etileno, o que permitiu que essas plantas mantivessem seu metabolismo equilibrado mesmo sob estresse devido à deficiência de Mn, observado aqui pelos parâmetros de eficiência do uso de nutrientes e trocas gasosas.

Palavras Chaves: *Solanum lycopersicum*; Micro-Tom; *Never Ripe*; Estresse Nutricional.

IMPACT INDICATORS

This work represents an advance in the knowledge regarding the morpho-physiological, nutritional, and yield responses of tomato (*Never Ripe* mutant) to manganese absence. The obtained results contribute to understanding the role of ethylene perception and its impact on physiological responses, energy metabolism, and plant production under Mn deficiency conditions. Furthermore, a better understanding of nutritional stress responses in crops like tomato leads to improved management, which favors production in soils with nutritional restrictions, directly contributing to agricultural sustainability and food security. This work is of fundamental scientific nature, as it aims to expand theoretical knowledge about the physiological and nutritional mechanisms of ethylene-insensitive tomato, focusing not on immediate practical application but rather on unraveling underlying biological principles. Nevertheless, it possesses an indirect outreach character due to the potential application of its results in soils naturally deficient in manganese or where the management of this nutrient is a challenge. The benefited public includes researchers, plant breeders, interested in plant physiology and plant nutrition. No direct outreach activities with external society took place during the execution of the dissertation. Those involved in the research were the author (Tatiane Santos Correia), the advisor (Prof. Dr. Vitor de Laia Nascimento), and collaborators from UFLA. The impacts of the work can be classified primarily under thematic area 7 - Technology and Production. The research contributes directly to the advancement of knowledge in plant physiology and plant nutrition, with the objective of improving agricultural production, specifically tomato cultivation, which can lead to technologies and practices that optimize management and productivity. The dissertation's impacts are also aligned with the United Nations Sustainable Development Goals (SDGs), contributing to the 2030 Agenda, especially: SDG 2: Zero Hunger and Sustainable Agriculture: By seeking to understand the impacts of Mn deficiency to optimize tomato productivity and resilience, the work contributes to increasing food supply and food security, promoting more sustainable agricultural practices through a better understanding of plant nutrition. SDG 12: Responsible Consumption and Production: The knowledge generated can lead to more efficient resource use (such as manganese fertilizers) and more sustainable production practices in agriculture. Finally, the dissertation's impacts are also aligned with SDG 15: Life on Land. Research in plant physiology deepens the understanding of plant health and its interaction with the environment, contributing to the protection and recovery of terrestrial ecosystems, especially agricultural ones.

INDICADORES DE IMPACTOS

Este trabalho representa um avanço nos conhecimentos sobre as respostas morfofisiológicas, nutricionais e de rendimento do tomateiro (*Never Ripe*) à ausência de manganês. Os resultados contribuem para a compreensão do papel da percepção do etileno e seu impacto nas respostas fisiológicas, no metabolismo energético e na produção vegetal em condições de deficiência de Mn. Além disso, a melhor compreensão das respostas a estresse nutricional, em culturas como o tomate, leva a um manejo aprimorado, o que favorece a produção em solos com restrições nutricionais, contribuindo diretamente para a sustentabilidade da agricultura e a segurança alimentar. Este trabalho possui natureza científica fundamental, pois visa expandir o conhecimento teórico sobre os mecanismos fisiológicos e nutricionais do tomateiro insensível ao etileno, sem focar em uma aplicação prática imediata, mas sim em desvendar os princípios biológicos subjacentes. No entanto, possui um caráter extensionista indireto pelo potencial de aplicação dos seus resultados em solos naturalmente deficientes em manganês. O público beneficiado inclui pesquisadores, melhoristas de plantas, interessados em fisiologia vegetal e nutrição de plantas. Não houve ações extensionistas diretas com a sociedade externa durante a execução da dissertação. Os envolvidos na pesquisa foram a autora (Tatiane Santos Correia), o orientador (Prof. Dr. Vitor de Laia Nascimento) e colaboradores da UFLA. Os impactos podem ser classificados na área temática 7 - Tecnologia e produção, devido a aplicação do conhecimento gerado para o aprimoramento de técnicas agrícolas, a pesquisa contribui diretamente para o avanço do conhecimento em fisiologia vegetal e nutrição de plantas, com o objetivo de melhorar a produção agrícola, especificamente a cultura do tomate, o que pode levar a tecnologias e práticas que otimizem o manejo e a produtividade. Os impactos também estão alinhados com os Objetivos de Desenvolvimento Sustentável (ODS) da ONU, contribuindo para a Agenda 2030, em especial: ODS 2: Fome Zero e Agricultura Sustentável: ao buscar entender os impactos da deficiência de Mn, o trabalho contribui para o aumento da oferta de alimentos e para a segurança alimentar, promovendo práticas agrícolas mais sustentáveis através do melhor entendimento da nutrição de plantas. ODS 12: Consumo e Produção Responsáveis: o conhecimento gerado pode levar a um uso mais eficiente dos recursos (como fertilizantes de manganês) e a práticas de produção mais sustentáveis na agricultura. Por fim, os impactos da dissertação também estão alinhados com a ODS 15: Vida Terrestre: a pesquisa em fisiologia vegetal aprofunda o entendimento sobre a saúde das plantas e a interação o ambiente, contribuindo para a proteção e recuperação dos ecossistemas terrestres e agrícolas.

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LIST OF ABBREVIATIONS AND ACRONYMS

A	Net CO_2 assimilation rate
AFE	Specific Leaf Area
APX	Ascorbate Peroxidase
B	Boron
Ca	Calcium
$Ca(NO_3)_2.4H_2O$	Calcium Nitrate Tetrahydrate
CAT	Catalase
Ci	Internal CO2 Concentration
cm	Centimeters
CO_2	Carbon Dioxide
Cu	Copper
$CuSO_4.5H_2O$	Copper Sulfate Pentahydrate
DNS	3,5-Dinitrosalicylic Acid
E	Transpiration
EDTA	Ethylenediaminetetraacetic Acid
ETR	Electron Transport Rate
EUA	United States
F	Steady-state Fluorescence Before Saturating Pulses
F0	Initial Fluorescence
Fe	Iron
$FeSO_4.7H_2O$	Iron (II) Sulfate Heptahydrate
Fm	Maximum Fluorescence
Fm’	Maximum Fluorescence Under Light-Adapted Conditions
Fv	Variable Fluorescence
GRW	Greenwich Meridian
gs	Stomatal conductance
h	Hours
H_2O	Water
H_2O_2	Hydrogen peroxide
H_3BO_3	Boric acid
K	Potassium
KH_2PO_4	Potassium dihydrogen phosphate
KNO_3	Potassium nitrate
KOH	Potassium hydroxide
LA	Leaf Area
Mg	Magnesium
MG	Minas Gerais
mg	Milligram
$MgSO_4.7H_2O$	Magnesium sulfate heptahydrate
ml	Milliliter
mM	Millimolar
mmol	Millimole
Mn	Manganese
$MnSO_4$	Manganese sulfate
N	Nitrogen
Na_2MoO_4	Sodium molybdate
NaOH	Sodium hydroxide
NBT	Nitroblue Tetrazolium Chloride

NH_4NO_3	Ammonium nitrate
NPQ	Non-photochemical quenching
Nr	*Never ripe*
ºC	Degrees Celsius
P	Phosphorus
pH	Potential of Hydrogen
PSII	Photosystem II
PVPP	Polyvinylpolypyrrolidone
qP	Photochemical quenching
ROS	Reactive Oxygen Species
S	Sulfur
SOD	Superoxide dismutase
UFLA	Universidade Federal de Lavras
WT	Wild type
Zn	Zinc
$ZnSO_4.7H_2O$	Zinc sulfate heptahydrate
µmol	Micromole
µL	Microliter

SUMMARY

1. INTRODUCTION

Ethylene is a phytohormone that modulates a wide range of physiological process of plants, it is a hydrocarbon gas with the chemical formula C_2H_4 (or $H_2C=C_2H$) and is synthesized in all parts of a plant (Iqbal et al., 2017; Dubois et al., 2018). Biosynthesis of ethylene occurs in several moments of the plant development, such as germination, fruit ripening, leaf abscission, and flower senescence, in addition to the role in several stress responses, with highlights to abiotic stresses as drought, waterlogging, salt, temperature and nutritional (Iqbal et al., 2017; Dubois et al., 2018). The ethylene is produced in the Yang cycle and using the amino acid methionine as precursor, this is then converted into 1-aminocyclopropane-1-carboxylic acid (ACC) by the enzyme ACC synthase, and subsequently into ethylene by the enzyme ACC oxidase (ACO) (Yang; Hoffman 1984).Moreover, ethylene is perceive by receptors in the endoplasmic reticulum (ER) as ETR leading to the activation of transcription factors (EIN) (Binder, 2020).

Plants require elements called essential nutrients to complete their life cycle and these nutrients are constituents of metabolites and/or required for the action of a metabolic process (Engels et al., 2012; Kumar et al., 2021). Under nutritional stress conditions (both excess or absence), plants develop morphological and physiological responses, for instance investment in their root architecture and/or the fine adjustment of gas exchange and transpiration rates, aimed at facilitating the absorption and control mobilization of the limiting nutrient (García et al., 2015). Once absorbed in sufficient quantity, these responses need to be turned off to avoid toxicity and conserve energy (Garcia et al., 2015).

Manganese (Mn) is one of the most common so-called micronutrients (which one that plants require in low amounts) in tropical soils, with levels in rocks commonly ranging between 350 mg kg^{-1} and 2,000 mg kg^{-1} (Prado, 2008; Miyazawa et al., 2024). In plants, Mn participates in metabolic process, acting mainly in the Oxygen-Evolving Complex (OEC) of photosynthesis and as an enzymatic cofactor for several important enzymes. Some factors can contribute to absence Mn, such as soils with a pH greater than 6.5 and the presence of other nutrients, such as calcium (Ca), iron (Fe), zinc (Zn), and magnesium (Mg), in which the presence of these elements can inhibit its absorption and even its transport partly due to competition for the same membrane transporters (Millaleo et al., 2010). Because manganese plays a critical role in plant nutrition, research into its level variations is paramount for advancing nutrient management techniques.

The interaction between Mn homeostasis and phytohormones such as ethylene is not very clear and is still poorly investigated regarding the impact of this interaction on energy

metabolism and plant production. One way to explore the interaction of Mn with ethylene in controlling growth and development is using tomato (Solanum lycopersicum L.), since hormonal mutants in different pathways are already available. Among the cultivars, the dwarf cultivar Micro-Tom is the most advantageous for studies, due to its reduced size/height (15-25 cm) and rapid cycle (70-90 days). The ethylene-insensitive Never ripe (Nr) in tomato has lost the ability to respond to ethylene due to mutation in the ETR3 receptor due to an amino acid substitution within the sensor domain of the Nr-SlETR3 protein, which prevents ethylene binding, consequently inhibiting its signaling cascade and response (Lanahan et al., 1994; Wilkinson et al., 1995; Chen et al., 2020; Khan et al., 2024); this leads to a series of different characteristics not only in the fruits but in the whole plants (Lanahan; Giovannoni et al., 1994; Nascimento et al., 2021). Furthermore, studies with this same ethylene-insensitive, under nutritional stress conditions due to phosphorus (P) deficiency and ammonium (NH_4^+) stress, demonstrate that the Nr plants can tolerate this nutritional stress with better development and productivity. In addition to being more photochemically efficient and presenting higher levels of nitrogen (N) -related compounds (as amino acids and proteins), overall nutrient levels in the leaves, and better nutrient utilization efficiency (Garcia, 2023; Souza, 2023).

2. LITERATURE REVIEW

Plants are sessile organisms that constantly need to adapt their growth and development to frequent environmental changes, and to achieve this, they use efficient mechanisms to interpret and differentiate prolonged signals from transient noise (Jaillais; Chory, 2010). One of these mechanisms involves plant hormones, which are non-nutrient organic compounds produced and perceived by plants. They act in low concentrations, regulating growth and development by participating in the control and coordination of cell division, growth, cell differentiation, and the regulation of stress responses (Hooley, 1994; Wolters, Jurgens 2009).

Plant hormones (or phytohormones) are classified into nine main groups: auxins, gibberellins, cytokinin, ethylene, abscisic acid, brassinosteroids, jasmonates, salicylic acid, and strigolactones. These hormones are chemical messengers that act in response to a signal; the hormone produced from this signal is transported to the site of action, where it is perceived by cell receptors that are linked to signal transduction pathways, generating a physiological response (Muller; Munné-Bosch et al., 2021).

Nutrient deficiency is one of the factors that can serve as a signaling cue for the biosynthesis and perception of hormones. Ethylene is one of the hormones involved in responses to nutritional stress (10.1104/pp.15.00708). Ethylene plays a role in regulating physiological and morphological responses to various nutrient deficiencies, mediating ammonium toxicity, nitrogen uptake, regulation of sulfur acquisition by controlling the expression of sulfate transporter genes, and adaptation of root system architecture (García et al., 2015, Ma et al., 2023, Murad et al., 2024).

Ethylene signaling plays a role in the interaction between the deficiency responses of P, S, and Fe in Arabidopsis roots, potentially increasing the specificity and absorption of nutrients (Garcia et al., 2024). However, the role of ethylene perception and its impact on physiological responses, especially in relation to micronutrients such as Mn, need to be further explored.

2.1. Ethylene biosynthesis and signaling

Ethylene (C_2H_4) is a gaseous hydrocarbon with a significant role in plant growth and development and in multiple stress responses (Baharudin; Osman, 2023; Huang et al., 2023). It is commonly associated with fruit ripening but plays an important role throughout the plant's life, acting as a regulator of seed germination, seedling growth, leaf and petal abscission, organ senescence, stress responses, and responses to pathogens (Schaller; Kieber

2002; Baharudin; Osman, 2023). The action of ethylene in various plant development processes is frequently related to its interaction with other hormones, such as auxins, cytokinins, and gibberellins (Munné-Bosch; Müller, 2013).

The ethylene biosynthesis pathway begins with the conversion of the amino acid methionine into S-adenosylmethionine (AdoMet) by the enzyme AdoMet synthetase. AdoMet is then converted into 1-aminocyclopropane-1-carboxylic acid (ACC) by the enzyme ACC synthase, and subsequently into ethylene by the enzyme ACC oxidase (ACO) (Adams; Yang 1979). For ethylene signal transduction and responses to occur, membrane receptors (ETR) linked to an ethylene repressor protein (CTR) must be deactivated, leading to the activation of transcription factors (EIN) (Schaller; Kieber 2002). In the absence of ethylene, the receptors maintain CTR1 in an active state, repressing the hormone's responses. When ethylene is present, the receptors are inactivated, which leads to the inactivation of CTR1. As a result, EIN is activated, and a transcriptional cascade involving transcription factors is initiated (Schaller; Kieber 2002) 2.2. Hormonal mutants insensitive to ethylene

Different hormonal mutants for ethylene have already been described. In Arabidopsis, there are mutants that produce ethylene in excess (eto1 and eto3) and ethylene-insensitive mutants (ETR1) (Ogawara et al., 2003; Chang et al., 1993; Schaller & Bleecker, 1995). In Medicago truncatula, the skl mutant is insensitive to ethylene (10.3390/ijms21134657). In Solanum lycopersicum L., there are the Never-ripe (Nr) mutants that are insensitive to ethylene and the Epinastic mutants that produce ethylene in excess. The hormonal mutant Nr has lost the ability to respond to ethylene generated endogenously or applied exogenously. This leads to a series of different characteristics in the fruits, because the fruits of these mutants do not undergo many of the processes associated with normal ripening (Lanahan; Giovannoni et al., 1994).

Tomato plants have seven ethylene receptors (SlETR1, SlETR2, SlETR3, SlETR4, SlETR5, SlETR6, and SlETR7) (Klee, 2002; Chen et al., 2020). However, Nr mutants have a defect in the SlETR3 receptor, due to an amino acid substitution within the sensor domain of the Nr-SlETR3 protein, which prevents ethylene binding, consequently inhibiting its signaling cascade and response (Lanahan et al., 1994; Wilkinson et al., 1995; Chen et al., 2020; Khan et al., 2024). Furthermore, the binding of ethylene to the receptor is mediated by the cofactor copper (Cu). Nr genotype plants do not exhibit binding to the Cu cofactor, preventing binding with ethylene. The receptor also shows a strong affinity for silver (Ag), which is an inhibitor of ethylene uptake and acts on the receptor complex by displacing Cu from the active site (Rodriguez et al., 1999; Khan et al., 2024).

2.3. Effect of the Nr mutation on plant growth and metabolism

Studies show that ethylene leads to direct changes in plant metabolism. Conversely, low ethylene perception leads to changes in fruit ripening, increased vegetative growth, increased fruit set and yield, and improved tolerance to nutritional stresses (Nascimento et al., 2021; Garcia, 2023; Souza 2023). Investigation of the morphophysiological and biochemical responses of tomato plants to exogenous ethylene application demonstrates that ethylene disrupts the ideal structure of tomato leaves. In general, high concentrations of ethylene were observed to impair growth, while low concentrations can induce plant growth (Nascimento et al., 2021).

The same was observed in tomato mutants with low ethylene perception (Nr), where Nr plants exhibit greater vegetative growth (Garcia, 2023; Souza 2023). Studies with hormonal mutants aimed at understanding the interaction with nutritional balance and low ethylene perception demonstrate that mutant plants have an affinity for specific chemical forms of nitrogen and greater tolerance to nutritional stress from phosphorus (P) (Garcia, 2023; Souza, 2023).

Nr and wild-type (WT) genotypes subjected to different chemical forms of nitrogen (ammonium (NH_4^+) and nitrate (NO_3^-)) show a greater affinity for NO_3^-; both genotypes did not differ statistically with the NO_3^- form. However, in both sources, the Nr mutant showed better development and productivity, in addition to being more photochemically efficient (Garcia, 2023). Under nutritional stress conditions due to the deficiency of P, the Nr mutant did not perceive the nutritional stresses with the same intensity as the WT genotype. The main changes were observed in biometric parameters, with the Nr genotype showing better phenotypic stability, as well as better levels of nitrogen compounds (amino acids and proteins), nutrient levels in the leaves, and better P utilization efficiency. In P excess treatment, the WT genotype showed greater phenotypic stability, and the Nr genotype showed greater damage (Souza, 2023).

The role of ethylene as a growth-regulating hormone and its involvement in various plant development processes is well-defined (Nascimento et al., 2021). However, despite these advances in studying the interaction of ethylene with nutritional stresses, further research is needed to understand the relationship of ethylene with other plant nutrients, which will lead to greater clarification of the role of ethylene in modulating carbon assimilation and central metabolism under nutritional stress conditions.

2.4. Manganese as a plant nutrient

The first evidence of Mn in plant tissue was described in vegetable ash more than 200 years ago (Saussure, 1804). It was only in the early 1900s, in studies with wheat, that it was documented as essential for plant growth and development, and its essentiality remains evident today. It is a micronutrient, required in small amounts, but as critical for growth and development as other nutrients (McHargue, 1919; Alejandro et al., 2020; Lilay et al. 2024).

Mn supports metabolic functions within different cell compartments. In the soil, Mn is found in minerals along with other elements. The active forms include three groups of compounds: water-soluble, exchangeable, and easily recoverable Mn, among which there is a mobile equilibrium (Perfileva; Krutovsky 2024). Plants mainly use water-soluble and exchangeable Mn from the soil in the form of Mn^{2+} ions, which is the form most easily transported to root cells and translocated to the aerial part, while the oxidized species Mn^{3+} (e.g. Mn_2O_3) and Mn^{4+} (e.g., MnO_2) (III) and Mn (IV) form insoluble oxides that quickly sediment (Alejandro et al., 2020). Mn uptake by roots occurs through root interception and diffusion. Once Mn reaches the symplast, it is translocated and distributed towards the xylem, transferred to the phloem, and translocated to different tissues (Li et al., 2017; Alejandro et al., 2020).

Mn absorption is regulated by transporters located in the root plasma membrane; however, most proteins that transport Mn are not specific to the metal. The transporters can transport other divalent cations into the plant, such as Fe^{2+}, Zn^{2+}, Cu^{2+}, Cd^{2+}, Ca^{2+}, Co^{2+}, and Ni^{2+} (Chu et al., 2017; Alejandro et al., 2020). For example, the NRAMP (Natural Resistance-Associated Macrophage Protein) transporter family plays an important role in the transport of a wide range of divalent metals, including Fe^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+}, and Cd^{2+}. They can be found in the plasma membrane (for nutrient uptake from the soil) and in the membranes of internal organelles (for metal storage and homeostasis). They function by transporting divalent cations across cell membranes driven by a proton gradient (Gao et al., 2017). Another family, IRT1 (Iron-Regulated Transporter 1), is a high-affinity transporter for iron (Fe^{2+}), located in the plasma membrane of root epidermal and endodermal cells. This family also exhibits low specificity and can transport other divalent cations such as Mn^{2+}, especially under iron deficiency conditions (Long et al., 2017).

After being absorbed, Mn homeostasis is regulated by Mn importer and exporter proteins. Importer proteins transfer Mn to the extracellular space or from internal compartments to the cytosol, while exporters are responsible for excluding Mn from the cytosol to intracellular compartments or to the apoplast (Alejandro et al., 2020). After Mn

enters the cell, it is moved to target supply sites or for storage. In the cell, Mn is present in all cell compartments, including the endoplasmic reticulum, Golgi complex, mitochondria, plastids, and peroxisomes, where it performs specific cellular functions (Alejandro et al., 2020).

Fertilization with Mn increases photosynthesis, net assimilation, and relative growth and yield (Barros et al., 2019). Soils with low Mn content cause fewer chloroplasts, lower net photosynthetic efficiency, and a decrease in chlorophyll content, resulting in decreased plant growth, biomass, and productivity. Generally, Mn absence occurs in soils with a pH greater than 6.5, and its critical deficiency level for most plant species is in the range of 10 to 20 mg kg^{-1} (Mousavi et al., 2011; Tao et al., 2023; Zheng et al., 2024).

One way to solve the problem of Mn absence is through fertilization with different Mn sources. Some studies show that the use of Mn carbonate promoted a greater number of tillers in rice (Barros et al 2019). The use of Mn-chelate increased both yield and net return of wheat (Dhaliwal et al., 2023). In contrast, fertilization with Mn sulfate and Mn carbonate significantly improved the growth and yield of beans (Ozbahce; Zengin 2014).

On the other hand, excess Mn results in the degradation of lipids, proteins, carbohydrates, and nucleic acids, impairing cellular metabolism and causing cell death in some cases (Kishchenko et al., 2023; Silva et al., 2022). Generally, Mn toxicity occurs in soils with a pH less than 5.5, being considered the second major limiting metal for agricultural production, second only to aluminum. Its toxicity and tolerance limits to excesses depend on plant species, as well as varieties or genotypes (Fageria et al., 2008). It is important to emphasize that due to increased cultivation, soil pH is progressively decreasing, which may result in greater Mn toxicity. Studies on variations in Mn levels are necessary for better management of this important micronutrient (Yang et al., 2013; Zheng et al., 2024).

The interaction between Mn and phytohormones is not very clear; so far, we know that there may be some influence of gibberellins on the absorption of this nutrient in rice plants (Guo et al., 2015). Other evidence demonstrates that the inhibitory effect of Mn toxicity on root growth is mediated by auxin biosynthesis and transport (Zhao et al., 2017). Finally, in addition to this link between Mn metabolism and some hormones, the interaction with ethylene, and the impact of this interaction on energy metabolism and plant production, is still poorly investigated, making it an interesting target for current research.

2.5. Influence of manganese on photosynthesis

The first evidence of the importance of Mn in photosynthetic processes was observed in plants and algae, which showed an inability to release O_2 in the deficiency of Mn in their growth medium (Pirson et al. 1937; Pirson et al. 1951). Currently, it is known that it is an essential cofactor for the Oxygen-Evolving Complex (OEC) of photosynthetic machinery. The first stage of photosynthesis begins with a photolysis reaction of water, through the luminous energy of photosystem II (PSII). This reaction is dependent on Mn, which aids in breaking down water molecules, releasing electrons, protons, and oxygen. The protons produce ATP, the electrons are transported in PSII producing $NADPH_2$, and oxygen can be released into the atmosphere in the form of O_2 (Rana et al., 2023).

In addition, Mn also influences the biochemical phase of photosynthesis by replacing Mg at the active site of some enzymes, including Rubisco. This change in the active site can alter the functional role of these enzymes. Binding with Mg^{2+} favors carboxylation over oxygenation, while binding with Mn^{2+} retards carboxylation until oxygenation proceeds at approximately the same rate. This means that CO_2 affinity is decreased approximately 4 times, and O_2 affinity is not affected, becoming non-specific for either substrate (Bloom; Kameritsch, 2017; Voland et al., 2024). This relationship has a strong influence on the relationship between photosynthesis and photorespiration, where increased photorespiration decreases photosynthesis and the production of biomass and productivity.

2.6. Manganese as an enzymatic cofactor

Under stress conditions, there is an increase in the production of Reactive Oxygen Species (ROS), such as superoxide anion, hydrogen peroxide, hydroxyl radical, and singlet oxygen (Gong et al., 2020). In plant cells, ROS are mainly formed in chloroplasts, mitochondria, peroxisomes, and the cytosol. The increase in ROS causes oxidative damage to cell constituents, such as lipids, proteins, and nucleic acids, which leads to metabolic dysregulation, affecting photosynthesis and mitochondrial function, resulting in damage to growth and productivity, and in the long term, potentially leading to the organism's death (Araz et al., 2022).

Superoxide dismutase (SOD) is one of the essential enzymes that regulate ROS, but for the reaction to occur, a multivalent metal ion, such as $Cu^{1+/2+}$, $Mn^{2+/3+}$, $Fe^{2+/3+}$, and Ni^{2+}, must bind to the active site (Jiang et al., 2019). In plants, there are three forms of the enzyme, classified by their active site metal ion: Cu/Zn, Mn, and Fe forms. Mn-SOD is located in mitochondria and peroxisomes, where it catalyzes the dismutation of superoxide (O_2^-) by converting these compounds into less reactive compounds, such as hydrogen peroxide (H_2O_2)

and O_2, which can be broken down by other enzymes or non-enzymatic antioxidants, thus reducing their harmful effects (Bowler et al., 1994; Corpas et al., 2017; Kidwai et al. 2020; Jiang et al., 2019). When Mn binds to the active site of SOD, in the first reaction, the ROS superoxide anion binds to the available site of the Mn metal cofactor. When the superoxide anion is oxidized by the catalyst, it produces neutral O_2. When the superoxide anion is reduced by the catalyst and reacts with hydrogen ions, it produces H_2O_2 (Srnec et al., 2009).

In general, SOD is composed of subunits with 150 to 220 amino acid residues, which bind to form protein complexes in the form of homodimers, tetramers, or hexamers. MnSOD is tetrameric in mitochondria and peroxisomes and dimeric in the cytosol. These subunits are responsible for coordinating specific metal cofactors; through the redox mechanism, the metal in the active site can transition between its reduced and oxidized forms (Shams et al., 2024). The enzyme is formed by two domains: the N-terminal domain binds to Mn and Zn ions, and the C-terminal domain forms the protein's core. SOD exhibits activity regardless of whether the metal center is in an oxidized or reduced state. When Mn binds to the active site, it acts as a cofactor, accelerating activity and enhancing the reduction of damage to cellular components (Shams et al., 2024).

In the apoplast, mainly of monocotyledonous plants, oxalate oxidase is located, another Mn-dependent enzyme that catalyzes the oxidation of oxalate to CO_2 and the reduction of O_2 to H_2O_2. It is possible that disease resistance is associated with oxalate oxidase activity, since the H_2O_2 produced can be used in lignin cross-linking (Schmidt; Husted 2019). Mn also acts as a cofactor for enzymes involved in isoprenoid biosynthesis. This occurs because Mn and Mg are two of the main cofactors of terpene synthases; therefore, different plant terpene patterns are correlated with Mg/Mn ratios (Farzadfar et al., 2017). In addition to Mg, Mn can serve as a cofactor for phenylalanine ammonia-lyase, a key enzyme in the phenylpropanoid pathway to produce monolignols, which are precursors of lignin. Lignin can also be synthesized from monolignols, which are oxidized by Mn^{3+} into monolignol radicals, which can then be added to existing phenolic groups to form lignin polymers (Önnerud et al., 2002; Alejandro et al. 2020).

3. AIMS

3.1. General aim

To investigate how the ethylene insensitivity of *Never ripe* tomato mutant influences plant physiology (growth, nutrition, and metabolism) and productivity under manganese (Mn) absence conditions.

3.2. Specific aims

- To determine the interactive effects of the ethylene insensitivity and Mn absence on the **growth and productivity** of tomato plants.
- To determine the interactive effects of the ethylene insensitivity and Mn absence on the **photosynthetic parameters** of tomato plants.
- To determine the interactive effects of the ethylene insensitivity and Mn absence on the **carbohydrate and nitrogen compound metabolites** of tomato plants.
- To determine the interactive effects of the ethylene insensitivity and Mn absence on the **activity of antioxidant enzymes** in tomato plants
- To determine the interactive effects of the ethylene insensitivity and Mn absence on the **elemental composition and nutrient use efficiency** of tomato plants.

4. HYPOTHESIS

The ethylene insensitivity of *Never ripe* (*Nr*) mutant tomato plants enhances tolerance to Mn absence by modulating physiological responses, leading to improved growth, photosynthetic efficiency, nutrient utilization, and fruit productivity compared to wild-type plants under same conditions.

5. MATERIAL AND METHODS

5.1. Plant material, growth conditions and experimental design

Tomato plants (*Solanum lycopersicum* L. cv Micro-Tom) of the wild type (WT) and the ethylene-insensitive mutant *Never ripe* (*Nr*) were grown in a greenhouse of the Plant Physiology Sector of Department of Biology - Institute of Natural Sciences of the Universidade Federal de Lavras (UFLA), in the municipality of Lavras – Minas Gerais. Tomato seeds were sown in trays with sand and irrigated daily until cotyledon protrusion. Afterwards, the seedlings were transferred from the trays to 1000 mL capacity pots containing sand and nutrient solution in a fertigation system, with the application of a nutrient solution adapted from Hoagland (**Table 1**) (Hoagland; Arnon, 1938). The stock solutions present the following salts and their respective concentrations: KH_2PO_4 (1M), KNO_3 (1M), NH_4NO_3 (1M), Ca $(NO_3)_2.4H_2O$ (1M), $MgSO_4.7H_2O$ (1M), $ZnSO_4.7H_2O$ (1 mM), $CuSO_4.5H_2O$ (0.25 mM), $FeSO_4.7H_2O$ – Na_2EDTA (64 mM), H_3BO_3 (12.5 mM), Na_2MoO_4 (0.25 mM) and $MnSO.4H_2O$ (1 mM) (**Table 1).**

Table 1. Concentrations of nutrient sources in the nutrient solutions per 1 L of solution.

Nutrient Sources	Mn absence	Control
KH_2PO_4	2 mM	2 mM
KNO_3	4 mM	4 mM
NH_4NO_3	4 mM	4 mM
$Ca(NO_3)_2.4H_2O$	4 mM	4 mM
$MgSO_4.7H_2O$	1 mM	1 mM
$ZnSO_4.7H_2O$	0.002 mM	0.002 mM
$CuSO_4.5H_2O$	0.005 mM	0.005 mM
$FeSO_4.7H_2O$ – Na_2EDTA	0.064 mM	0.064 mM
H_3BO_3	0.025 mM	0.025 mM
Na_2MoO_4	0.005 mM	0.005 mM
$MnSO_4.H_2O$	-	0.002 mM

Source: Author (2025).

The nutrient solution was initially applied at half ionic strength until the complete acclimatization. During this period, the plants were subjected to the nutritional treatments (Mn absence and control). After acclimatization, the solution was applied at its full ionic strength, pH 5.5 ± 0.5, with 100 mL per pot. The plants were watered daily with distilled water to avoid excess Mn, and the nutrient solution was applied every 7 days. The experiment

was conducted in a completely randomized design, comparing two genotypes (WT and *Nr*) in two nutritional conditions (Mn absence and control), totaling four treatments. The experiment evaluated how ethylene perception can act directly in the response to Mn absence describing the impact of these changes is on plant physiology. The experiment was conducted with 5 replicates per treatment and repeated four times to confirm the results obtained.

5.2. Photosynthetic parameters

At the end of the vegetative stage of the plants, 30 days after transplanting, gas exchange and chlorophyll *a* fluorescence parameters were measured in fully expanded leaves, which were also collected and used for biochemical analyses. The net CO_2 assimilation rate (*A*, μmol CO_2 m^{-2} s^{-1}), stomatal conductance (g_s, mol H_2O m^{-2} s^{-1}), transpiration (*E*, mmol H_2O m^{-2} s^{-1}) and internal CO_2 concentration (C_i, μmol CO_2 mol air^{-1}) were measured using an infrared gas analyzer (IRGA), model LI6400XT (LI-COR Biosciences Inc., Nebraska, USA). The measurements were taken in the morning (8:00 AM to 12:00 PM) under saturating artificial light of 1.500 μmol photons m^{-2} s^{-1} and ambient CO_2 concentration (C_a), temperature, and H_2O vapor. The reference air was collected 50 cm above the ground and homogenized in a 10 L container before reaching the leaf chamber. From the measured data, the intrinsic (A/g_s) and extrinsic (A/E) water use efficiencies (WUE) were calculated. For each plant, 10 readings per leaf were taken, one every 12 s, and their average was considered one measurement ((Farquhar, Richards 1984; Melo et al., 2009; Medlyn et al., 1998).

The chlorophyll *a* fluorescence parameters were obtained using a MINI-PAM modulated portable fluorometer (Walz Inc., Effeltrich, Germany). After thirty minutes of dark adaptation of the leaves, the initial fluorescence (F_0) and maximum fluorescence (F_m) parameters will be obtained, and from these, the values of variable fluorescence ($F_v = F_m-F_0$) and the potential quantum yield (F_v/F_m) of photosystem II (PSII) will be calculated (Abadía et al., 1999). The same leaves were exposed to incident radiation, and eight increasing pulses of light were applied to determine the steady-state fluorescence parameters before the saturation pulses (F) and the maximum fluorescence under light conditions (F_m'). From these values, the photochemical quenching (q_P), $q_P = (F_m'- F)/(F_m'-F_0)$, (Van Kooten; Snel, 1990), non-photochemical quenching (NPQ), $NPQ = (F_m-Fm')/F_m'$ (Bilger; Björkman, 1990), and the electron transport rate (ETR) were determined (Strong et al., 2000).

5.3. Growth and productivity analysis

At the end of the experiment, 90 days after transplanting to the pots, the height (cm) and root length (cm) were measured with the a millimeter ruler, and the stem diameter (mm) was measured with a digital caliper. For the determination of dry mass, the organs were placed in a forced-air oven and kept at 70 ºC for 72 h until constant weight. With the dry mass of the organs, the allocation of dry matter to the root, stem, leaf, and fruits was determined, in addition to the root-to-shoot ratio (Hunt, 1982; Potter; Pother-mann 1992). The leaf area (LA) data will be quantified using the ImageJ software (version 1.52a) (Schneider et al., 2012). The leaf area (LA) was obtained by the formula LA = L x W, where (L) is the leaf length and (W) leaf width. From these values, the specific leaf area (SLA) was quantified, calculated using the formula: SLA (cm^2 g^{-1}) = LA / dry mass of the leaf (Hunt, 1982). The biometric and production (yield) parameters evaluated were total number of leaves, fruit diameter, number of inflorescences, number of flowers, number of fruits per plant, fruit set, fresh and dry mass of fruits, and total fresh and dry mass of fruits. Furthermore, the harvest index was calculated as the ratio between the dry mass of the fruits and the total dry mass of the plant (Singh; Stoskopf 1971).

5.4. Biochemical analyses

The same leaves in which photosynthetic parameters were measured were collected for biochemical analyses. The leaves were frozen in liquid N (N_{liq}) and stored in an freezer at -20 ºC until the analyses. The samples were subjected to hot ethanolic extraction (Andrade et al., 2024) and in the ethanol-soluble fraction, the levels of total soluble sugars (TSS), reducing sugars (RS), and non-reducing sugars (NRS), and total soluble amino acids were determined. In the insoluble fraction, the levels of starch and proteins were determined (Miller, 1959; Dische, 1962; Lichtenthaler, 2001; López-Hidalgo et al., 2021). For the determination of protein levels, quantification was performed using the Bradford method (1976). Starch extraction was carried out as proposed by Hendriks (2003) and Zanandrea et al. (2010). The levels of photosynthetic pigments (chlorophylls *a* and *b*, and carotenoids), were determined according to the equations of Lichtenthaler (2001). Total phenolic compounds were determined using the Folin-Ciocalteu colorimetric method (López-Hidalgo et al., 2021). The quantification of malondialdehyde (MDA) was determined by the thiobarbituric acid (TBA) extraction method (Lopez-Hidalgo et al., 2021). The quantification of hydrogen peroxide (H_2O_2) was determined by the method proposed by Velikova et al. (2000). All metabolites were expressed per mg g^{-1} of fresh mass (FM).

For the extraction of the enzymatic antioxidant system components, catalase (CAT), superoxide dismutase (SOD), and ascorbate peroxidase (APX), approximately 100 mg of fresh matter will be ground in liquid N and polyvinylpyrrolidone (PVPP), homogenized in an extraction medium containing 100 mM potassium phosphate buffer (pH 7.8) supplemented with 10 mM ethylenediaminetetraacetic acid (EDTA) and 200 mM ascorbic acid. The reaction medium for CAT was prepared according to Anderson et al. (1995), SOD activity was measured as described by Giannopolitis and Ries (1977), and APX activity was determined using the method described by Nakano and Asada (1981).

5.5. Nutrient analysis in plant tissues

For the evaluation of the nutritional status, the same plant material (dried material) used to growth analysis (described before) were used. The levels of phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), sulfur (S), copper (Cu), iron (Fe), Mn, and zinc (Zn) were determined according to the methodology proposed by Malavolta et al. (1997). For the determination of total N levels, the Kjeldahl method was used (Feema, 1983). Mn accumulation was calculated by multiplying the dry mass of leaves, stem, and root by the Mn concentration in these respective parts (Santos et al., 2020). The Mn utilization efficiency (MnUE) was calculated according to the proposal by Osborne and Rengel (2002).

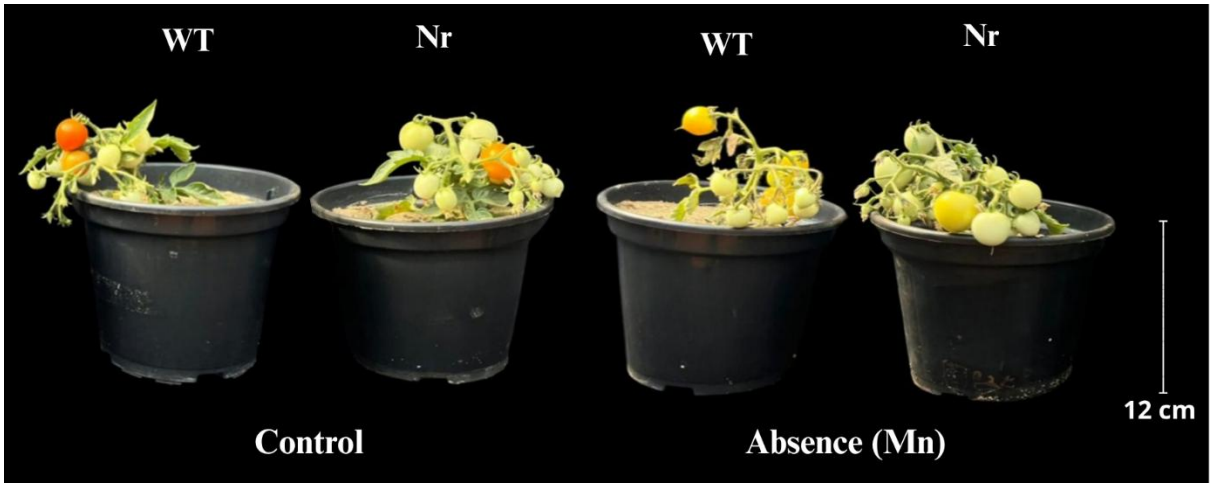
5.7. Data analysis

The data obtained will be analyzed using the R software, latest version (R Core Team, 2023). The evaluated characteristics will be subjected to analysis of variance (ANOVA) by the F test. When a significant effect was observed in the analyzed parameters, the Scott-Knott test ($p \leq 0.05$) will be applied, using the ExpDes.pt package (Ferreira et al., 2013). The graphs will be prepared using the SigmaPlot software.

6. RESULTS

Overall, tomato plants of the *Nr* mutant and WT genotypes showed did not show significant changes when exposed to Mn absence. However, the *Nr* mutant genotype presented the highest results in most of the analyzed parameters (**Figure 1**). The content, accumulation, and utilization efficiency of Mn (**Figure 2**), growth parameters (**Figure 3**), dry biomass (**Figure 4),** production (**Figure 5**), chlorophyll fluorescence (**Figure 6**), gas exchange (**Figure 7**), pigments (**Figure 8**), carbohydrate and nitrogen metabolites (**Figure 9**), stress-related metabolites (**Figure 10**), and enzymatic activity (**Figure 11**) were evaluated. Furthermore, the values referring to the overall nutrient content in the root, stem, and leaves were also evaluated (**Figures 12** to **15**).

Figure 1. Plants of the genotypes wild-type (WT) and *Never ripe* (*Nr*) under Mn absence and control.

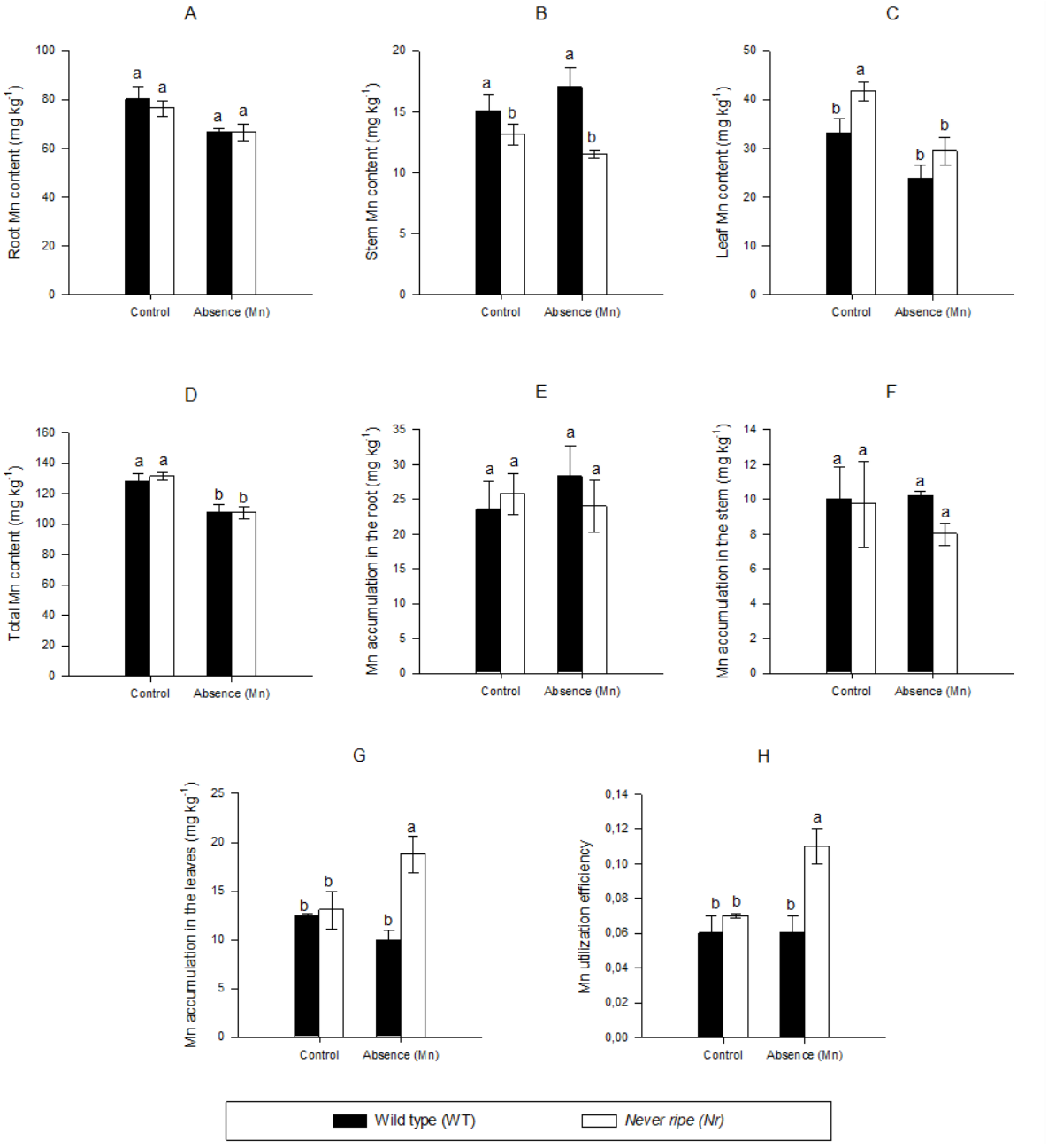


Source: Author (2025).

Regarding the Mn content in the different plant parts, the Mn absence treatment did not interfere with the Mn content in the root for the genotypes (**Figure 2A**). The Mn content in the stem of *Nr* genotype plants under Mn absence was 32.4% lower than in WT plants. Under optimal Mn conditions (control), the Mn content was 12.6% lower (**Figure 2B**). The Mn content in the leaves of *Nr* genotype plants under control was 25.8% higher than in WT plants, but under Mn absence, the genotypes did not differ from each other (**Figure 2C**). The total Mn content in both genotypes was higher under control (**Figure 2D**). The Mn absence did not interfere with Mn accumulation in the root and stem in either genotype (**Figures 2E** and **2F**). The accumulation of Mn in the leaves of *Nr* genotype plants under Mn absence was 100% higher than in WT plants, but in control, the genotypes did not differ from each other

(**Figure 2G**). The Mn utilization efficiency in *Nr* genotype plants under Mn absence was 83.3% higher than in WT plants but under control, the genotypes did not differ from each other (**Figure 2H**).

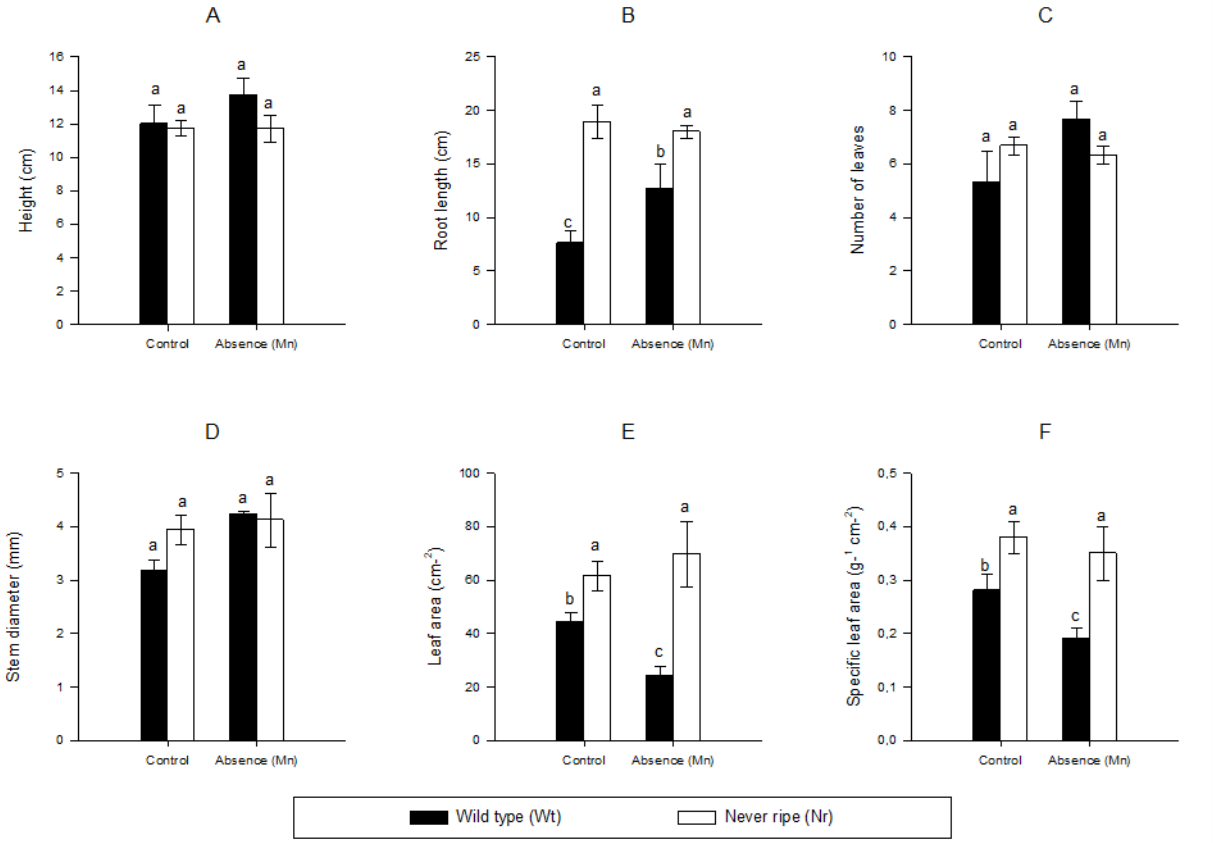
Figure 2. Root (**A**), stem (**B**), leaves (**C**) and total (**D**) manganese (Mn) contents, Mn accumulation in the root (**E**), stem (**F**), leaves (**G**) and Mn utilization efficiency (**H**) of tomato plants cv. Micro-Tom from wild type (WT) and *Never ripe* (*Nr*) genotypes at different Mn levels (control and absence).



Means of variables followed by the same letter do not differ statistically by the Scott-Knott test at $p \leq 0.05$, n = 6 ± SE. The black bar represents WT and white bar represents *Nr*. Source: Author (2025).

Regarding the growth parameters, it was observed that the WT and *Nr* genotypes did not differ in plant height, number of leaves, and stem diameter values (**Figures 3A**, **3C** and **3D**). However, the *Nr* genotype showed higher root length values, wherein under Mn absence conditions, *Nr* plants were 42% longer than the WT genotype, while control, this difference increased to 149% (**Figure 3B**). *Nr* plants under Mn absence and control showed larger LA, being 189% and 39.2% larger compared to WT plants (**Figure 3E**). The same was observed regarding SLA, wherein under Mn absence and control, *Nr* plants were 84.2% and 30.7% larger compared to WT plants (**Figure 3F**).

Figure 3. Height (**A**), root length (**B**), number of leaves (**C**), stem diameter (**D**), leaf area (**E**) and specific leaf area (**F**) of tomato plants cv. Micro-Tom from wild type (WT) and *Never ripe* (Nr) genotypes at different Mn levels (control and absence).

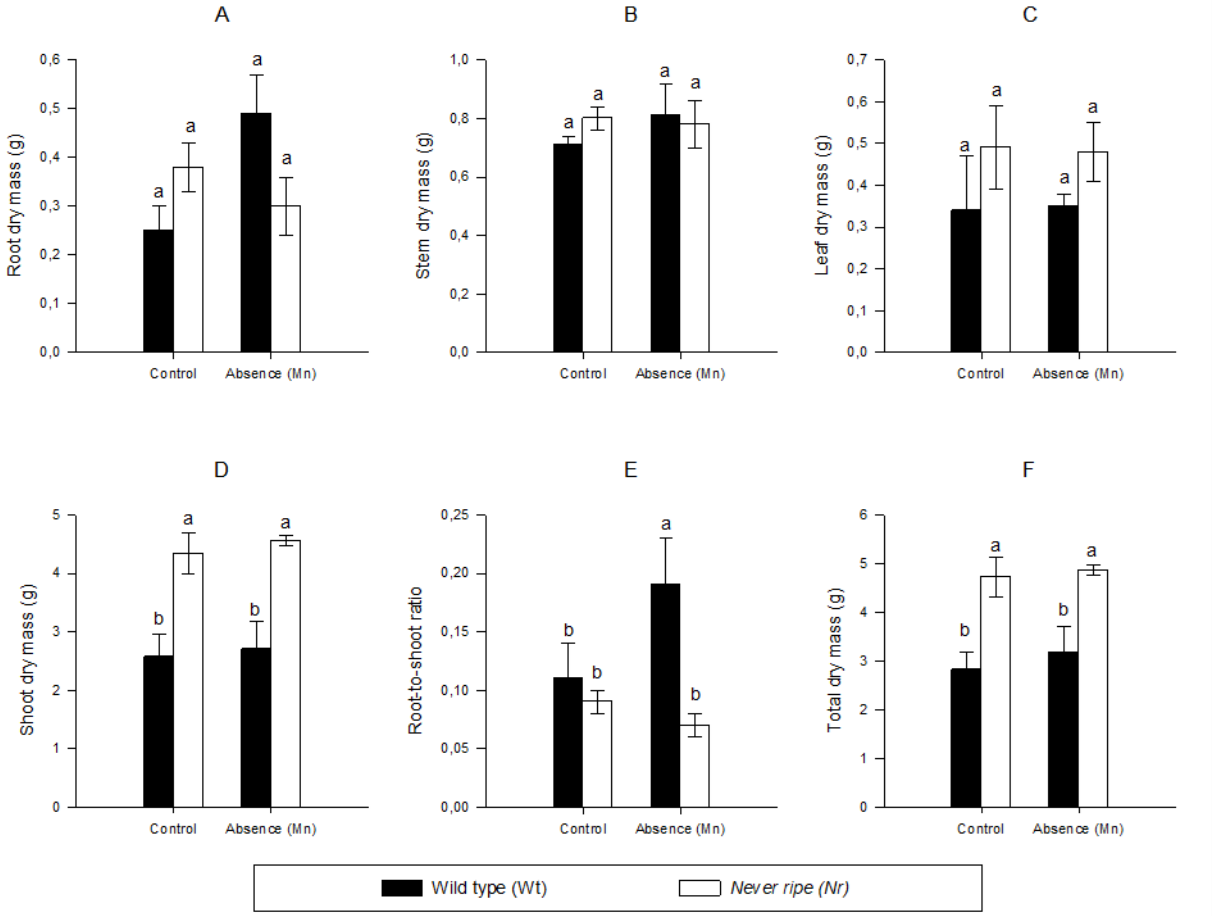


Means of variables followed by the same letter do not differ statistically by the Scott-Knott test at $p \leq 0.05$, $n = 6 \pm SE$. The black bar represents WT and white bar represents Nr. Source: Author (2025).

Regarding dry biomass, plants of the WT and *Nr* genotypes did not differ in root, stem, and leaf dry biomass (**Figures 4A**, **4B** and **4C**). *Nr* plants under Mn absence and control conditions showed better results for shoot dry mass, being 68.9% higher than WT plants

(**Figure 4D**). The same pattern was observed for total dry mass values, where *Nr* plants showed the highest values, being 68.8% (Mn absence) and 67.38% (control) higher than WT plants (**Figure 4F**). The opposite was observed regarding the root-to-shoot ratio, where the *Nr* genotype, under Mn absence, was 94% lower than WT plants (**Figure 4E**).

Figure 4. Root dry mass (**A**), stem dry mass (**B**), leaf dry mass (**C**), shoot dry mass (**D**), root-shoot ratio (**E**) and total dry mass (**F**) of tomato plants cv. Micro-Tom from wild type (WT) and *Never ripe* (Nr) genotypes at different Mn levels (control and absence).

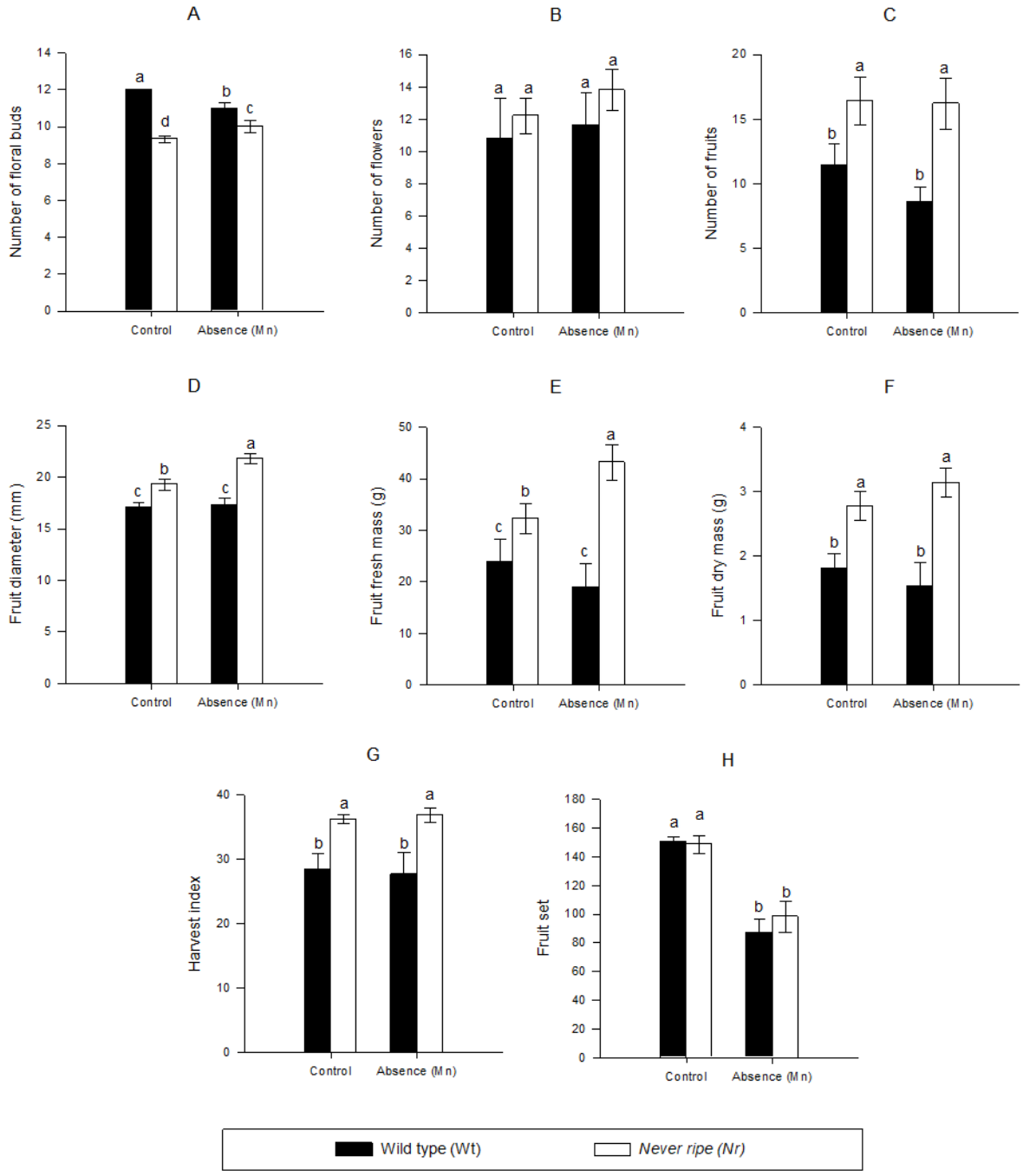


Means of variables followed by the same letter do not differ statistically by the Scott-Knott test at $p \leq 0.05$, n = 6 ± SE. The black bar represents WT and white bar represents Nr. Source: Author (2025).

Regarding production (yield) parameters, plants of the *Nr* genotype showed highest values in most parameters, even in the Mn absence. The same was observed under control (**Figure 5**). *Nr* plants under Mn absence showed a reduction in the number of flower buds, being 9.1% lower than *Nr* plants under control; moreover, *Nr* plants under control were 22.5% lower than WT plants under control (**Figure 5A**). Both genotypes did not differ in the number

of flowers (**Figure 5B**). However, under Mn absence, a difference was observed in the number of fruits (88.3%), fruit diameter (26%), fruit fresh mass (127%), fruit dry mass (104%), and harvest index (33.3%) (**Figures 5C** to **5G**). Under control, *Nr* plants showed the best values compared to WT plants for the number of fruits (43.7%), fruit diameter (13.4%), fresh mass (34.9%), dry mass of fruits (6.06%), and harvest index (27.4%) (**Figures 5C** to **5G**). The fruit set index in both genotypes was higher under control (**Figure 5H**)

Figure 5. Number of floral buds (**A**), number of flowers (**B**), number of fruits (**C**), fruit diamtrer (**D**), fruit fresh mass (**E**), fruit dry mass (**F**), harvest index (**G**) and fruit set (**H**) of tomato plants cv. Micro-Tom from wild type (WT) and *Never ripe* (Nr) genotypes at different Mn levels (control and absence).

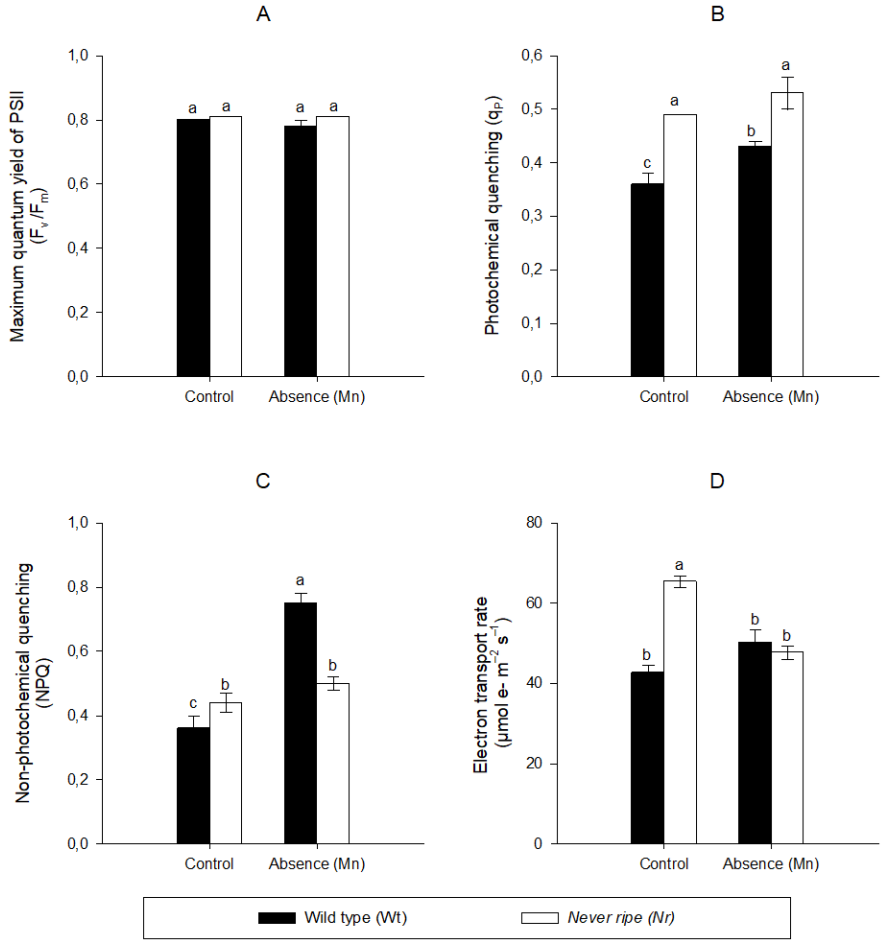


Means of variables followed by the same letter do not differ statistically by the Scott-Knott test at $p \leq 0.05$, n = 6 ± SE. The black bar represents WT and white bar represents Nr. Source: Author (2025).

The chlorophyll *a* fluorescence results show that after the stress, the genotypes did not exhibit changes in the potential quantum yield of PSII (**Figure 6A**). *Nr* plants under Mn absence and control conditions showed better q_P results, being 23.2% and 36.1% higher than WT plants (**Figure 6B**). However, when evaluating NPQ, under Mn absence, the *Nr* genotype showed lower values, 33.3% lower than WT plants and under control, the *Nr* genotype was 22.2% higher than WT plants (**Figure 6C**). The *Nr* genotype under control showed the best

results for electron transport rate, being 52.9% higher than WT plants, but under Mn absence, the genotypes did not differ from each other (**Figure 6D**).

Figure 6. Maximum quantum yield of PSII (**A**), photochemical quenching (**B**), Non-photochemical quenching (**C**) and electron transport rate (**D**) of tomato plants cv. Micro-Tom from wild type (WT) and *Never ripe* (Nr) genotypes at different Mn levels (control and absence).

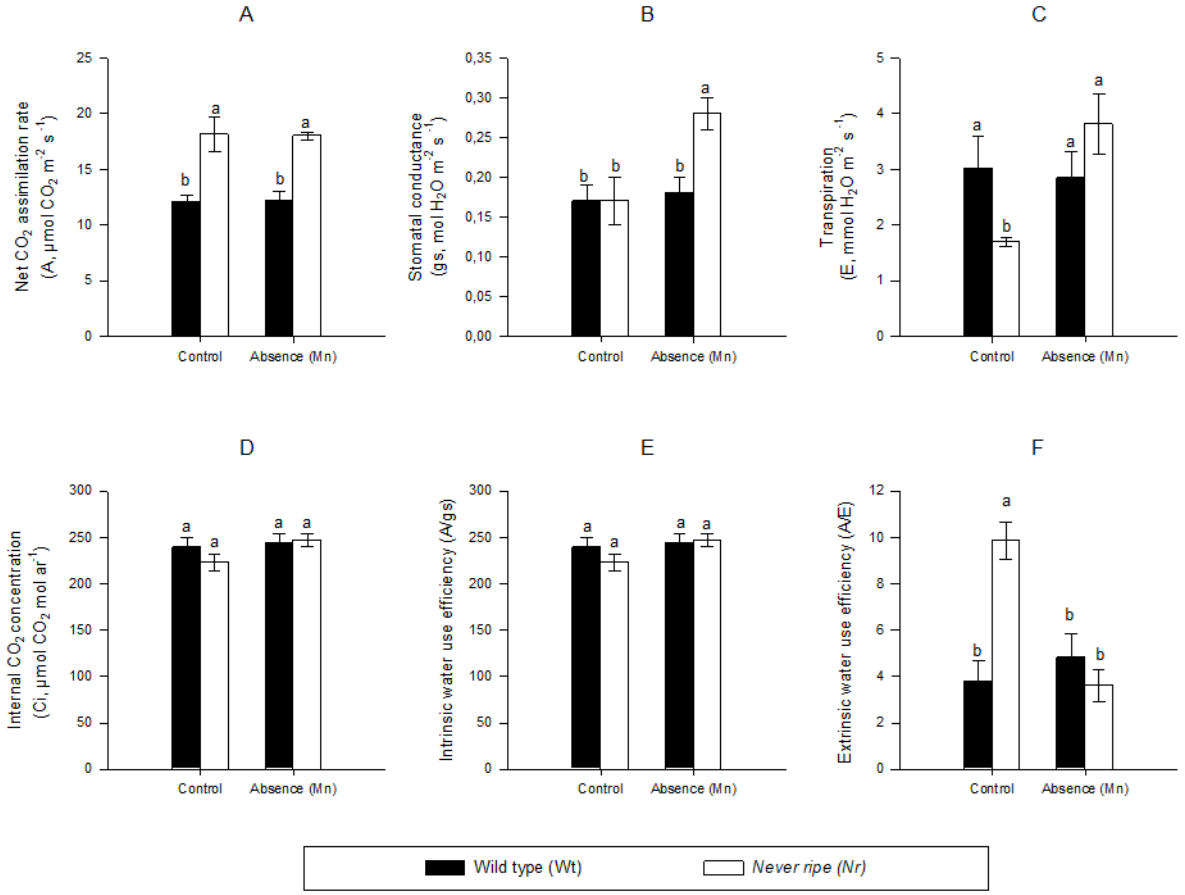


Means of variables followed by the same letter do not differ statistically by the Scott-Knott test at $p \leq 0.05$, n = 6 ± SE. The black bar represents WT and white bar represents Nr. Source: Author (2025).

Regarding the gas exchange parameters, the genotypes did not show any difference in the internal CO_2 concentration and intrinsic water use efficiency (**Figures 7D** and **7E**). The net CO_2 assimilation in *Nr* plants under Mn absence was 47.8% higher than the WT and 50.3% higher under control (**Figure 7A**). However, the *Nr* genotype, under Mn absence, showed higher stomatal conductance values (33.8%) when compared to the WT genotype and the control plants, however, did not differ from each other (**Figure 7B**). Plants of the *Nr* genotype, under control showed lower transpiration values, 44% lower than WT plants and

under Mn absence the genotypes did not differ (**Figure 7C**). The *Nr* genotype under control showed the best results for extrinsic water use efficiency, being 162% higher than WT plants and under Mn absence the genotypes did not differ from each other (**Figure 7F**).

Figure 7. Net CO_2 assimilation rate (**A**) stomatal conductance (**B**), transpiration (**C**), internal CO_2 concentration (**D**), intrinsic water use efficient (**E**) and extrinsic water efficient (**F**) of tomato plants cv. Micro-Tom from wild type (WT) and *Never ripe* (*Nr*) genotypes at different Mn levels (control and absence).

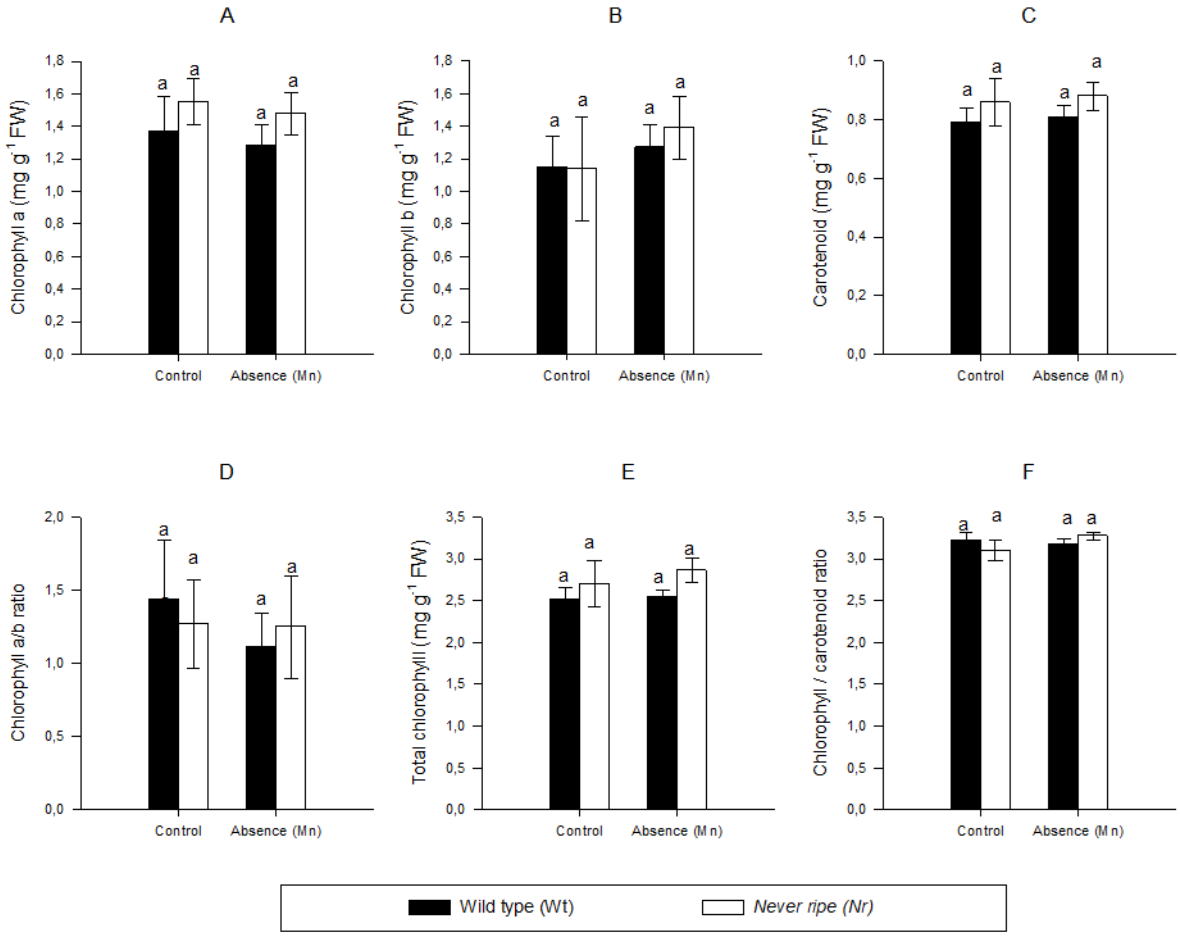


Means of variables followed by the same letter do not differ statistically by the Scott-Knott test at $p \leq 0.05$, n = 6 ± SE. The black bar represents WT and white bar represents *Nr*. Source: Author (2025).

The WT and *Nr* genotypes did not show significant interference in pigment values with the imposition of Mn absence treatment (**Figure 8**). The levels of chlorophyll *a*, *b*, and carotenoid, as well as the chlorophyll *a*/*b* ratio, total chlorophyll, and chlorophyll/carotenoid ratio were evaluated (**Figure 8**).

Figure 8. Chlorophyll a (**A**), chlorophyll b (**B**), carotenoid (**C**), chlorophyll a/b ratio (**D**), total chlorophyll (**E**) and chlorophyll/carotenoid ratio (**F**) of tomato plants cv. Micro-

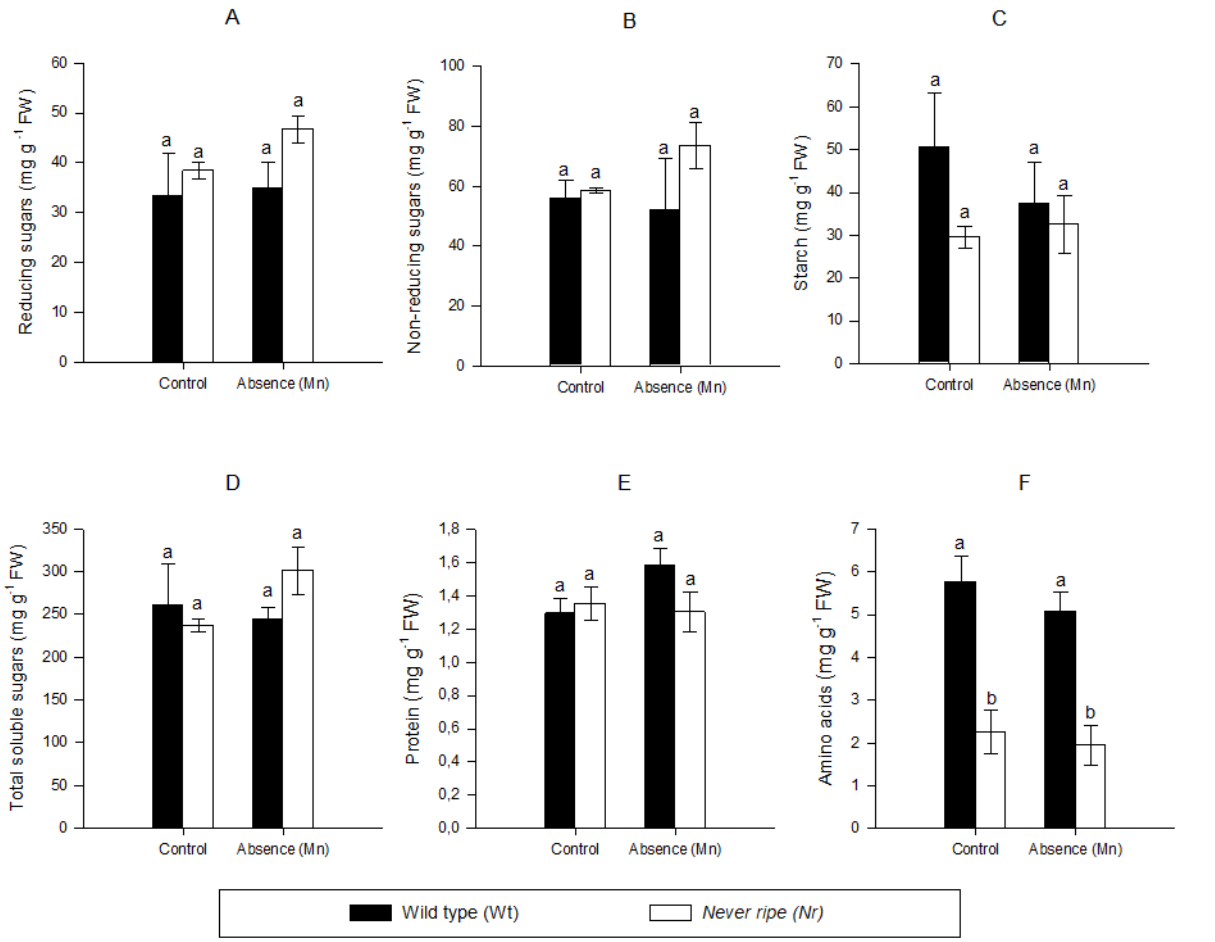
Tom from wild type (WT) and *Never ripe* (*Nr*) genotypes at different Mn levels (control and absence).



Means of variables followed by the same letter do not differ statistically by the Scott-Knott test at $p \leq 0.05$, n = 6 ± SE. The black bar represents WT and white bar represents *Nr*. Source: Author (2025).

Regarding the carbohydrate metabolism, our results reveal that Mn absence did not interfere with the levels of reducing sugars, non-reducing sugars, starch, and total soluble sugars (**Figures 9A** to **9D**). However, when analyzing the metabolites of N-related metabolism, we observed that both genotypes did not show any difference in protein levels (**Figure 9E**); however, plants of the *Nr* genotype, under Mn absence and control, showed lower amino acid levels, being approximately 61.6% and 60.9% lower than the WT genotype (**Figure 9F**).

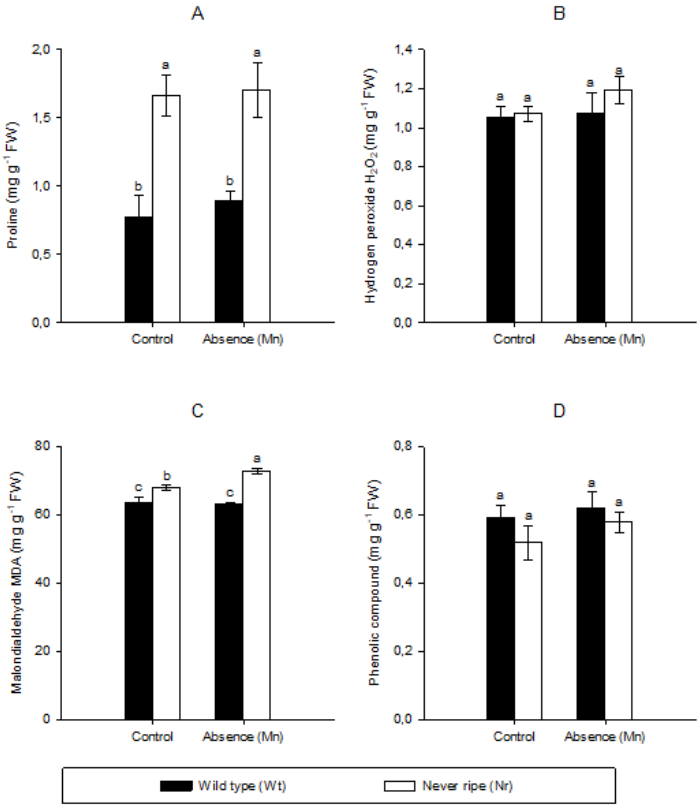
Figure 9. Reducing sugars (**A**), non-reducing sugars (**B**), starch (**C**), total soluble sugars (**D**), protein (**E**) and amino acids (**F**) of tomato plants cv. Micro-Tom from wild type (WT) and *Never ripe* (*Nr*) genotypes at different Mn levels (control and absence).



Means of variables followed by the same letter do not differ statistically by the Scott-Knott test at $p \leq 0.05$, n = 6 ± SE. The black bar represents WT and white bar represents *Nr*. Source: Author (2025).

Regarding stress-related metabolites, both genotypes did not show significant alterations in the levels of H_2O_2 and phenolic compounds (**Figures 10B** and **10D**). However, regarding the content of proline and MDA, an interesting result was observed, wherein the *Nr* mutant showed elevated values even under control. The proline levels in *Nr* plants under Mn absence was 91% higher than the WT and 116% higher under control (**Figure 10A**). The MDA levels in xi plants under Mn absence was 15.4% higher than the WT, and 7.5% higher under control (**Figure 10C**).

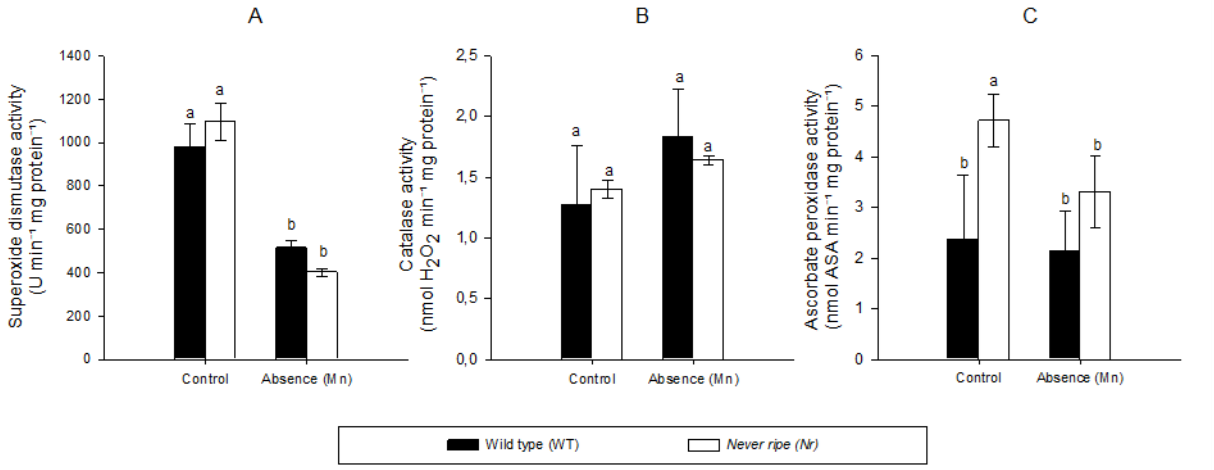
Figure 10. Proline (**A**), hydrogen peroxide (**B**), molondialdehyde (**C**) and phenolic compound (**D**) of tomato plants cv. Micro-Tom from wild type (WT) and *Never ripe* (Nr) genotypes at different Mn levels (control and absence).



Means of variables followed by the same letter do not differ statistically by the Scott-Knott test at $p \leq 0.05$, n = 6 ± SE. The black bar represents WT and white bar represents Nr. Source: Author (2025).

Regarding enzymatic activity of the antioxidant system, SOD activity in both genotypes was strongly affected by the Mn absence, with reductions of 63.3% in *Nr* and 47.6% in WT (**Figure 11A**). The opposite was observed for CAT activity, where the Mn absence did not interfere with the activity (**Figure 11B**). However, *Nr* plants, under control, had higher APX activity values, being 98.7% higher than the WT and when under Mn absence, the WT and *Nr* genotypes did not differ from each other (**Figure 11C**).

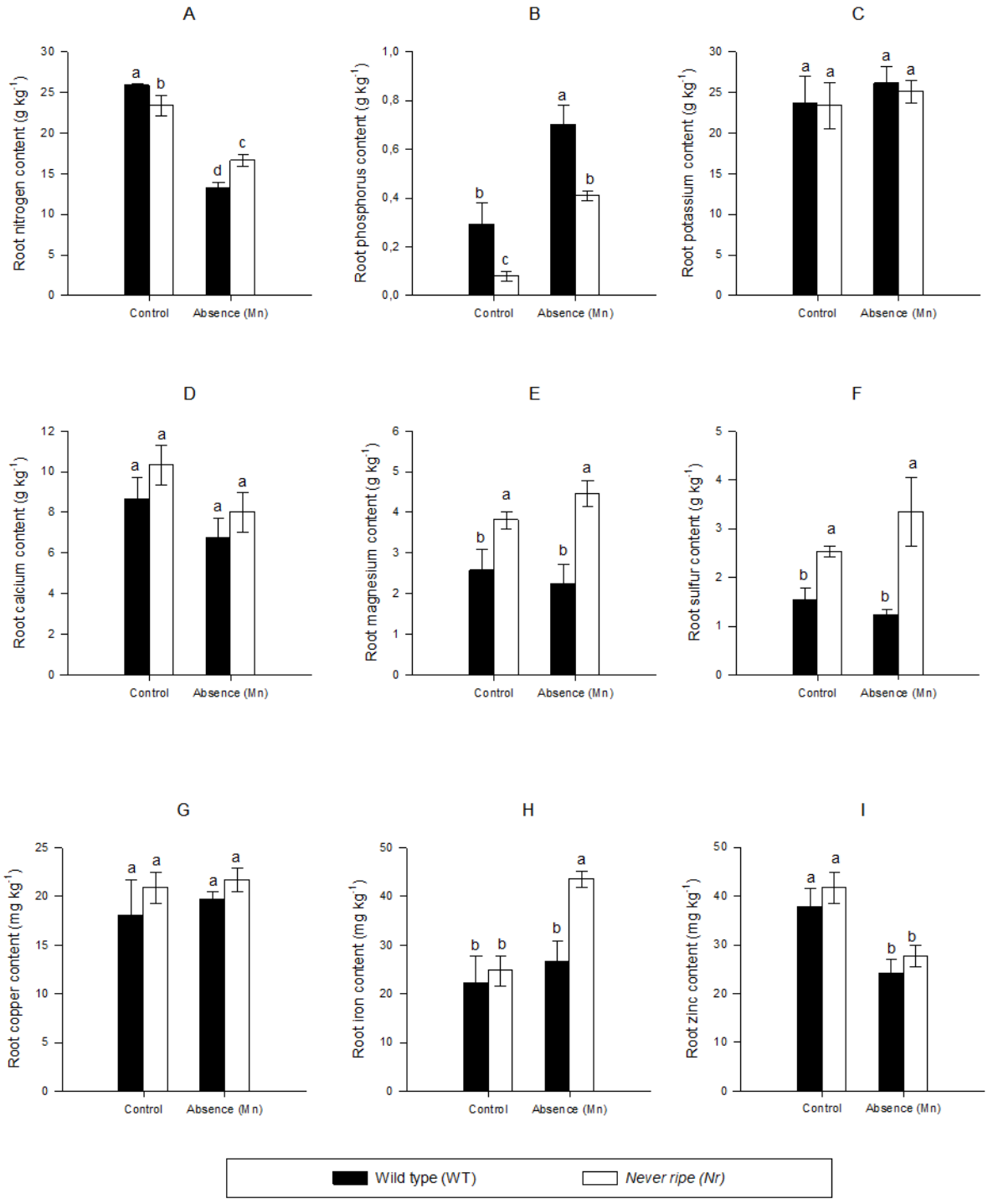
Figure 11. Superoxide dismutase activity (**A**), catalase activity (**B**) and ascorbate peroxidase activity (**C**) of tomato plants cv. Micro-Tom from wild type (WT) and *Never ripe* (Nr) genotypes at different Mn levels (control and absence).



Means of variables followed by the same letter do not differ statistically by the Scott-Knott test at $p \leq 0.05$, n = 6 ± SE. The black bar represents WT and white bar represents Nr. Source: Author (2025).

Regarding the nutrient content in the root, the Mn absence treatment did not interfere with the content of K, Ca, and Cu in either genotype (**Figures 12C**, **12D** and **12G**). The N content in *Nr* genotype plants, when under Mn absence, was 25.9% higher than WT plants, and under control the N content was 9.6% lower (**Figure 12A**). The P content in *Nr* genotype plants, under Mn absence, was 52.3% lower than WT plants and 68.9% lower under control (**Figure 12B**). The Mg content in *Nr* genotype plants under Mn absence was 99.1% higher than WT plants and 48.2% higher under control (**Figure 12E**). The S content in *Nr* genotype plants, under Mn absence, was 171% higher than WT plants and 66.4% higher under control (**Figure 12F**). The Fe content in *Nr* genotype plants, under Mn absence, was 64.1% higher than WT plants but under control, the genotypes did not differ from each other (**Figure 12H**). The Zn content in both genotypes was higher under control (**Figure 12I**).

Figure 12. Root nitrogen (**A**), phosphorus (**B**), potassium (**C**), calcium (**D**), magnesium (**E**), sulfur (**F**), copper (**G**), magnesium (**H**) and zinc (**I**) content of tomato plants cv. Micro-Tom from wild type (WT) and *Never ripe* (Nr) genotypes at different Mn levels (control and absence).

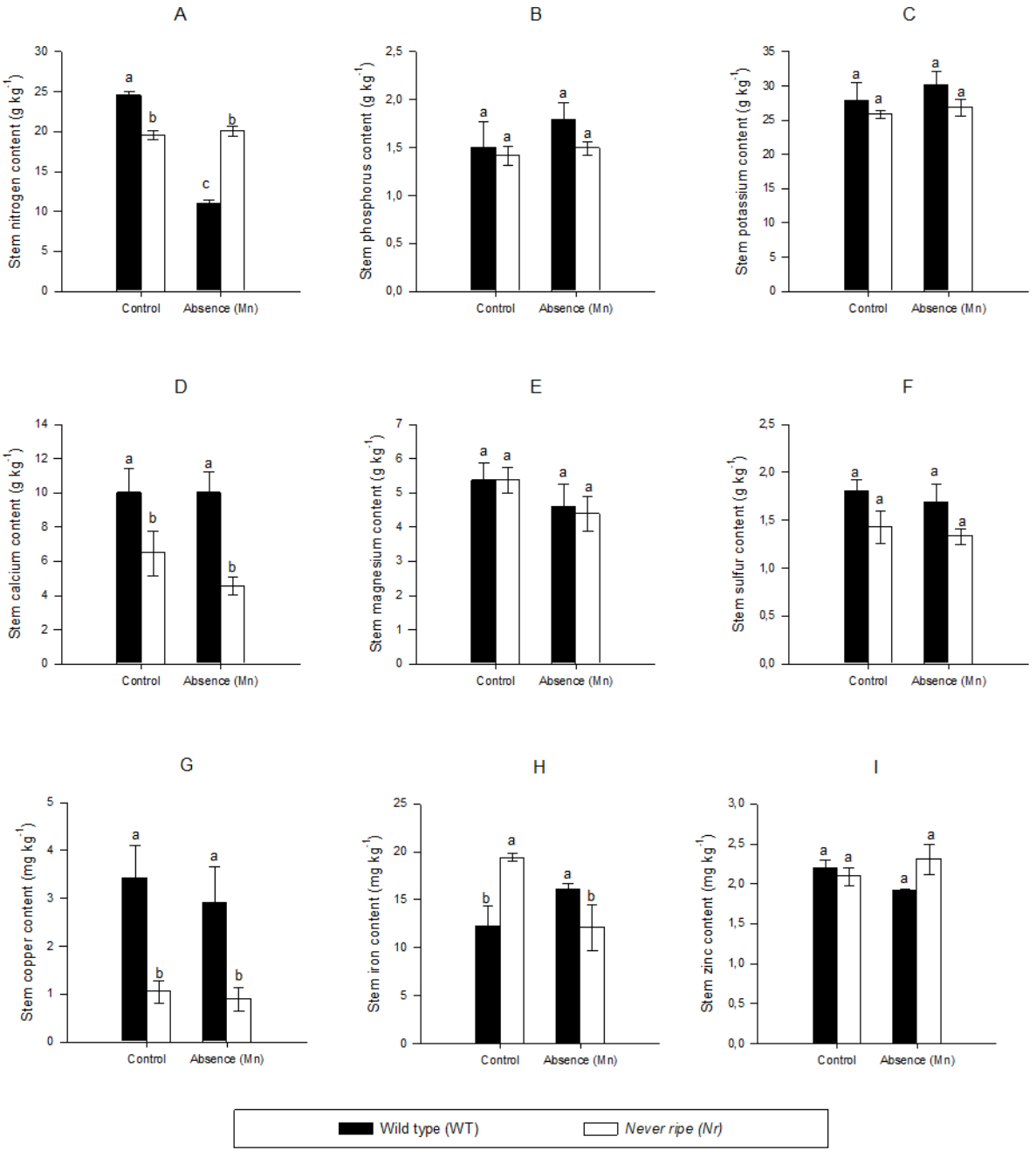


Means of variables followed by the same letter do not differ statistically by the Scott-Knott test at $p \leq 0.05$, n = 6 ± SE. The black bar represents WT and white bar represents Nr. Source: Author (2025).

Regarding the nutrient content in the stem, the Mn absence treatment did not interfere with the content of P, K, Mg, S, and Zn in either genotype (**Figures 13B**, **13C**, **13E**, **13F** and **13I**). The N content in *Nr* genotype plants, when under Mn absence, was 81.7% higher than WT plants and under control the N content was 20.2% lower than WT plants (**Figure 13A**). The Ca content in *Nr* genotype, under Mn absence, was 54.3% lower than WT plants and

35.2% lower under control (**Figure 13D**). The Cu content in *Nr* genotype, under Mn absence, was 69% lower than WT plants and 69.3% lower under control (**Figure 13G**). The Fe content in *Nr* genotype, when under Mn absence, was 24.4% lower than WT plants and under control the Fe content was 58.9% higher than WT plants (**Figure 13H**).

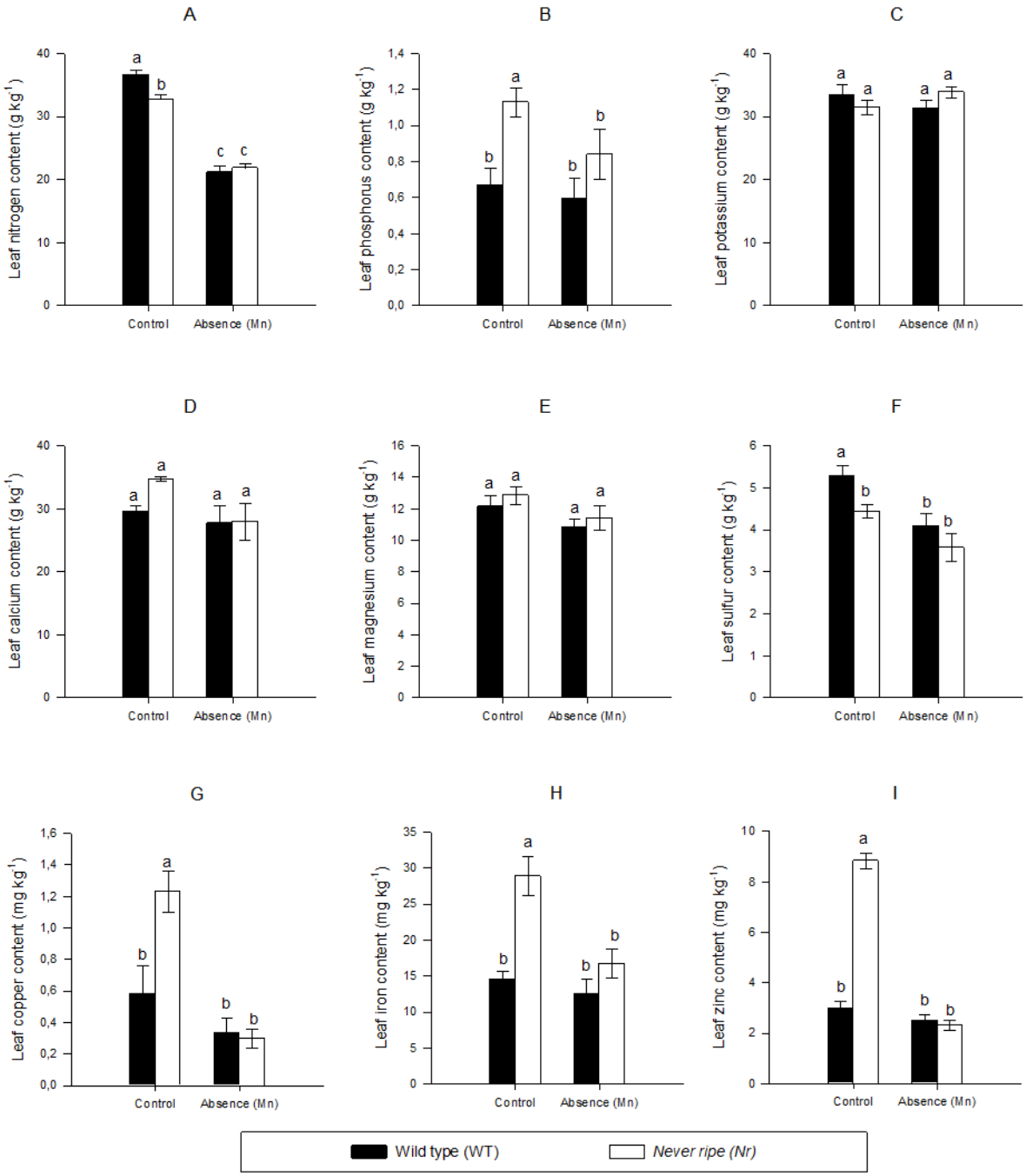
Figure 13. Stem nitrogen (**A**), phosphorus (**B**), potassium (**C**), calcium (**D**), magnesium (**E**), sulfur (**F**), copper (**G**), magnesium (**H**) and zinc (**I**) content of tomato plants cv. Micro-Tom from wild type (WT) and *Never ripe* (Nr) genotypes at different Mn levels (control and absence).



Means of variables followed by the same letter do not differ statistically by the Scott-Knott test at $p \leq 0.05$, n = 6 ± SE. The black bar represents WT and white bar represents Nr. Source: Author (2025).

Regarding the nutrient content in the leaves, the Mn absence treatment did not interfere with the content of K, Ca, and Mg in either genotype (**Figure 14C** to **14E**). The N content under control was 10.7% lower in *Nr* than WT plants and under Mn absence, the genotypes did not differ from each other (**Figure 14A**). The P content in *Nr* genotype under control was 42.4% higher than WT plants., but under Mn absence the genotypes did not differ from each other (**Figure 14B**). The S content in *Nr* genotype under control was 16.1% lower than WT plants, but under Mn absence the genotypes did not differ from each other (**Figure 14F**). The Cu content in *Nr* genotype under control was 107% higher than WT plants, but under Mn absence the genotypes did not differ (**Figure 14G**). The Fe content in *Nr* genotype under control was 98.9% higher than WT plants, but under Mn absence, the genotypes did not differ from each other (**Figure 14H**). The Zn content in *Nr* genotype under control was 195% higher than WT plantsbut under Mn absence the genotypes did not differ from each other (**Figure 15I**).

Figure 14. Leaf nitrogen (**A**), phosphorus (**B**), potassium (**C**), calcium (**D**), magnesium (**E**), sulfur (**F**), copper (**G**), magnesium (**H**) and zinc (**I**) content of tomato plants cv. Micro-Tom from wild type (WT) and *Never ripe* (Nr) genotypes at different Mn levels (control and absence).

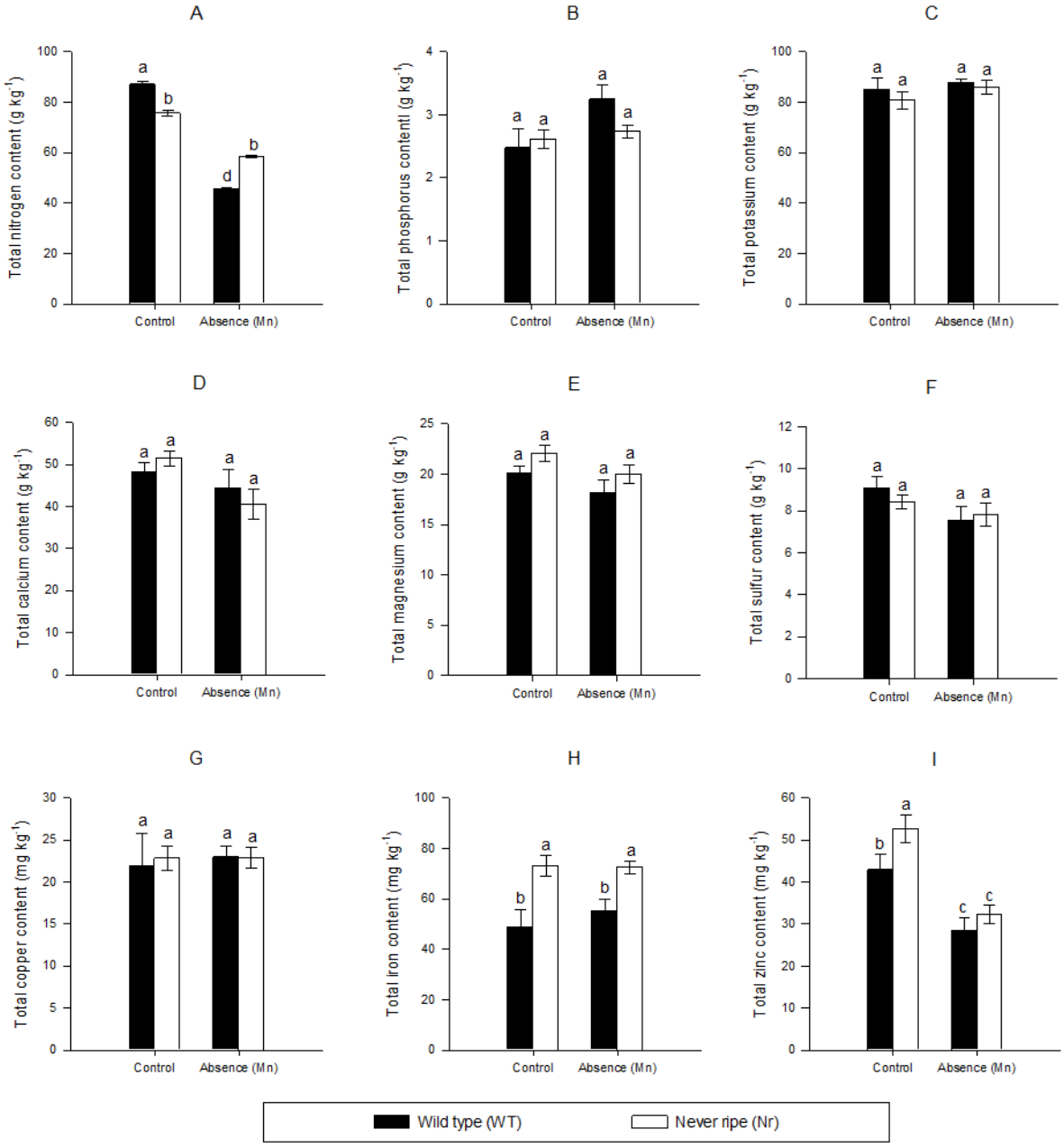


Means of variables followed by the same letter do not differ statistically by the Scott-Knott test at $p \leq 0.05$, $n = 6 \pm$ SE. The black bar represents WT and white bar represents Nr. Source: Author (2025).

The Mn absence did not interfere with the total concentration of P, K, Ca, Mg, S, and Cu in either genotype (**Figure 15B** to **15G**). The total N content in *Nr* genotype, when under Mn absence, was 28.8% higher than WT plants and under control the total N content was 13% lower than WT plants (**Figure 15A**). The total Fe content in *Nr* genotype under Mn absence was 31.5% higher than WT plants and 49.6% higher under control (**Figure 15H**). The total Zn

content in *Nr* genotype under control was 23% higher than WT plants, but under Mn absence, the genotypes did not differ from each other (**Figure 15-I**).

Figure 15. Total nitrogen (**A**), phosphorus (**B**), potassium (**C**), calcium (**D**), magnesium (**E**), sulfur (**F**), copper (**G**), magnesium (**H**) and zinc (**I**) content of tomato plants cv. Micro-Tom from wild type (WT) and *Never ripe* (Nr) genotypes at different Mn levels (control and absence).



Means of variables followed by the same letter do not differ statistically by the Scott-Knott test at $p \leq 0.05$, n = 6 ± SE. The black bar represents WT and white bar represents Nr. Source: Author (2025).

7. DISCUSSION

Our results show that WT plants presented more stress-related responses than *Nr* in the condition of Mn absence with several changes in its physiology. Basically, *Nr* plans performed better than WT in the Mn absence. In total, 94 parameters of WT and *Nr* plants subjected to Mn absence were analyzed, wherein 45 of the analyzed parameters, the ethylene insensitivity in association with the Mn absence did not interfere with different responses in the tomato plants. In 14 parameters, the responses were constitutive of the genotypes, meaning these are characteristics that are always present and active in the *Nr* genotype, regardless of a specific external stimulus, for instance total dry mass (**Figures 4D** and **4F**), amino acid content (**Figure 9F**) and proline content (**Figure 10D**). Other parameters were influenced by the Mn absence treatment, where plants under control showed higher values, for instance total Mn content (**Figure 2D**), fruit set (**Figure 5H**), SOD activity (**Figure 11A**), and Zn content in the root (**Figure 12I**). In another 31 parameters, the genotypes showed a different response pattern to the Mn absence treatment supporting our hypothesis that the ethylene insensitivity in *Nr* tomato plants enhances Mn absence tolerance, improving growth, photosynthesis, nutrient use, and fruit yield compared to WT.

7.1 Manganese absence: ethylene insensitivity enhances stress tolerance and alters physiological and morphological responses in tomato

7.1.1 Root system modifications

Ethylene is one of the main hormone related to the stress response; under nutritional stress conditions, plants develop morphological and physiological responses, seeking ways to tolerate the stress and avoid major damage to metabolism (Baharudin; Osman, 2023). In general, WT plants showed some changes in physiology due to Mn absence, for instance these alterations were observed in increases of root length (Figure 3B), root-to-shoot ratio (Figure 4E), and non-photochemical quenching (Figure 6C). These parameters are mainly related to stress responses (Mehra et al., 2025), and indicate that WT plants were more affected by the Mn absence than Nr plants, which did not show changes in these parameters. The primary root is the basic component of root systems; under nutritional absence, one of the main strategies is increased root growth to enhance the water and nutrient absorption (Mehra et al., 2025). Thus, once that this response pattern was not observed in the ethylene-insensitivity Nr plants, we can infer that the increase in root length in WT plants under Mn absence is a stress tolerance response mediated by ethylene, to increase the area for Mn acquisition in the soil. On the other hand, Nr plants, under Mn absence and control, showed greater root length than WT plants (Figure 3B), and this response is not direct related to the imposition of the Mn

absence treatment but may be related to ethylene-insensitivity per se. The use of ethylene led to an the inhibition of primary root growth, by inhibition in cell proliferation and elongation in the root meristem zone in Arabidopsis and rice (Qin et al., 2019). The inhibition of ethylene in primary root elongation is mediated by interactions with phytohormones, such as auxin, abscisic acid, gibberellin, cytokinin, jasmonic acid, and brassinosteroids and it is also known that low ethylene sensitivity affects auxin transport, which becomes less sensitive to ethylene, contributing to the increase in adventitious root growth (Negi et al., 2010). Thus, we can attribute that the greater root length in Nr plants is related to low ethylene sensitivity.

7.1.2 Root-to-shoot ratio as a stress indicator

Under Mn absence the WT plants showed higher root-to-shoot ratio values compared to WT plants under control (Figure 4E), and a reduction in the root-to-shoot ratio indicates that the plant is likely growing under more favorable conditions; conversely, an increase in the root-to-shoot ratio is almost always in response to less favorable conditions (Harris, 1992; Zhao et al., 2021). In our study, we observed that the WT plants showed an increase in the root-to-shoot ratio under Mn absence (**Figure 4E**), an expected result under these conditions, where plants seek ways to tolerate the stress (Zhao et al., 2021). The opposite was observed in plants of the *Nr* plants, which showed a reduction in the root-to-shoot ratio, suggesting that ethylene-insensitivity leads these plants to maintain their metabolism functioning normally even under adverse conditions (in our case, the Mn absence).

7.1.3 Impact on photosystem functioning

The interaction between Mn absence and ethylene-insensitivity led to alterations in the photosystem functioning, where WT and *Nr* plants showed different responses pattern (**Figure 6C**). The Mn absence led WT plants to optimize the photochemical quenching (qP), the proportion of absorbed light energy used in photochemical reactions of photosynthesis (Murakami et al., 2024); however, the Mn absence also caused a significant loss of energy in the form of heat (NPQ) (**Figures 6B** and **6C**). The opposite occurred in plants of the *Nr* plants, which, compared to WT plants, showed a reduction in NPQ under Mn absence and an increase under control. NPQ represents the set of mechanisms that plants have developed for dissipating excess absorbed energy, that is, the portion dissipated as heat (Baker, 2004; Flexas et al., 2002; Murakami et al., 2024). WT plants, under Mn absence, may have dissipated the increased excitation energy as heat (NPQ) to protect the photosynthetic apparatus from damage, something that did not occur in *Nr* plants, as they maintained the same behavior under both conditions. This behavior reinforces the idea that the ethylene-insensitivity does

not lead to changes in stress response, but instead they already have constitutive changes that allow greater tolerance to stress.

7.2 Metabolic changes and nutrient utilization in *Nr* under manganese absence

The ethylene-insensitive *Nr* mutant also showed changes in metabolism due to Mn absence, with main alterations observed in the Mn accumulation and utilization efficiency (**Figures 2G** and **2H**), number of flower buds (**Figure 5A**), fruit diameter (**Figure 5D**), fruit fresh and (**Figures 5E**), g_S (**Figure 7B**), *E* (**Figure 7C**), P and Fe content in the root (**Figures 12B** and **12H**) and MDA (**Figure 10C**). However, a different pattern emerged, as these parameters, except for MDA, are not typical stress-related responses, indicating that *Nr* plants were less affected by the Mn absence than WT plants. The Nr plants under Mn absence showed higher values of Mn accumulation and utilization efficiency, wherein the higher efficiency of Mn use in Nr plants under Mn absence may be related to the higher values of the number of flower buds, fruit diameter, and fruit fresh and dry mass. This occurs because, in the chloroplast, Mn acts in the water photolysis as part of the cluster with Mn at oxygen-evolution center (OEC) (Rana et al., 2023), after the breakdown of the water molecule, the released electrons are transferred to photosystem II. This process leads to greater efficiency of photosynthesis, which generates substrate for respiration (Malavolta, 2006; Rana et al., 2023). Nr plants showed higher values of shoot dry biomass even under Mn absence, a constitutive response because of the ethylene-insensitivity mutation. However, we can still attribute the higher values of flower buds, fruit diameter, and fruit fresh and dry mass even under Mn absence to the higher Mn utilization efficiency.

7.3 Manganese absence: ethylene insensitivity optimizes gas exchange, water use, and fruit development

Nr plants, under Mn absence, also showed higher values of gS and E, parameters thar are entirely related, once that plants open their stomata to absorb CO2 and, during this process, H2O is released through transpiration. Stomatal regulation plays a fundamental role in maintaining the balance between water loss and carbon gain. This occurs because under adverse conditions, plants tend to close their stomata (Davidson et al., 2023). However, WT plants did not show differences in gS and E under Mn absence (Figures 7B and 7C), indicating that this treatment was not sufficient to interfere with these parameters. The opposite observed in Nr plants may be related to ethylene-insensitivity; however, the higher values under Mn absence show a different behavior from the pattern that tends to lower gS values under Mn absence conditions (Figure 7B).

Under control, Nr plants showed a reduction in E and increase in the intrinsic water use efficiency (Figure 7C). Better water use efficiency in plants can be related to genetic, physiological, and agronomic strategies, which may include the manipulation of gS and Increased root growth (Leakey et al., 2019); furthermore, Mn absence can lead to lower water use efficiency due to increased E (Hebbern et al., 2009). Thus, we can conclude that the higher water use efficiency value in Nr plants is related to higher Mn efficiency use and higher root length. Other parameters, such as Mn content (Figure 2), fruit set (Figure 5H), SOD activity (Figure 11A), and Zn content in the root (Figure 12I), were strongly affected by the Mn absence. The fruit set index refers to the proportion between fruits and flowers (Stanton et al., 1987; An et al., 2020). Fruit set is mainly regulated by hormones such as auxin, gibberellins, and ethylene, which activate signaling pathways to promote ovary development into fruit (An et al., 2020). Gibberellin mainly acts on cell expansion and both auxin and ethylene stimulate the development of female reproductive organs, while the development of male reproductive organs is stimulated by auxin, and ethylene pulls it down (An et al., 2020). This hormonal relationship acting on the fruit set index explains a higher number of fruits in Nr plants because the negative regulation of male organ formation, which is mediated by ethylene, does not occur. Our results indicate that the Mn absence can strongly affect the fruit set index, reducing it . Despite WT plants showing a lower number of fruits under control these plants maintained the same fruit set values as Nr plants (Figure 5H), suggesting that the action of ethylene promoting ovary development into fruit was essential to maintain the fruit set index equal to that of Nr plants. Furthermore, these plants showed an increase in amino acid and total N content (Figures 9F and 12A). The N supply influenced the fruit set index in peppers; this was mainly related to an increase in amino acid content, which was also observed in WT plants under control (Silva et al., 2019). Additionally, the higher fruit set value in both genotypes under control may be related to the higher content of N, Mn, and Zn in these plants, since the higher concentration of these nutrients improves fruit yield and quality (Silva et al., 2019; Hasani et al., 2012).

7.4 Manganese absence and ethylene insensitivity: impacts on nutrient homeostasis and photosynthetic efficiency

Regarding the nutritional homeostasis we highlight the variation of P levels in plants because when under Mn absence the WT and Nr genotypes showed different behaviors regarding P content in the roots and leaves (Figures 12A and 14B). The Mn absence favored P content in the root in both genotypes; however, WT plants did not differ in P content in the leaves, the opposite observed in Nr plants under control suggests that the presence of Mn

favored the transport of P to the leaves, while its deficiency favors P content retained in the roots (Figures 12B and 14B). P is a macronutrient involved in plant growth and development. It can be separated into five distinct fractions: inorganic-P, lipid-P, metabolite-P (sugar phosphates and ATP), nucleic acid-P (RNA and DNA), and residual-P (phosphoproteins) (Kedrowski, 1983; Hidaka and Kitayama, 2011; Veneklaas et al., 2012; Jeong et al., 2017; Yan et al., 2019). The higher values of Mn accumulation and utilization efficiency in Nr plants under Mn absence (Figure 2G and 2H) may have interfered with the P content values in the root (Figure 12B), since the accumulation of Mn in the leaves leads to greater efficiency in P acquisition (Lambers et al., 2015). Our results corroborate those described in the literature, as Nr plants under Mn absence showed greater Mn accumulation in the leaves and higher P values in the root. The higher P content in the root of WT plants may be related to the higher Mn content in the stem that these plants presented.

The higher P content in the leaves of Nr plants under control indicates a relationship between Mn and improved P transport. The relationship between Mn and P has been described previously, where high concentrations of Mn in the soil solution increased P concentrations in shoot tissue (Kuo; Mikkelsen, 1981; Yan et al., 2024). Our results show that WT plants did not differ in P content in leaves values with the Mn absence (Figure 12B), and the opposite observed in Nr plants shows that ideal Mn concentrations are already sufficient to increase of P content in the leaves of Nr plants (Figure 14B). Furthermore, it is known that plants utilize different strategies for P mobilization, one of these strategies being the release of carboxylates, during this release process, carboxylates mobilize Mn and P from the soil (Lambers et al., 2015). Thus, we can infer that the presence of Mn is linked with the transport of P to leaves of Nr plants. However, the P content in the leaves of WT plants did not differ with the Mn absence, thus, we can infer that the higher P content in the leaves of Nr plants may be an indication of better efficiency in P transport in Nr plants when under control.

Another important nutrient is Fe, and Nr plants under Mn absence showed higher Fe values in the root compared to plants under control (Figure 12H). Fe is an important micronutrient for plants; in metabolism, Fe participates in essential processes such as photosynthesis, respiration, and N assimilation (Angulo et al., 2021). The relationship between Fe and Mn has been previously reported, where high Mn concentrations decrease Fe absorption and content in plant due to competitive interactions between the two metals, that can utilize the same transporter, which affects their uptake and translocation in plants (Kuo; Mikkelsen, 1981; Long et al., 2017). The Mn absence may have favored the higher Fe concentrations in these plants. However, under control, Nr plants also showed higher Fe

values in the stem and Fe and Mn in the leaves. Our results indicate that despite the Mn absence having favored a higher Fe content in the root, the presence of Mn did not interfere with Fe absorption under control.

Studies show a relationship between N and Zn, where an increase in N concentration improves Zn absorption by the root and Zn transport from root to shoot (Nie et al., 2017). Higher Zn concentrations can also lead to increased concentrations of Mn and Fe, improving the photochemistry of PSII (Moustakas et al., 2022). Nr plants showed better efficiency in the photosystem than WT plants (Figure 7A); this increase is likely related to the greater efficiency of Nr plants in optimizing PSII based on higher concentrations of Fe and Mn. However, WT plants, even with higher concentrations of these nutrients, did not show an increase in PSII efficiency, suggesting an influence of ethylene on PSII regulation.

7.5 Stress-related responses: MDA and APX activity

Regarding the stress-related responses, WT plants did not differ in malondialdehyde (MDA) values, however, here, the Mn absence led to an increase in MDA in Nr plants (Figure 10C). MDA is a common product of lipid peroxidation and is used to identify lesions caused by oxidative stress (Yasar et al., 2022). WT and Nr plants subjected to cadmium and sodium stress showed no differences in MDA values between WT and Nr (Monteiro et al., 2011). A reduction in MDA content would be expected in the Nr plants, since previous studies have shown that membrane peroxidation is induced by ethylene (Flors et al., 2007; Hodges, 2000; Sylvestre; Paulin, 1987; Riyazuddin et al., 2020). However, the higher MDA results in Nr plants under Mn absence indicate a limited role of ethylene signaling in lipid peroxidation (Monteiro et al., 2011). In another stress-related parameters, Nr plants showed higher values under control, and WT plants did not differ, for instance, the Nr plants under control showed increased APX activity (Figure 11C). In many cases, APX activity is the first enzyme to change under stress conditions because it is one of the first steps in oxidative stress metabolism, transforming hydrogen peroxide (H2O2) into water and oxygen (O2) (Silva et al., 2018); however, no influence of ethylene-insensitivity and Mn absence was observed here on H2O2 values and we can infer that the higher APX activity was not a direct response to increased H2O2 but rather due to other factors. Unfortunately, based on our data, it is still difficult to trace any response connecting the ethylene-insensitivity and antioxidant enzymatic activity.

7.6 Photosynthetic electron transport rate (ETR):

The Nr genotype, under control, showed alterations in ETR, with higher values(Figure 6D), that is a measure referring to the electron flow that occurs in the thylakoid membrane

and is an important indicator of the efficiency of the light-dependent reactions of photosynthesis (Rana et al., 2023). The first step of photosynthesis begins with a water photolysis reaction; Mn ions are essential for the function of the tetramanganese-calcium cluster (Mn4CaO5), responsible for water oxidation, the source of electrons for photosynthesis (Rana et al., 2023). When plants are exposed to Mn absence, the quantity of PSII supercomplexes is drastically reduced (Schmidt et al., 2015). The Mn absence in WT plants did not interfere with ETR, showing that this treatment was not sufficient to cause interference in this photochemical parameter (Figure 6D). Furthermore, we can infer that the interaction with ethylene in WT plants was essential to maintain electron transport even under Mn absence. Moreover, the increase in ETR in Nr plants under control is an indication that these plants obtained better performance from the Mn incorporated into the tetramanganese-calcium cluster, releasing more electrons and the Mn absence is one factor that can negatively interfere with this important step of photosynthesis. Despite Nr plants under Mn absence showing better performance in Mn use then WT, the increase in ETR values in Nr plants under control shows that these plants exhibited greater Mn efficiency use under control for ETR maintenance.

7.7 Superoxide dismutase (SOD) activity:

In both genotypes, SOD activity was strongly reduced with the Mn absence (**Figure 11A**). SOD is an important enzyme of the antioxidant system that acts by breaking down ROS into other less reactive compounds, such as H_2O_2 (Silva et al., 2018). In plants, there are three isoforms of this enzyme, classified by their metal ion at the active site: Cu/Zn, Mn, and Fe isoforms. The Mn-SOD form is in the mitochondria and peroxisomes, where it acts in these superoxide (O_2^-) catalysis processes (Bowler et al., 1994; Corpas et al., 2017; Kidwai et al., 2020; Jiang et al., 2019). Our results reveal that the Mn absence strongly influenced the activity of the enzyme in the Mn-SOD form, which led to lower activity in both genotypes.

9. CONCLUSION

While manganese absence causes few biometric and production changes in tomato, our results confirm that ethylene insensitivity in the *Never ripe* (*Nr*) mutant significantly enhances tolerance to this absence. This manifests as improved growth, greater photosynthetic and nutrient utilization efficiency, and superior fruit productivity compared to wild-type (WT) plants under the same conditions.

The low ethylene perception in Nr plants allows them to maintain their metabolism fully functional even under Mn absence, resulting in higher Mn utilization efficiency and an absence of typical stress responses. Conversely, WT plants exhibit more pronounced stress tolerance responses, such as increased root length, a higher root-to-shoot ratio, and elevated NPQ, indicating ethylene's role in their ability to tolerate Mn absence.

From a nutritional standpoint, Mn absence disrupted nutrient homeostasis in both genotypes. In the long term, this could lead to significant metabolic imbalances, although not observed in tomato plants due to their short life cycle. Furthermore, we infer that the better performance of Nr plants in circumventing Mn absence affected the content of other leaf micronutrients (Cu, Fe, Zn).

Our findings represent an advance in studies of ethylene's interaction with plant nutrition. Despite consistent results from previous repetitions of this experiment, we highlight the need for further experimental repetitions to reaffirm and deepen our understanding of ethylene's role in Mn stress and its interaction with other plant nutrients.

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