Université Catholique de Louvain
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Soil Salinity Effects on the Morphology, Physiology and Nutritional Quality of Seed and Leaf Cultivars of Red Amaranth (*Amaranthus cruentus*)

Paul-Henry Minguet

Thesis submitted in partial fulfillment of the requirements for the Degree of
Master of Science in Biology (Organisms and Ecology)
in the Science Faculty at the University of Louvain

Supervised by:
Dr. Muriel Quinet
Professor, UCLouvain
Adrien Luyckx
Ph.D. Candidate, UCLouvain

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Supervisor:
Dr. Muriel Quinet
Co-supervisor:
Adrien Luyckx

Jury:
Dr. Guillaume Lobet
Assistant Professor, UCLouvain
Researcher, Forschungszentrum Jülich
Dr. Stanley Lutts
Professor, UCLouvain
Dr. Renate Wesselingh
Professor, UCLouvain

Abstract

The challenges arising from soil salinity and the imperative of ensuring global food security are significantly influenced by global change. A viable approach to mitigate these challenges involves the cultivation of salt-resistant plants. Amaranths, distinguished by properties akin to cereals despite taxonomic distinctions, represent C_4 plants well-adapted to thrive under adverse environmental conditions. This study focused on four cultivars belonging to the *Amaranthus cruentus* species, comprising two recognized for leaf production and two for seed production. These cultivars, namely Locale and Rouge, and, Don Leon and Montana-5, underwent a two-month exposure to salt stress through watering a 75 mM NaCl solution, in a semi-controlled greenhouse environment.

An extensive analysis encompassed the morphological and physiological at the whole-plant level, as well as nutritional aspects of seeds, across the cultivars. Despite the inclusion of both salt-resistant and salt-sensitive profiles, all four cultivars exhibited analogous responses to salt exposure, occasionally deviating from anticipated outcomes based on prior studies. Notably, various parameters such as plant height, leaf production, biomass, water potential, and photosynthetic efficiency were negatively impacted under salt-induced stress. This observation held true across all cultivars studied, potentially highlighting resilience in the face of challenging conditions. The findings of this study contribute valuable insights into the complex interplay between salt stress and the physiological responses of diverse *Amaranthus cruentus* cultivars, thereby enhancing our understanding of sustainable agriculture practices under changing environmental dynamics.

Les défis liés à la salinité des sols et l'impératif d'assurer la sécurité alimentaire mondiale sont fortement influencés par les changements climatiques. Une approche viable pour atténuer ces défis implique la culture de plantes résistantes au sel. Les amarantes, se distinguant par des propriétés similaires aux céréales malgré des distinctions taxonomiques, et représentant des plantes fixant leur carbone en C_4, sont bien adaptées pour prospérer dans des conditions environnementales difficiles. Cette étude s'est concentrée sur quatre cultivars appartenant à l'espèce *Amaranthus cruentus*, comprenant deux reconnus pour la production de feuilles et deux pour la production de graines. Ces cultivars, à savoir Locale et Rouge, et, Don Leon et Montana-5, ont été soumis pendant deux mois à un stress salin via l'arrosage d'une solution 75 mM de NaCl, dans un environnement de serre semi-contrôlé.

Une analyse approfondie englobant les aspects morphologiques et physiologiques à l'échelle de la plante entière, ainsi que les aspects nutritionnels des graines, à travers les cultivars a été réalisée. Malgré l'inclusion de profils résistants et sensibles au sel, les quatre cultivars ont présenté des réponses analogues à l'exposition au sel, déviant parfois des résultats anticipés basés sur des études antérieures. Notamment, divers paramètres tels que la hauteur de la plante, la production de feuilles, la biomasse, le potentiel hydrique et l'efficacité photosynthétique ont été impactés négativement sous stress salin. Cette observation s'est vérifiée pour tous les cultivars étudiés, soulignant une potentielle résilience face à des conditions difficiles. Les conclusions de cette étude apportent des éléments précieux sur l'interaction complexe entre le stress salin et les réponses physiologiques de divers cultivars d'*Amaranthus cruentus*, contribuant ainsi à notre compréhension des pratiques agricoles durables dans un contexte de dynamiques environnementales.

Keywords: *Amaranthus cruentus*, soil salinity, vegetative phase, food security, nutritional quality

Table of Contents

Abstract **5**
State of the Art **9**
1 • Soil Salinity and Food Security 9
1 • 1 • Soil Salinity and Its Causes 9
1 • 2 • Food Security 10
2 • Soil Salinity and Its Impacts on Plants 10
2 • 1 • Salt Tolerance 10
2 • 2 • Osmotic Stress 11
2 • 3 • Ionic Stress 12
2 • 5 • Plant Salinity Stress Response 13
2 • 6 • Salinity's Impact on Plant Yield 14
3 • Amaranths 14
3 • 1 • Amaranthus cruentus 16
Study Objectives **19**
Materials and Methods **21**
1 • Plant Material 21
2 • Impact of Salinity on the Vegetative Phase 21
2 • 1 • Growing Conditions 21
2 • 2 • Plant Growth and Development 22
2 • 2 • 1 • Plant Height and Leaf Count 22
2 • 2 • 2 • Fresh and Dry Mass 22
2 • 3 • Physiology 23
2 • 3 • 1 • Chlorophyll Content 23
2 • 3 • 2 • Water Potential and Gas Exchanges Measurements 23
2 • 3 • 3 • Mineral Content 24
3 • Impact of Salinity on Seed Nutritional Quality 24
3 • 1 • Plant Material 24
3 • 2 • Minerals 24
3 • 3 • Total Soluble Sugars and Starch 24
3 • 4 • Proteins 25
4 • Statistical Analyses and Graphical Representation 25
Results **27**
1 • Impact of Salinity on the Vegetative Phase 27
1 • 1 • Morphological Measurements 27
1 • 2 • Biomass, Water Content and Water Potential 28
1 • 3 • Mineral Content 30
1 • 4 • Photosynthesis-Related Parameters 31
2 • Impact of Salinity on Seed Nutritional Quality 33
2 • 1 • Mineral Content 33
2 • 2 • Soluble Sugars, Starch and Proteins Concentrations 34
Discussion **37**
1 • Impact of Salinity on the Vegetative Phase 37
2 • Impact of Salinity on Seed Nutritional Quality 40

Conclusions and Perspectives..... 43
Acknowledgements..... 44
References..... 45
References: R Packages..... 54
Appendices..... 57

State of the Art

1 • Soil Salinity and Food Security

1 • 1 • Soil Salinity and Its Causes

Soil salinity is characterized by the presence of surplus ions, including among others Na^+, Cl^-, K^+, SO_4^{2-}, and Mg^{2+}, which impede plant function and growth (Keller *et al*., 1986). Saline soils can also be defined by their electrical conductivity (EC) surpassing 4 $dS.m^{-1}$ at a temperature of 25°C (Richards, 1954), which is equivalent to 40 mM of NaCl (Munns and Tester, 2008). Among the various salts found in salt-altered soils, NaCl and Na_2SO_4 are the predominant ones (Bado *et al*., 2016).

Sodicity, another significant concern associated with salts, influences soil quality (Corwin *et al*., 2003). It considers the ratio of Na^+ ions to other cations, mainly Ca^{2+} and Mg^{2+}, and can contribute to the degradation of soil characteristics (Tanji and Wallender, 2011). As a consequence, sodic soils encounter limited aeration and restricted water movement, resulting in a decrease of agricultural productivity when compared to soils affected solely by salinity (FAO, 2021). From an agronomic perspective, sodic soils are characterized by an exchangeable sodium percentage (ESP) exceeding 15% (Abrol *et al*., 1988) and an EC below 4 $dS.m^{-1}$ (Sparkz, 2003). This surplus of Na^+ ions disrupts the arrangement of soil clay particles, causing their separation and dispersion (Nouri *et al*., 2017) or causing them to slake and swell (Quirk, 2001). Certain scientists also recognize a pH value greater than 8.5 as a distinctive characteristic of sodic soils (Ahmad *et al*., 2013).

According to FAO and ITPS (2015), approximately $1x10^9$ hectares of land are impacted by salt. This accumulation of salts in soil can be attributed to two causes: primary and secondary salinization. While the former, also known as dryland salinity, results from a natural soluble salts build-up from either saline material or saline groundwater, the latter is referred to as irrigation salinity and is a direct consequence of human activities (Artzy and Hillel, 1988; Rengasamy, 2010; Stavi *et al*., 2021).

Leading sources of soluble salts associated with anthropogenic activities include the usage of low-quality waters (Minhas *et al*., 2019), the misutilization of both organic and inorganic fertilizers as well as the introduction of brines from oil and gas fields mining operations (Suarez and Jurinak, 2011; Litalien and Zeeb, 2019). Arid and semiarid zones with a low rainfall and high evapotranspiration rates tend to favor salinization (Richards, 1954). Often occurring on irrigated lands lacking adequate drainage (Artzy and Hillel, 1988), soil salinity can pose serious threats for both economic growth and food security (Butcher *et al*., 2016).

1 • 2 • Food Security

The term ‘food security’ can be characterized as the condition wherein individuals consistently have access to the requisite physical, social, and economic resources to acquire adequate, safe, and nutritious food that aligns with their dietary requirements, allowing them to maintain an active and healthful lifestyle (Gibson, 2012; Berry *et al*., 2015).

Approximately, 80% of proteins and lipids primarily derived from livestock (Steinfeld *et al*., 2006) and 90% of food calories (Cassidy *et al*., 2013), are attributed to the contributions of agricultural land (Stephens *et al*., 2018). This sector is known to be impacted by climate change and unpredictable weather events (Blanc and Reilly, 2017). Climate change and its alterations in global rainfall patterns have the potential to affect both the quantity and distribution of precipitation (USGCRP, 2017). According to prediction models, drier regions of the world are likely to experience reduced annual average precipitation, particularly in arid zones (IPCC, 2022). These impacts can result in negative consequences for crop yield, water utilization, and soil health (Jarvis *et al*., 2010; Corwin, 2020).

However, it is noteworthy that certain aspects of climate change, such as the increasing atmospheric concentration of CO_2, can also have positive effects. For instance, increased levels of CO_2 can contribute to the enrichment of organic matter in soil and promote both growth and improved water use efficiency (Poorter and Navas, 2003; Körner, 2006).

Other consequences of climate change, including the emergence of water scarcity linked to rising temperatures (Gosling and Arnell, 2016), have the potential to exert influence on soil salinization through risen demands for irrigation water (Elnashar and Elyamany, 2022), especially when considering low-quality waters (Minhas *et al*., 2019). Within food production, plants are the first organisms to suffer from soil salinity (Otlewska *et al*., 2020).

2 • Soil Salinity and Its Impacts on Plants

2 • 1 • Salt Tolerance

The effects of salinity can vary depending on plant species, soil conditions, and even geographic location (Tang *et al*., 2015). In response to saline stress, plants exhibit diverse behaviors to withstand and thrive, resulting in their classification as either glycophytes (from the Greek word *glyco*, ‘sweet’) or halophytes (Taiz *et al*., 2015; Acosta-Motos *et al*., 2017) (from the Greek word *halo*, ‘salty’).

While halophytes are plants characterized by their adaptability to high salt levels and their capability to complete their life cycle in saline environments (Mishra and Tanna, 2017), glycophytes have adapted to low sodium conditions (Assaha *et al.*, 2017), hence considered salt-sensitive plants.

The precise definition of salt-tolerant plants within the halophyte classification remains a subject of debate (Xu *et al*., 2016). While some researchers propose that a plant must be capable of completing its lifecycle in an environment with a salt concentration exceeding 0.5% NaCl (Chapman,

1942), others propose that these plants should withstand salt stress during a critical phase of their life, a period that proves lethal for most plants (Stocker, 1928).

Nevertheless, the classification of plants as either glycophytes or halophytes is contingent upon their specific responses to salt stress, including a range of factors such as variations in photosynthesis-related mechanisms, ion exclusion and/or compartmentalization at both cellular and whole-plant levels, the control of ion uptake (Isayenkov and Maathuis, 2019) at the root level and subsequent transport to leaves. Other factors include the modifications in membrane structure, as well as the induction of antioxidative enzymes, plant hormones (Munns, 2005; Parida and Das, 2005; Koyro, 2006; Stepien and Johnson, 2009; Tang *et al*., 2015) and osmoregulation (Hellebusi, 1976).

The tolerance to salinity varies between 50 mM for monocotyledonous and between 100 to 200 mM for dicotyledonous species, exhibiting greater variations among themselves (Munns and Tester, 2008; Glenn *et al*., 1999; Flowers and Colmer, 2008). Interestingly, certain dicotyledonous halophytes require salt concentrations exceeding 100mM for optimal growth (Flowers *et al*., 1977).

Saline stress can be divided into two components: the water-deficit effect (Parihar *et al*., 2014) and the ion-toxicity effect (Greenway and Munns, 1980), respectively known as osmotic and ionic stress.

2 • 2 • Osmotic Stress

An increased soil salt concentration poses challenges for water uptake by plant roots, leading to osmotic stress (Galvan-Ampudia and Testerink, 2011). The decrease in soil hydric potential caused by soil salinity causes water to move out from the plant tissues (Lopez and Hall, 2023) as the high salt concentration outside the root cells creates an osmotic gradient. Most plants experience a significant decline in shoot growth when the osmotic stress threshold of approximately 40mM is reached (Munns and Tester, 2008). Consequently, leaf expansion is hindered, new leaf emergence is delayed, and lateral bud development is slowed or may even remain dormant, resulting in reduced lateral shoots or branches (Munns and Tester, 2008).

Certain plants, on the contrary, exhibit tolerance to osmotic stress, resulting in enhanced leaf growth and increased stomatal conductance (Hasanuzzaman *et al*., 2023). However, in water-limited environments, the expansion of leaf area may not confer benefits (Munns and Tester, 2008) as larger leaf area corresponds to higher water demand through transpiration (Ritchie, 1972).

Water deficiency will trigger the accumulation of abscisic acid, leading to increased stomatal closure (Lim *et al*., 2015). Consequently, gas exchanges will be reduced, resulting in a decline in photosynthesis (Gahir *et al*., 2021).

Additionally, this dehydration causing oxidative stress will stimulate the production of reactive oxygen species (ROS) through their upregulation (Cruz de Carvalho *et al*., 2012). For instance, in mitochondria, untransferred free electrons generated during mitochondrial respiration will not be

transferred to NADP+ (Taiz *et al*., 2015) but to molecular oxygen, partially reducing it to a precursor of most ROS (Turrens, 2003).

Signaling mechanisms governing osmotic tolerance (Ismail *et al*., 2007) result in a rapid reduction in stomatal conductance (Roy *et al*., 2014; Yeo *et al*., 1991; Chazen *et al*., 1995). Halophytes and glycophytes both exhibit salt tolerance mechanisms, with halophytes having developed additional adaptations to thrive in high-salinity environments, such as employing osmoprotection through ion membrane transport or osmotic adjustment, it being the active buildup of cellular solutes in response to a decreased soil water potential (Morgan, 1984), to preserve turgor and cellular osmotic pressure (Van Zelm *et al*., 2020). Leaf turgidity can also be preserved through alterations in cell wall elasticity and enhanced apoplastic water content, thereby mitigating saline stress (Hasanuzzaman and Fujita, 2022).

These cellular solutes include in particular amino acids, glycerin, sugars, and inorganic ions such as Na^+, K^+, Ca^{2+}, and Cl^- (Chen and Jiang, 2010). Proline and glycine betaine are major organic osmolytes believed to enhance plant growth and crop yields during periods of abiotic stress by neutralizing ROS, keeping membrane integrity, and preserving enzymes and proteins (Ashraf and Foolad, 2007).

2 • 3 • Ionic Stress

The regulation and compartmentalization of ions play a crucial role in promoting plant growth by maintaining cellular homeostasis (Parida and Das, 2005). The process of compartmentalizing these ions within the vacuole or other tissues serves the plants to prevent excessive accumulation of salts in the cytoplasm (Reddy *et al*., 1992; Zhu, 2003). The enzymatic process responsible for the regulation of sodium ions involves the activity of a salt-inducible enzyme called Na^+/H^+ antiporter (Apse *et al*., 1999). Plants also respond to salt stress by regulating the expression and activity of K^+ and Na^+ transporters and H^+ pumps, allowing them to maintain high cytosolic K^+ concentrations and low Na^+ concentrations (Zhu *et al*., 1993).

In addition, alternative mechanisms such as salt secretion, selective salt accumulation, and salt exclusion are employed by plants (Parida and Das, 2005). Through the development of distinctive cellular structures known as salt glands (Dassanayake and Larkin, 2017) or bladders, which are modified trichomes and epidermal cells respectively, their secretion facilitates the release of salt, predominantly NaCl, from leaves thereby regulating lower internal ion concentrations (Flowers *et al*., 1986; Hogarth, 2015). In contrast, salt exclusion is facilitated through the roots of numerous halophytes, allowing them to regulate the salt concentration in their leaves (Levitt, 1980). Moreover, through selective accumulation of ions or solutes, plants facilitate osmotic adjustments (Parida and Das, 2005).

As older leaves cease to expand, they tend to accumulate high levels of salts (Taiz *et al*., 2015). This accumulation occurs because the halted leaf growth prevents the continuation of diluting

incoming salts, ultimately leading to cell death and leaf shedding. If the rate of leaf mortality surpasses the emergence of new leaves, it can result in a photosynthetic issue. Insufficient carbohydrate supply to meet the plant's needs further reinforces the decline in growth rate (Munns and Tester, 2008).

The cytotoxicity of ions, closely associated with decreased water potential and cellular dehydration, can give rise to secondary effects, including diminished cell and leaf growth, stomatal closure, leaf shedding hence cell death, cytorrhysis, cavitation, and the generation of ROS (Taiz *et al*., 2015). It is important to note that the osmotic phase and the ionic phase are temporally distinct from each other (Munns *et al*., 1995), mainly when salinity levels are not high with the osmotic stress occurring beforehand (Munns and Tester, 2008).

In most plants, the primary site of Na^+ toxicity is the leaf blade, where Na^+ accumulates via the transpiration stream rather than in the roots (Munns, 2002), with a significant amount retained in the shoot. This accumulation process is mainly regulated by the net delivery of Na^+ into the root xylem (Tester, 2003). On a cellular scale, leaves can endure the influx of substantial quantities of Na^+ and $Cl^–$ (Munns and Tester, 2008).

2 • 5 • Plant Salinity Stress Response

Overall, salinity-induced stress has an array of consequences impeding not only the stomatal conductance, photosynthetic rate, and activity of pivotal enzymes in plants (Fu and Yang, 2023) but also other metabolic and physiological processes previously discussed.

Signaling pathways such as the Salt Overly Sensitive (SOS) signaling pathway serves as a regulatory mechanism in order to maintain osmotic homeostasis in plants exposed to high levels of salt (Ma *et al.*, 2019), particularly through a plasma membrane Na^+/H^+ antiporter (Shi *et al.*, 2000) exporting sodium ions out of the cells. In addition, this protein's activity is combined with a Ca^{2+} binding protein and a serine/threonine protein kinase that sense calcium levels in the cytosol, preventing the accumulation of Na^+ (Ali *et al.*, 2023). Functional homologs of SOS proteins have been observed in other plant species (Martínez-Atienza *et al.*, 2007; Tang *et al.*, 2010; Sathee *et al.*, 2015 ; Fahmideh and Fooladvand, 2018). Other potassium transporters, such as *Arabidopsis* K^+ Transporter 1 (AKT1) and High-Affinity K^+ Transporter 1 (HKT1), have been identified to participate in salinity-induced stress response (Fu and Yang, 2023).

The sequestration of Na^+ ions in the vacuole can be achieved through the activity of a Na^+/H^+ antiport, known as Na^+/H^+ Exchanger 1 (NHX1), located in the tonoplast's plasma membrane (Apse *et al*., 1999). In addition, Cl^- ions sequestration in the vacuole via Cl^- Channels (CLC) could be involved in salt tolerance (Wu and Li, 2019).

2 • 6 • Salinity's Impact on Plant Yield

In general, soil salinity has a detrimental impact on the growth and yield of most plant species (Munns and Gilliham, 2015).

Miscanthus x giganteus, renowned for its high yield as a C_4 crop (Lewandowski *et al*., 2000), experiences a reduction in yield productivity when exposed to salt concentrations exceeding 100 mM (Sun *et al*., 2014; Stavridou *et al*., 2016). In other cases such as for beans, complete yield inhibition has been observed when root salinity reaches 50 mM (Volkmar *et al*., 1998). Barley, when subjected to salinity stress, exhibits a gradual decrease in leaf size over time (Munns *et al*., 1988), impacting biomass production. Decreased grain yield in some cases is attributed to a reduction in fertile tillers on plants (Maas and Grieve, 1990).

Saline soils engender mechanisms such as leaf necrosis, altered phenology, and plant mortality (Volkmar *et al*., 1998), further compromising cultivation productivity.

3 • Amaranths

Amaranthus sprouts

The *Amaranthus* L. genus in *Amaranthaceae* family comprises more than seventy species globally, with the majority native to the American continents (Waselkov *et al*., 2018). These plants are renowned for their cultivation as grains, leafy vegetables, dye-plants, and even ornamentals (Sauer, 1950; Trucco and Tranel, 2011). Amaranths fall under the category of pseudo-cereals (Baraniak and Kania-Dobrowolska, 2022), hence not belonging to *Poaceae*, and are the most widely studied pseudo-cereals, along with quinoa, chia and buckwheat (Morales *et al*., 2021). Nonetheless, they share similar properties and applications with cereals (Morales *et al*., 2021). *Amaranthus* species are characterized, but not exclusively, by their annual or short perennial life cycles, alternate leaves, as well as terminal and axillary or exclusively terminal inflorescences. Depending on the specific species, *Amaranthus* can be either monoecious or dioecious (that have respectively, separate male and female flowers on the same plant or distinctive male and female plants), both with unisexual flowers (Mosyakin and Robertson, 2003). Every species within the genus utilizes the C_4 photosynthetic

pathway (Sage *et al*., 2007). This pathway enables these plants to achieve higher photosynthetic efficiency compared to C_3 crops (Kajala *et al*., 2011) in drought and/or high temperature conditions. Unlike C_3 plants, which exclusively employ the Calvin cycle for CO_2 fixation happening in the chloroplasts of mesophyll cells, C_4 plants have photosynthetic activities that occur in different cellular locations (Wang *et al*., 2012), leading to an enhanced availability of CO_2 at the catalytic site of RuBisCO, ultimately reducing the rate of photorespiration. Also known for their enhanced water use efficiency (Wolosik and Markowska, 2019), amaranths display notable resistance to challenging environmental conditions such as drought, high temperatures, pest infestations (National Research Council, 1984; Olufolaji *et al*., 2013) and moderate saline stress (Luyckx *et al.*, 2021).

In terms of nutritional composition, *Amaranthus* grain species are characterized by their high content of soluble fiber (Early and Early, 1987; USDA, 2019) and a protein concentration exceeding 12%, particularly rich in methionine and lysine (Teutonico and Knorr, 1985; Becker *et al*., 1981) which are present in poorer concentration in conventional cereals, whereas particularly poor in leucine and are gluten-free (NRC, 1984). They can be considered to possess a high-quality amino acid profile and excellent digestibility (Martinez-Lopez *et al*., 2019; Wolosik and Markowska, 2019). The lipid content of amaranth varies widely depending on the species and genotype (Caselato-Sousa and Amaya-Farfán, 2012), typically falling within the range of 5 to 13% (Martinez-Lopez *et al*., 2019). Additionally, these plants possess significant amounts of essential nutrients, including riboflavin, niacin, ascorbic acid, as well as minerals such as calcium and magnesium (Singhal and Kulkarni, 1988). Leaf amaranth species also exhibit high levels of proteins, vitamins, minerals (NRC, 1984) and nutritional value (Luyckx *et al.*, 2023). Furthermore, amaranth grain encompasses bioactive compounds that possess health-promoting effects (Karamać *et al*., 2019). While most *Amaranthus* species are safe for human consumption, *A. retroflexus*, *A. viridis*, and *A. spinosus* should be avoided (Caselato-Sousa and Amaya-Farfán, 2012). Certain grain species may contain additional compounds such as phytate, saponins, and tannins (Aderibigbe *et al*., 2020), which are recognized as antinutritional factors for human consumption (Phan *et al*., 2018; López-Moreno *et al*., 2022).

In terms of yield, grain *Amaranthus* species possess the capacity for significant seed production, with certain cultivations consistently achieving yields of 1.80 to 2.70 $t.ha^{-1}$ (NRC, 1984; Schulte auf'm Erley *et al.*, 2005) or surpassing this in intensive cultivation (Wu *et al*., 2000). Among the major contributors of grain amaranth such as China, the United States of America, India and Peru (Aderibigbe *et al*., 2020), the typical amaranth grain yield ranges from 2.00 to 5.50 $t.ha^{-1}$ (Cai *et al.*, 2004). The yield of *A. hypochondriacus* can range from 2.31 to 4.88 $t.ha^{-1}$ depending on the fertilizer used (Tyrus and Lykhochvor, 2022). FAOSTAT has not published any yield records for *Amaranthus* species (Rosentrater and Evers, 2018).

3 • 1 • *Amaranthus cruentus*

Cultivated throughout the southern Mexicano-Guatemalteco peninsula (Sauer, 1950), *Amaranthus cruentus* appears to have independently originated across that same region from a lineage of *A. hybridus* (Kietlinski *et al.*, 2014); Figure 1 shows the taxonomy of *A. cruentus*.

Taxonomic Classification of *A. cruentus* by the United States Department of Agriculture	
Domain	Eukaryota
↳Kingdom	Plantae
↳Subkingdom	Tracheobionta
↳Superdivision	Spermatophyta
↳Division	Magnoliophyta
↳Class	Magnoliopsida
↳Subclass	Caryophyllidae
↳Order	Caryophyllales
↳Family	*Amaranthaceae*
↳Genus	*Amaranthus* L.
↳Species	*Amaranthus cruentus* L.

Figure 1: The taxonomic classification of *Amaranthus cruentus* by the United States department of agriculture (USDA, 2013).

Amaranthus cruentus

The Flora of North America (FoNACE, 2015) describes *A. cruentus* as a nearly glabrous plant stranding upright reaching up to 120cm with green to red-purple stems and leaves. The latters have a rhombic-ovate shape standing with a petiole around half of their length. While the inflorescences and flowers have a dark red-purple color and are located at the apex of the stem, their seeds are in shades of white or occasionally red with a diameter of around 1.4mm (see Appendix 1 for further morphological details).

Amaranthus cruentus

Study Objectives

Amaranthus cruentus serves as the focal point of the study. Notably, it is regarded as one of the most adaptable amaranth species (NRC, 1984).

The research delves into the effects of saline stress, particularly at 75mM of NaCl, on the morphology, physiology, and nutritional quality of four *A. cruentus* cultivars. These cultivars encompass both grain and leaf utilization, as well as salt-tolerant and salt-sensitive characteristics. Specifically, the cultivars Rouge and Locale are utilized, with Rouge identified as a leafy salt-tolerant variety and Locale as salt-sensitive (Wouyou *et al*., 2017). Additionally, Don Leon and Montana-5, are respectively as sensitive as Rouge and Locale (Luyckx *et al.*, 2023) yet as grain varieties.

The primary aim of this thesis is to elucidate the salt resistance mechanisms intrisic in both grain and leafy cultivars involving a comparative analysis between salt-tolerant and salt-sensitive varieties. Additionally, this thesis aims to assess the influence of soil salinity on the nutritional composition of *Amaranthus cruentus* seeds.

Several hypotheses have been tested. First of all, salt-sensitive cultivars will exhibit a decline in plant growth, associated with reduced height as well as leaves amount and overall biomass, when exposed to salt-induced stress, compared to salt-resistant cultivars. Additionally, salt-sensitive cultivars will exhibit a reduction in physiological parameters such as photosynthetic rate and chlorophyll content, in contrast to salt-resistant cultivars. Furthermore, salt-sensitive cultivars will show decreased nutritional quality due to alterations in nutrient uptake and assimilation, while the resistant cultivars will keep better nutrient profiles under these same saline conditions.

To assess these variations, various morphological parameters were measured, including plant height and leaf production. Physiological parameters such as chlorophyll content, gaz exchanges measurements, and water potential were also evaluated. Additionally, the nutritional quality of seeds was determined by measuring concentrations of minerals (including Na^+ and K^+), proteins and soluble sugars.

Materials and Methods

1 • Plant Material

Four *A. cruentus* cultivars were used: Locale (**LO**) and Rouge (**RO**) as leafy types provided by Dr. Gandonou, C.B. (University of Abomey-Calavi, Cotonou, Benin), and Don Leon (**DL**) from Argentina and Montana-5 (**M5**) from the USA as grain type, respectively accessed in the Genebank of the Crop Research Institute (CRI, Prague, Czech Republic) with 01Z5200166 and 01Z5200032.

2 • Impact of Salinity on the Vegetative Phase

2 • 1 • Growing Conditions

Plants were cultivated in UCLouvain's greenhouses (SeFy) located in Louvain-la-Neuve, Belgium, under semi-controlled conditions, maintaining a day-to-night temperature cycle with average day and night temperatures of 24.6 ± 1.4°C and 22.3 ± 0.9°C, respectively, along with relative humidity (RH) of 55.6 ± 6.3% and 59.2 ± 5.4%.

Sowing took place on February 28th 2023, in plastic seed trays (20*35*6cm) for each cultivar in a soil composed of ⅔rd potting soil (DCM, Amsterdam, The Netherlands) and ⅓rd of river sand (MPRO Wavre, Belgium) in volume, covered with a glass sheet for 2 days and then watered every 2 to 4 days with rain water. Then the first transplanting occurred 14 days later on March 14th, in individual PVC containers (6*6*6cm) in a soil composed of ½ of perlite and ½ of vermiculite in volume for 22 plants of each variety. Saline stress was applied three days later, on March 17th using a nutritive solution (Hoagland) for control condition and salty nutritive solution (Hoagland + 75 mM NaCl) for stressed condition (Hoagland and Arnon, 1938). The solutions (see Appendix 2 for further details on their composition) were prepared in 25L containers and kept in the greenhouse. The second transplanting occurred on March 31st in plastic pots (11*11*15cm) with the same perlite/vermiculite ratio (1:1) for 20 plants of each variety, each placed following a split-plot design as shown in Figure 2, for a total of 4 plants per tray (12*100*2cm). Nutritive solutions were used to water the plants twice a week, around 1.75L each time, and trays were cleaned every 3 weeks to limit salt accumulation. Harvest was done on April 28th, 60 days after sowing.

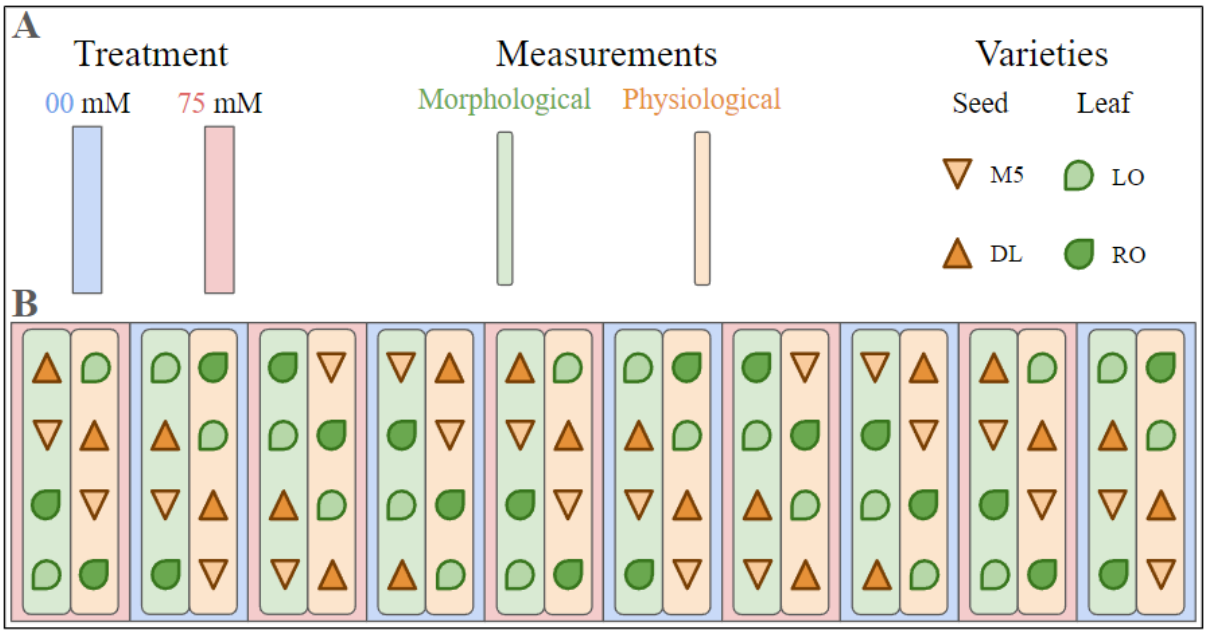

Figure 2: Spatial overview of both cultivars and treatments in the greenhouse. **A**) Treatments are represented in blue and red, respectively for control condition (0 mM) and saline stress condition (75 mM). Measurements taken on plants were distinguished between morphological (green) and physiological (orange) to limit nuisance, and each representing a tray. Cultivars are represented by a brown triangle (grain type) or green leaf (leaf type) and are of light intensity (salt-sensitive) or strong intensity (salt-tolerant). **B**) Spatial overview of which plant received which treatment along with which other plants.

2 • 2 • Plant Growth and Development

Morphological measurements included plant height and leaf count as well as the fresh and dry mass of leaves, stems and roots. A sample size of 5 was subjected to these measurements, with the exception of biomass for which n=4.

2 • 2 • 1 • Plant Height and Leaf Count

Plant height was taken perpendicular to the ground, from the cotyledon insertion to the apex of the youngest leaf. Leaves were considered, disregarding both cotyledon, once their blade was open, and were not considered once fallen off, on both the main stem and ramifications. Both plant height and leaf production were recorded on a weekly basis, seven times starting from the day saline stress was applied.

2 • 2 • 2 • Fresh and Dry Mass

On April 28th, fresh mass measurements were conducted. In addition to plant leaves mass, five leaves positioned 5 cm below the shoot apical meristem (SAM) were individually weighed per plant; these samples were earmarked for mineral content experiments. Stems were separated from both the leaves and the root system and weighed. The roots were cleaned with water, though the fresh mass of the root system was not quantified.

Once these plant organs were desiccated after three days at 70°C in a laboratory oven, dry mass measurements were taken. Water content for both leaves and stems were calculated as follows:

*((Fresh Mass - Dry Mass) / Fresh Mass) * 100*

2 • 3 • Physiology

Physiological measurements included leaf chlorophyll content, gas exchanges measurements as well as water potential.

2 • 3 • 1 • Chlorophyll Content

Seven days after the saline stress was applied, and on a weekly basis, the chlorophyll content in Soil Plant Analysis Development (SPAD) units was determined in leaves of equivalent age near the SAM, with a minimum midrib-to-perpendicular edge width of 1.5 cm and expressed in this study using the measured nitrogen content (1 SPAD unit ≅ 1.429±0.009 mg of N per g of plant). These measurements were performed using a Plant Nutritional Analyzer (PNA, Panomex Inc., India) on the upper half of the leaf blade (n=5).

2 • 3 • 2 • Water Potential and Gas Exchanges Measurements

On April 27th, water potential measurements were conducted. One leaf from each plant (n=6), located 5 cm below the SAM, was selected and inserted into the pressure chamber (1505D Pressure Chamber Instrument, PMS Instrument Company, Albany, Oregon, USA); the petiole tip on the stem side of the leaf protruded from the pressure chamber. Incremental pressure, in bar, was then applied, and with the help of a magnifying glass, pressure readings were taken upon the appearance of 0.5 mm bubbles.

On April 7th, 14th, 25th–27th, the plant steady-state photosynthetic CO_2 responses (A/Ci curves) and photosynthesis-irradiance (PI curves) were performed. Each day, one plant from each cultivar and treatment (n=1) were selected and a leaf located 10 cm below the SAM was put into the InfraRed Gas Analyser (IRGA; iFL Integrated Fluorometer Gas Exchange System, ADC BioScientific Ltd., Hoddesdon, United Kingdom) chamber, remaining on the plant. On April 7th, each plant was subjected to a 45-minute experimental session. At intervals of three minutes, totaling 15 iterations, irradiance was systematically elevated from 0 to 1750 photons µmol $m^{-2}s^{-1}$, with an incremental addition of 125 µmol photons $m^{-2}s^{-1}$ each time and the CO_2 concentration remaining at around 435 ppm. Concurrently, various plant responses, including net photosynthesis (A), transpiration (E), electron transport rate (J), stomatal conductance (g_s), and photosystem II quantum yield (PSII Φ), were recorded. Plants from the remaining experimental sessions were exposed to comparable conditions. However, in contrast to the previous session where irradiance varied, it was steadily maintained at 800 µmol photons $m^{-2}s^{-1}$. The fluctuation was done to A/Ci. The concentrations started at around 500 µmol $m^{-2}s^{-1}$, decreased to around 3 by 125 µmol $m^{-2}s^{-1}$ each decremental, then went from around 625 to end at approximately 1875, skipping 1000, with an incremental of 125 µmol $m^{-2}s^{-1}$.

2 • 3 • 3 • Mineral Content

Dry plant organs (see 2•2•2 Fresh and Dry Mass Measurements) were broken down into a fine powder using a mortar with liquid nitrogen. Minerals quantification involved the use of approximately 75 mg of dry powdered material (n=5) placed in 10 mL volumetric flasks. Afterwards, 4 mL of concentrated (68%) nitric acid were added. The flasks were positioned onto a sand bath beneath a fume hood for 24 hours at room temperature. The sand bath temperature was incrementally raised, starting at room temperature and then reaching nitric acid ebullition once the solution reached transparency. The flasks remained on the sand bath ensuring complete evaporation of the added nitric acid, then placed onto a glass plate to cool. Subsequently, the residues were dissolved in 2 mL of *aqua regia* (comprising 1.5 mL of concentrated (37%) HCl and 0.5 mL of concentrated (68%) nitric acid) and placed onto the warm sand bath until complete dissolution. After cooling, the flask volume was made up to 10 mL with purified water, and the resulting solution was filtered using Whatman Grade 1 filter papers into 15 mL Falcon tubes.

Each filtered sample was then diluted 10 and 100 times using $LaCl_3$ 10%. Finally, mineral quantification was obtained through atomic absorption spectrometry (AAS; ICE 3300, Thermo Scientific, Waltham, MA, USA) using adequate standards for each element. Leaves and roots were subject to the determination of K^+, Na^+ exclusively for a sample size of 5.

3 • Impact of Salinity on Seed Nutritional Quality

3 • 1 • Plant Material

Seed nutritional quality was assessed on seeds harvested from previous experiments (Cardoso de Lima, 2023; Kudryk, 2022) after 60 days of stress and upon seed maturation. Plants of the four cultivars (Locale, Rouge, Don Leon and Montana-5) were cultivated under salt stress (0 and 75 mM NaCl) as described in the present study (see 2•1 Growing Conditions).

3 • 2 • Minerals

Seeds were broken down into powder through cryogenic grinding using a cryomill (Cryomill. Retsch, Haan, Germany). The mineral analysis of seeds encompassed the determination of Na^+, K^+, Ca^{2+}, Cu^+, Fe^{2+}, Mg^+, and Zn^{2+} ions using the same protocol used for leaves and roots (see 2•3•3).

3 • 3 • Total Soluble Sugars and Starch

Soluble sugar and starch quantification involved respectively the use of approximately 250 and 125 mg of finely powdered seeds (n=5). This powder was then combined with 4 mL of 70% ethanol in a Falcon tube and homogenized. Following a 5-minute cooling period on ice, the Falcon tubes underwent centrifugation at 7084 g for 10 minutes at 4°C. The resulting supernatant was filtered into a new Falcon tube using Whatman Grade 1 filter papers. The remains from the initial centrifugation received an additional 2 mL of 70% ethanol, vortexed, and centrifuged under the same conditions.

This process was repeated thrice in total. After filtration, the volume was made up to 7 mL using 70% ethanol, and the samples were stored at -20°C for subsequent analysis. The pellet was employed for starch concentration determination.

To achieve this, 8 mL of 1M HCl was added to the pellet, which was then placed into a water bath at 95°C for 2 hours. The samples were vortexed every 15 minutes. After cooling, the liquid was filtered, and the Falcon tubes were rinsed with an additional 8 mL of 1M HCl, filtered and combined with the initial filtration. Then, 750 µL of the solution were transferred to 1.5 mL Eppendorf tubes for each sample. The pH was neutralized with 1M NaOH. Both the resulting volumes and the samples kept at -20°C were transferred to glass test tubes (3 aliquots per sample), to which 1 mL of anthrone solution (50 µL water, 1 mL concentrated H_2SO_4, 2 mg anthrone) was added. Following incubation at 100°C for 10 minutes, the mixture was transferred into spectrophotometer cuvettes, and concentrations were determined using a UV/Visible spectrophotometer (UV-1800 UV/Visible Scanning Spectrophotometer 115 VAC; Shimadzu, The Netherlands) at 625 nm.

Quantification of both total soluble sugars and starch measurements was performed using established standards ranging from 0 to 400 $mg.L^{-1}$ of D-glucose using ethanol 70%, each repeated thrice.

3 • 4 • Proteins

Protein quantification involved approximately 50 mg of powdered seeds (n=5) using the Bradford protein assay (Bradford, 1976). The samples were deposited into 2 mL Eppendorf tubes, and subsequently, 1.5 mL of phosphate-buffered saline (PBS; K_2HPO_4 and KH_2PO_4 at 54.7:45.3 mass, pH 7.4, 100mM) was added, followed by vortexing. The resulting mixture underwent centrifugation for 15 minutes at 12000 g and 4°C. 50 µL of the supernatant were then aliquoted to three separate glass test tubes for each sample. Following this, 1.5 mL of Bradford reagent was added; this reagent was obtained using 50 mL ethanol (95%) with 100 mg of Coomassie brilliant blue G-250, followed by its dissolution in H_3PO_4 (85%) with its volume made up to 1 L using purified water. After a 10-minute incubation period at room temperature, the samples were quantified using the UV/Visible spectrophotometer mentioned previously.

Quantification of protein concentration was performed using established standards ranging from 0 to 500 $µg.mL^{-1}$ of bovine serum albumin (BSA; 20 mg albumin in 20 mL of PBS) using PBS.

4 • Statistical Analyses and Graphical Representation

Statistical analyses were carried out using R version 4.3.2 (R Core Team, 2023) and the following packages: car, dplyr, emmeans, EMSaov, ggpubr, gridExtra, lme4, multcomp, RCurl, readxl, rstatix, tidyverse and visreg (see References: R Packages) were used to analyze and visualize the data. Normality of data and homogeneity of variances were checked using respectively Shapiro test [stats::shapiro.test()] and Levene test [car::leveneTest()]. Two-ways analysis of variance (anova)

[stats::aov()] was performed using both treatment and variety as explanatory variables and repeated for each day to detect significant changes ($p < 0.05$) in height and leaf production as well as nitrogen content over time. Student's t-test [stats::pairwise.t.test()] was performed to assess differences (ns: $p > 0.05$; *: $0.05 \geq p \geq 0.01$; **: $0.01 > p \geq 0.001$; ***: $p < 0.001$) between treatments of the same variety. Data were represented using bar charts alongside corresponding error bars (standard deviation) when the sample size was low ($n<5$). Alternatively, for cases where the sample size was equal or exceeded 5, boxplots were employed. A/Ci and Photosynthetic-Irradiance curves were obtained [RCurl::fit_AQ_curve()] and several of their parameters (Pmax: Maximum Photosynthetic Rate, α: Initial Slope of P-I Curve, Ik: Saturation Irradiance using α, Rd: Dark Respiration, Vcmax: Maximum Carboxylation Rate, Jmax: Maximum Electron Transport Rate) were then analyzed using Kruskal-Wallis test [stats::kruskal.test()] as a non-parametric alternative to the one-way anova, and post-hoc Dunn's test [rstatix::dunn_test()].

Results

1 • Impact of Salinity on the Vegetative Phase

1 • 1 • Morphological Measurements

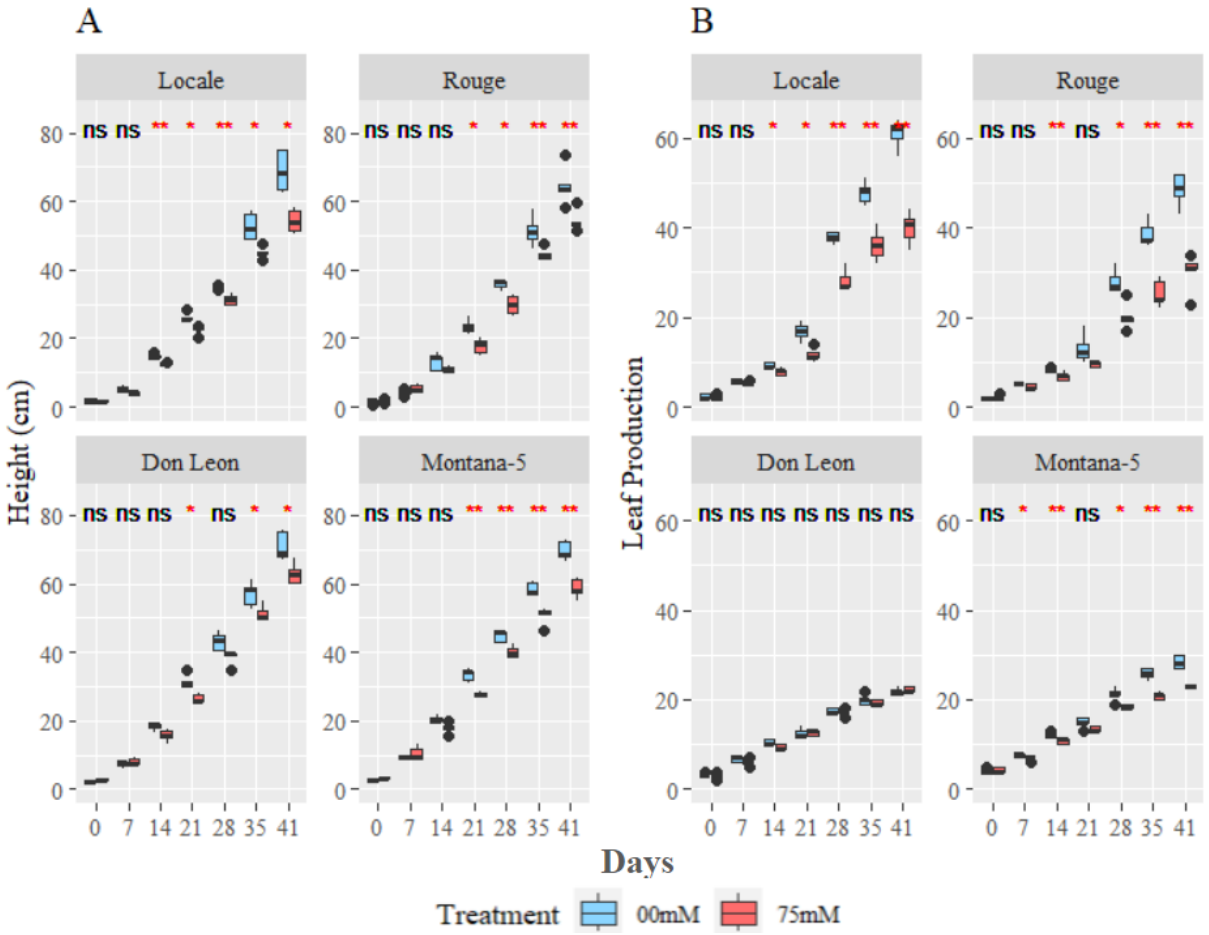

Figure 3: Effect of salinity (0 and 75 mM NaCl) on the height (**A**) and leaf production (**B**) of four *A. cruentus* cultivars expressed over time after stress initiation. Significance is represented below each variety for each day using Student's t-test.

Across the whole experiment (any day, both morphological measurements), differences between varieties were observed (Figure 3; Appendix 3a: anova – all p-values ≤ 0.001), with the treatment having a discernible impact starting from day 7 (on leaf production) and 14 (on height) of stress onwards (all $p < 0.001$). However, no interaction effect was discerned between the treatments and varieties on plant height (all $p > 0.05$) and only from day 21 onwards on leaf production (day 21: $p < 0.01$; day 28-41: $p < 0.001$).

On average, salinity led to a reduction in plant height for all four varieties (Figure 3, **A**; Appendix 3b). This decrease began as early as 14 days after stress for Locale ($p < 0.01$), but only manifested

after 21 days of stress for Rouge ($p < 0.05$), Montana-5 ($p < 0.01$), and Don Leon ($p < 0.05$). The latter did not exhibit a diminished impact on its height growth after 28 days ($p > 0.05$).

In line with the observed trends in plant height, leaf production (Figure 3, **B**; Appendix 3b) displayed a decline in leafy cultivars under stress conditions from day 7 (Locale) and day 14 (Rouge) onwards (all p-values < 0.05). The grain cultivar Montana-5 also exhibited a reduction from day 7 of stress onwards (all $p < 0.05$), except after 21 days ($p > 0.05$). While there was a discernible difference in the number of leaves between control and stressed conditions for the grain cultivar Montana-5, Don Leon did not demonstrate such an impact (all $p > 0.05$).

1 • 2 • Biomass, Water Content and Water Potential

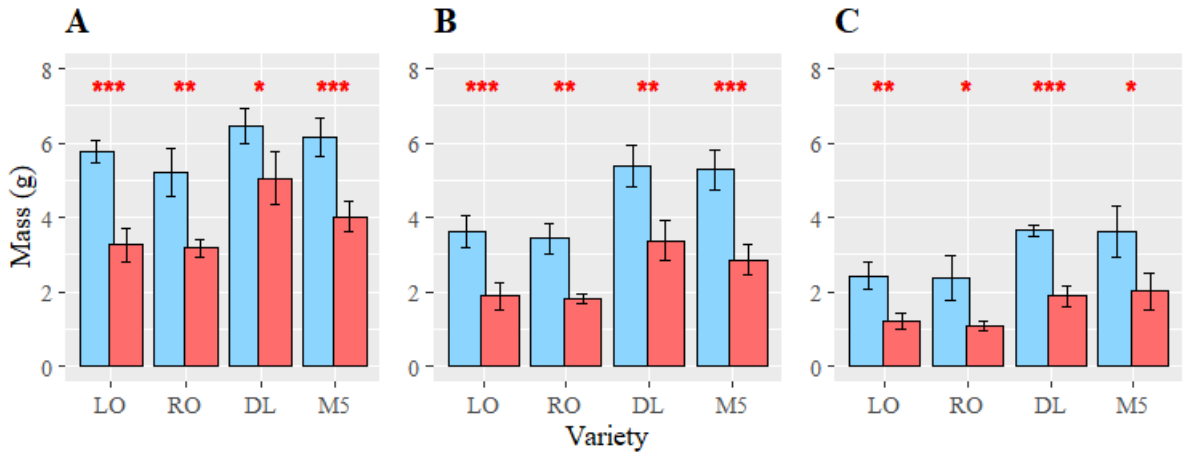

Figure 4: Effect of salinity (Blue:0 and Red:75 mM NaCl) on organs dry mass (**A**: Leaves; **B**: Stems; **C**: Roots) of four *A. cruentus* cultivars (LO: Locale, RO: Rouge, DL: Don Leon and M5: Montana-5) expressed in grams. Significance is represented using Student's t-test.

Salt concentration and plant variety had an impact on organ biomass (Figure 4; Appendix 4: anova – all $p < 0.001$). Additionally, leafy cultivars exhibited a lower biomass production compared to grain cultivars in all plant organs under both 0 mM and 75 mM NaCl (Figure 4). The interaction between the treatments and varieties did not have a significant impact on any of the plant organs (all three $p > 0.05$).

The exposure to 75 mM of NaCl had a detrimental impact on both plant variety and organ dry mass (Figure 4; Appendix 9). For instance, leafy cultivars and grain cultivars exhibited variations among each other. On average, the biomass of leafy cultivars under 0 mM of NaCl had lower mass than grain cultivars for leaves (5.50 ± 0.55 g *versus* 6.31 ± 0.49 g, respectively), stems (3.53 ± 0.39 g *versus* 5.32 ± 0.51 g, respectively), and roots (2.41 ± 0.46 g *versus* 3.64 ± 0.46 g, respectively). The same trend was observed under 75 mM of NaCl (leafy – leaves: 3.23 ± 0.34 g, stems: 1.86 ± 0.26 g, roots: 1.16 ± 0.19 g *versus* grain – 4.54 ± 0.76 g, 3.12 ± 0.51 g, 1.95 ± 0.38 g, respectively).

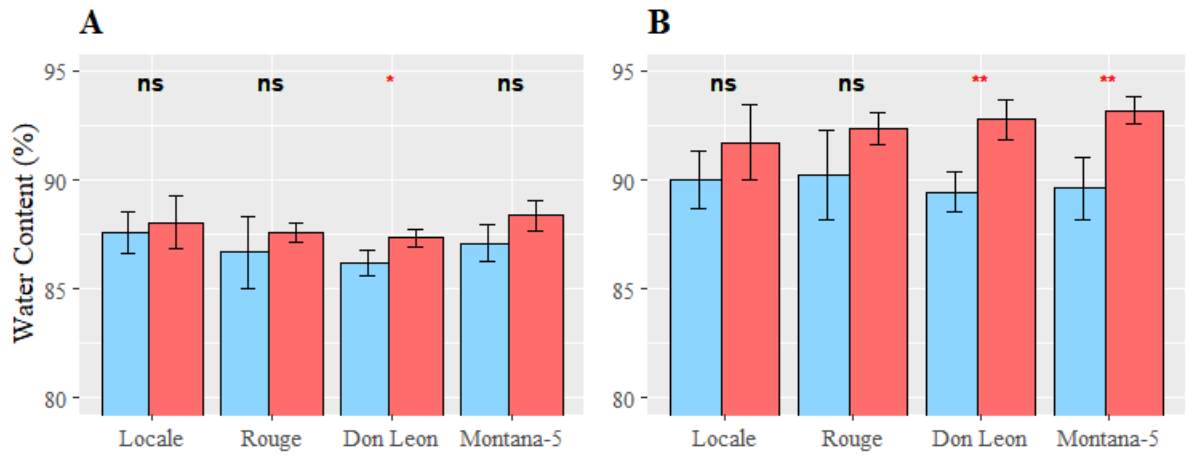

Figure 5: Effect of salinity (Blue:0 and Red:75 mM NaCl) on leaf (**A**) and stem (**B**) water content of four *A. cruentus* cultivars expressed in percentage. Significance is represented using Student's t-test.

Regarding the water content of both leaves and stems, respectively, only the treatment exhibited a significant impact (Appendix 4: $p < 0.01$ and $p < 0.001$) showing an increase of the water content with salinity (Figure 5).

However, further examination of inter-treatment comparisons within individual varieties revealed a significant increase in the stem water content of the grain cultivars (Figure 5; Appendix 9: $p < 0.01$ for both), and no significant differences were observed for the leaf water content, except for Don Leon ($p < 0.05$). On average, leafy cultivars showed greater variation among themselves for both organs (0 mM – leaves: ± 1.35%, roots: ± 1.63 % *versus* 75 mM – ± 0.88 %, ± 1.28%, respectively) than grain cultivars (0 mM – l: ± 0.83 %, r: ± 1.11 % *versus* 75 mM – ± 0.77 %, ± 0.75 %, respectively) while their water content mean remained similar.

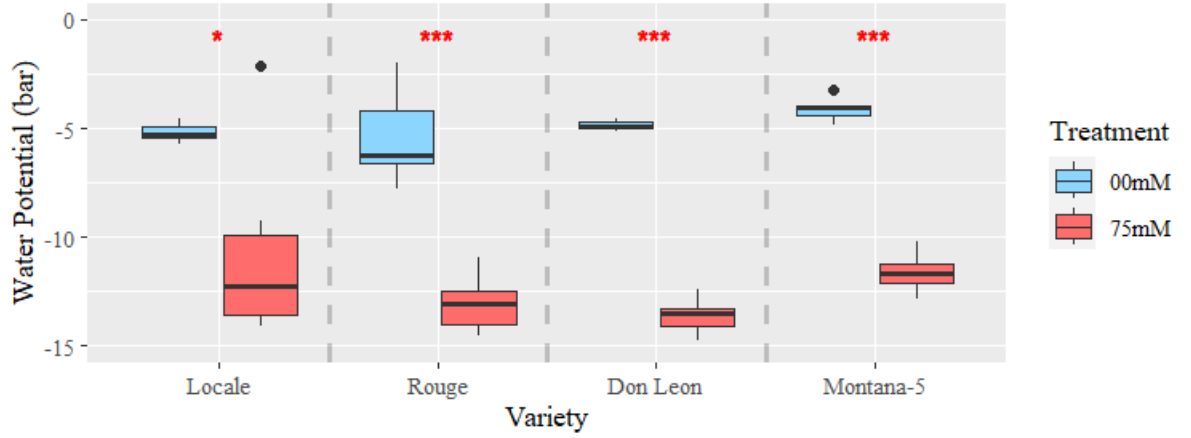

Figure 6: Effect of salinity (0 and 75 mM NaCl) on leaf water potential of four *A. cruentus* cultivars 59 days after sowing. Significance is represented using Student's t-test.

The water potential was significantly affected by the applied treatment (Figure 6; Appendix 4: $p < 0.001$), while neither the variety nor the interaction between the two did (both $p > 0.05$).

Indeed, salinity decreased the water potential in all varieties (Figure 6; Appendix 9: LO with $p < 0.05$, rest with $p < 0.001$).

1 • 3 • Mineral Content

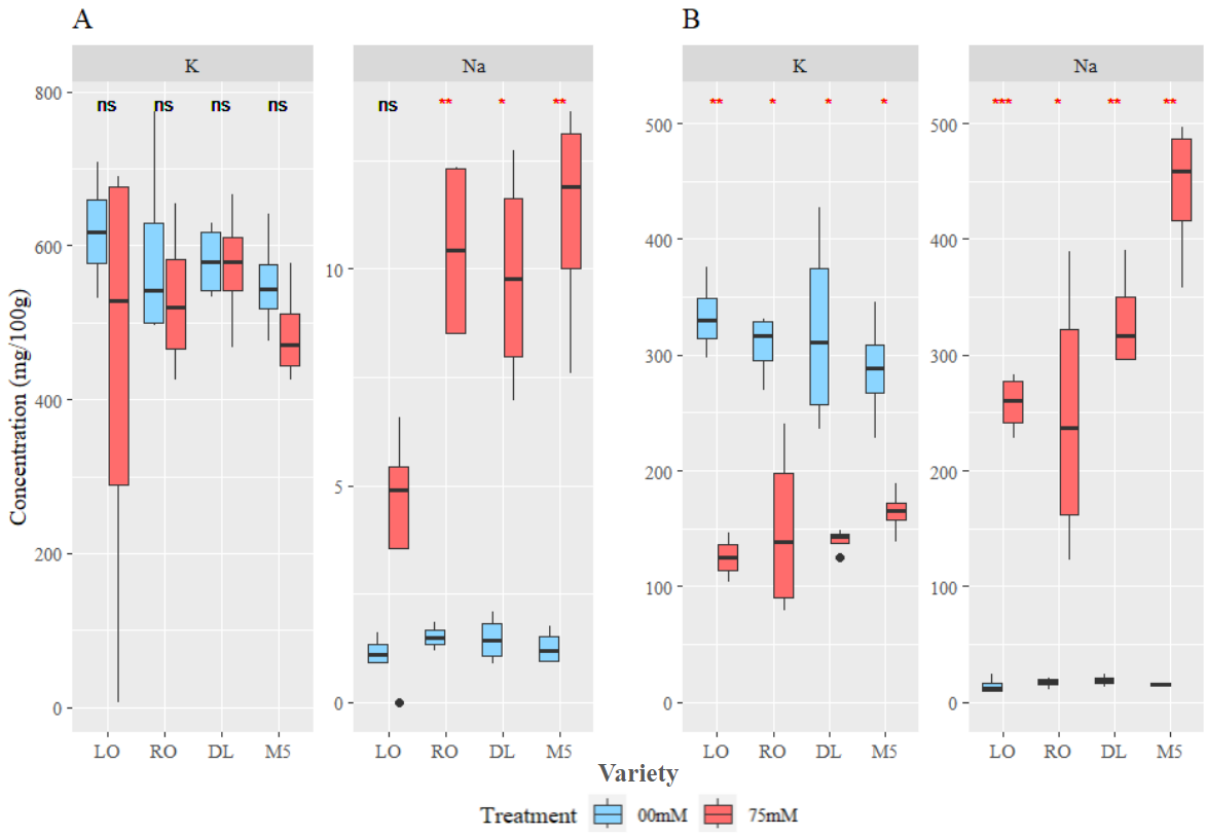

Figure 7: Effect of salinity (0 and 75 mM NaCl) on the leaves (**A**) and roots (**B**) mineral concentration of four *A. cruentus* cultivars (LO: Locale, RO: Rouge, DL: Don Leon and M5: Montana-5) after 60 days of growth, expressed in $mg.100g^{-1}$ of dry organ. Significance is represented below each mineral for each variety using Student's t-test.

Salt stress on leaves mineral content (Figure 7) resulted in significant differences between treatments and varieties for sodium (Appendix 5: both $p < 0.01$). The interaction between salt concentration and plant variety also had an impact on sodium concentration in leaves ($p = 0.004$).

Once comparing varieties within treatments, higher concentrations of sodium were observed in all cultivars leaves under salt stress (Appendix 6: all $p < 0.05$), except for Locale ($p = 0.112$). The latter result arised from an outlier; if removed, higher concentrations of sodium were also observed for Locale ($p < 0.05$).

The sodium concentration in the roots was impacted by both the salt concentration (Appendix 5; $p < 0.001$) and plant variety ($p = 0.003$). The interaction between the NaCl concentration and variety had an impact on sodium concentration ($p = 0.003$). Only the treatment had an impact on the potassium concentration ($p < 0.001$).

Similarly to leaves, higher concentrations of sodium were observed for the roots of every variety that underwent 75 mM NaCl (all $p < 0.05$). Contrary to leaves, root concentrations of potassium were also found in greater quantities (Figure 7) when plants were exposed to salt (Appendix 6: all $p < 0.05$).

1 • 4 • Photosynthesis-Related Parameters

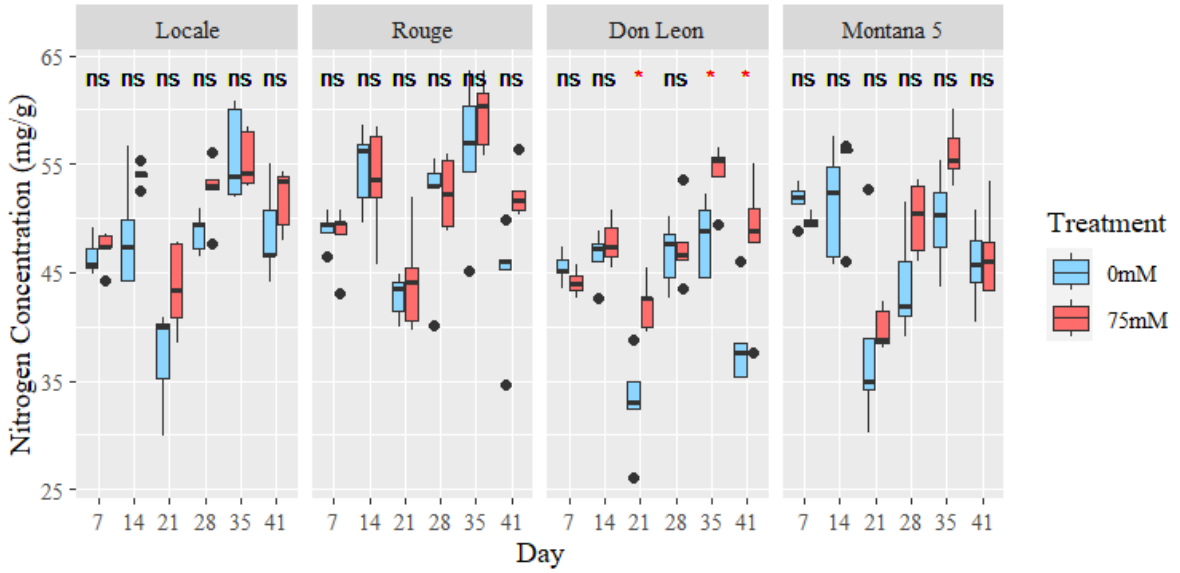

Figure 8: Effect of salinity (0 and 75 mM NaCl) on the leaf nitrogen content of four *A. cruentus* cultivars in days after stress initiation, expressed in $mg.g^{-1}$ of fresh leaf. Significance is represented below each variety for each day using Student's t-test.

Effects of the variety were observed upon each day plants were exposed to the experiment on the leaf nitrogen content (Figure 8; Appendix 7: all $p < 0.05$). However, the NaCl concentration only had an impact after 21 days onwards (all $p < 0.05$). The interaction between variety and salt concentration had no impact on the nitrogen content (all $p > 0.05$).

The varieties for which there was a difference between salt concentration in nitrogen content only were Don Leon on days 21, 35 and 41 (Figure 8; Appendix 8).

		Photosynthesis Irradiance			A/Ci Curves		
Variety	**Treatment**	**Pmax**	**α**	**I_k**	**Rd mean ± sd**	**Vcmax mean ± sd**	**Jmax mean ± sd**
LO	0mM	132.0	0.213	451.0	-18.9 **a** ± 7.9	149.5 **a** ± 104.8	197.9 **a** ± 107.7
LO	75mM	132.1	0.220	437.1	-23.5 **a** ± 4.0	185.4 **a** ± 118.3	193.3 **a** ± 82.2
RO	0mM	131.2	0.195	490.6	-17.1 **a** ± 5.3	109.0 **a** ± 67.6	238.0 **a** ± 80.8
RO	75mM	120.6	0.195	443.9	-22.8 **a** ± 4.7	226.9 **a** ± 169.2	209.8 **a** ± 46.9
M5	0mM	127.5	0.190	491.8	-18.3 **a** ± 6.1	192.6 **a** ± 50.1	306.4 **a** ± 156.8
M5	75mM	130.2	0.207	457.2	-28.0 **a** ± 12.0	101.8 **a** ± 118.6	1348.5 **a** ± 2113.3
DL	0mM	122.6	0.169	508.2	-11.5 **a** ± 4.6	113.4 **a** ± 59.9	464.6 **a** ± 402.3
DL	75mM	118.7	0.186	447.7	-23.5 **a** ± 16.7	71.5 **a** ± 89.5	193.1 **a** ± 129.0

Table 1: Effect of salinity (0 and 75 mM NaCl) associated with irradiance (n=1) and A/Ci curves (n=3) of four *A. cruentus* cultivars. **Pmax**: Maximum Photosynthetic Rate, **α**: Initial Slope of P-I Curve, **I_k**: Irradiance Saturation using α, **Rd**: Dark Respiration, **Vcmax**: Maximum Carboxylation Rate, **Jmax**: Maximum Electron Transport Rate. Significance is represented by a letter to differ groups using Dunn's test.

The salt concentration seemed to have had an impact on irradiance saturation (Table 1: lower under 75 mM NaCl) but not on the initial slope of the P-I curves nor the maximum photosynthetic rate. These results are to be taken with a grain of salt as the sample size was low (n=1).

Parameters obtained from A/Ci curves, including dark respiration, maximum carboxylation rate and maximum electron transport rate, were not impacted by either the salt concentration or the variety.

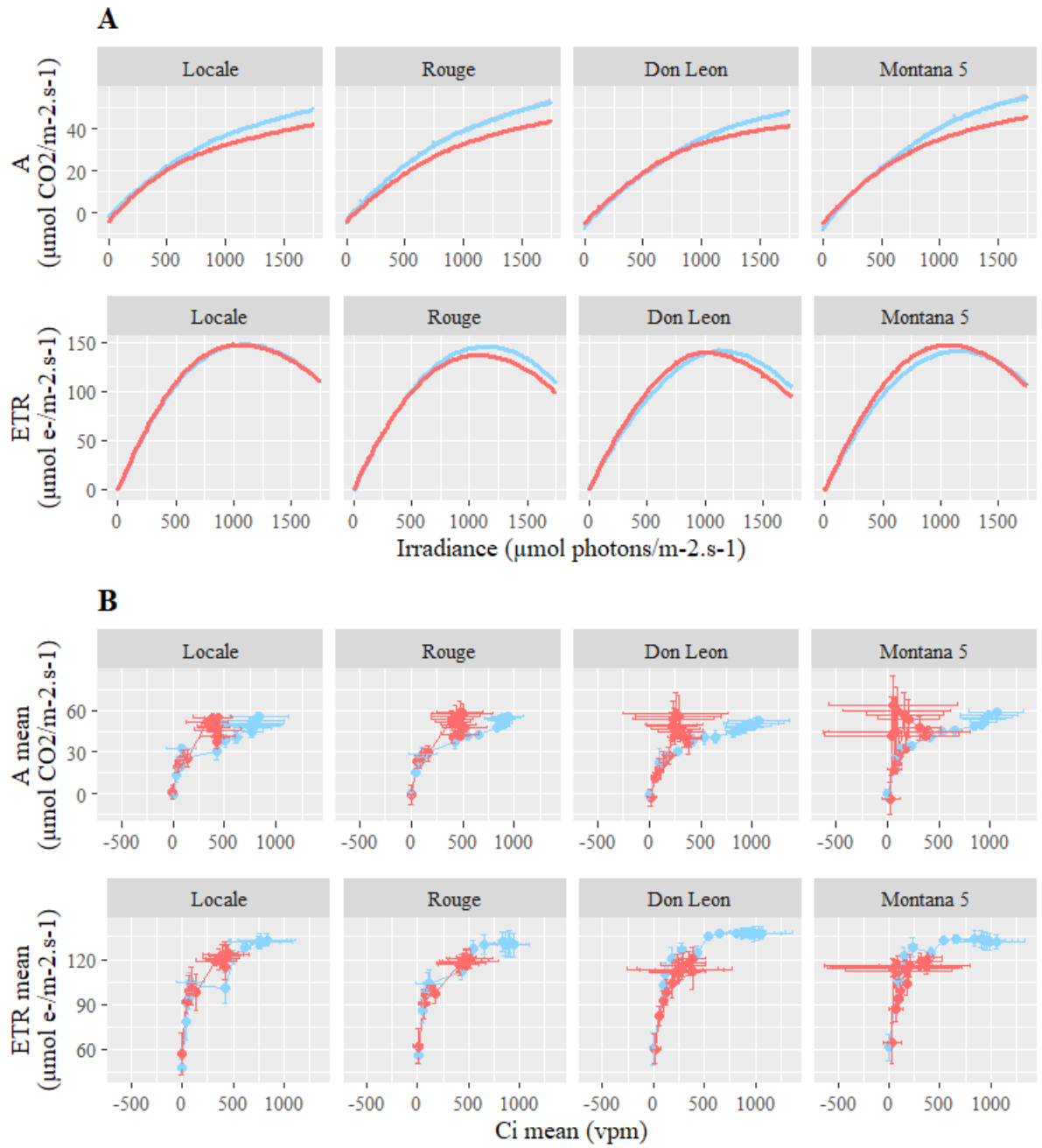

Figure 9: Effect of salinity (Blue:0 and Red:75 mM NaCl) combined with Irradiance (**A**) or Ci mean (**B**) of four *A. cruentus* cultivars on the photosynthesis rate (A) and electron transport rate (ETR). Lines represent the mean of sample trends, associated error bars are standard error.

Salinity stress appeared to have negatively impacted the photosynthetic rate (Figure 9, **A**) once irradiance reached 400 µmol $m^{-2}s^{-1}$ for leafy cultivars and above 700 for grain cultivars. This was also

observed depending on CO_2 concentration inside the plant (Figure 9, **B**; Appendix 10), with higher Ci in salt-free varieties for the same photosynthetic rate.

While the electron transport rate (ETR) was not impacted by salt stress when exposed to irradiance (Figure 9, **A**), ETR seemed to have been negatively impacted by 75 mM NaCl when CO_2 inside the plant reached 750 vpm and above (Figure 9, **B**).

2 • Impact of Salinity on Seed Nutritional Quality

2 • 1 • Mineral Content

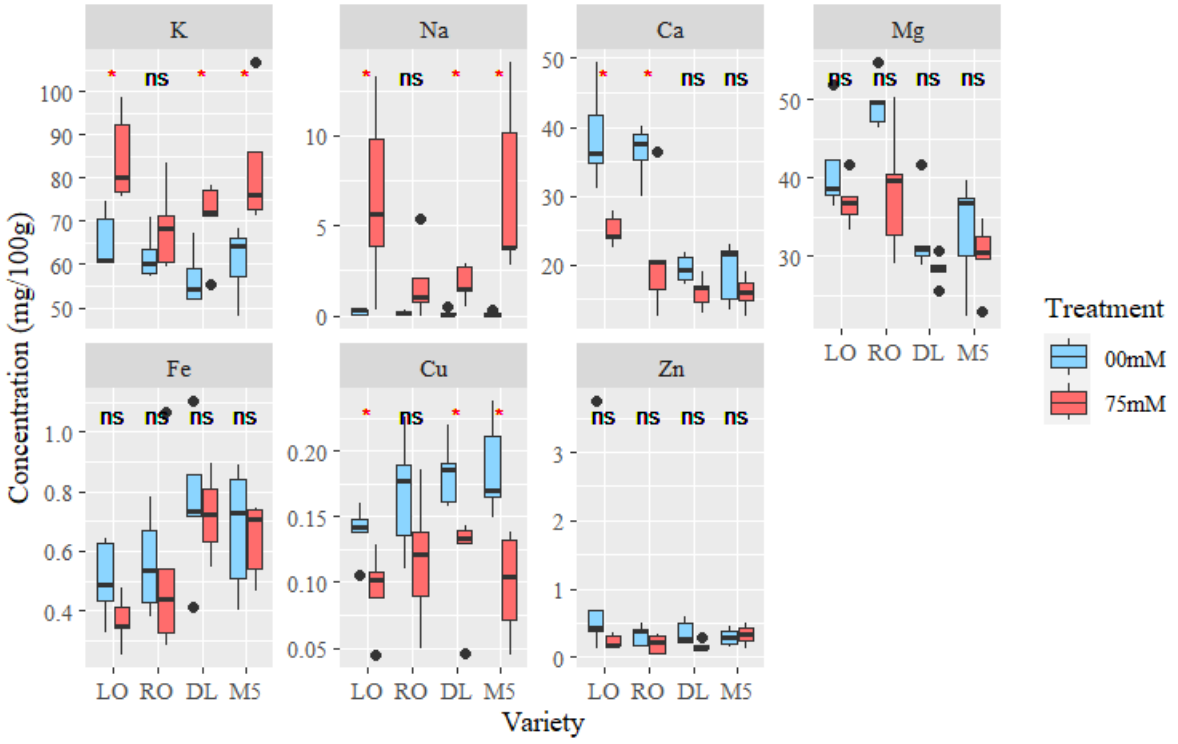

Figure 10: Effect of salinity (0 and 75 mM NaCl) on the seed mineral concentration of four *A. cruentus* cultivars expressed in $mg.100g^{-1}$ of dry powdered seeds. Significance is represented below each mineral for each variety using Student's t-test.

Salinity stress on seed mineral concentration (Figure 10; Appendix 5) resulted in significant differences between, respectively, treatments and varieties for sodium ($p < 0.001$ and $p < 0.05$), calcium (both $p < 0.001$), potassium ($p < 0.001$ and $p = 0.03$) and magnesium ($p = 0.002$ and $p < 0.001$). The interaction between the NaCl concentration and the plant variety had an impact on sodium and calcium concentration ($p = 0.049$ and $p = 0.010$, respectively). While there were differences between varieties for iron ($p = 0.007$) and treatments for copper ($p < 0.001$), no statistical significance had been observed for zinc (all $p > 0.05$).

Regarding the macronutrients, there was an augmentation of potassium and sodium for sensitive cultivars (Appendix 6: LO: $p = 0.048$ and $p = 0.046$; M5: $p = 0.033$ and $p = 0.034$, respectively) under 75 mM NaCl. While there was a reduction in calcium for leafy cultivars (both $p < 0.05$) under salt

stress, Don Leon had lower potassium (p = 0.024) and sodium (p = 0.033) concentration under 0 mM NaCl.

Except for copper concentration in grain cultivars, respectively Don Leon (p = 0.043) and Montana-5 (p = 0.035), micronutrients concentrations were not impacted by the variety and salt concentration (all p > 0.05).

2 • 2 • Soluble Sugars, Starch and Proteins Concentrations

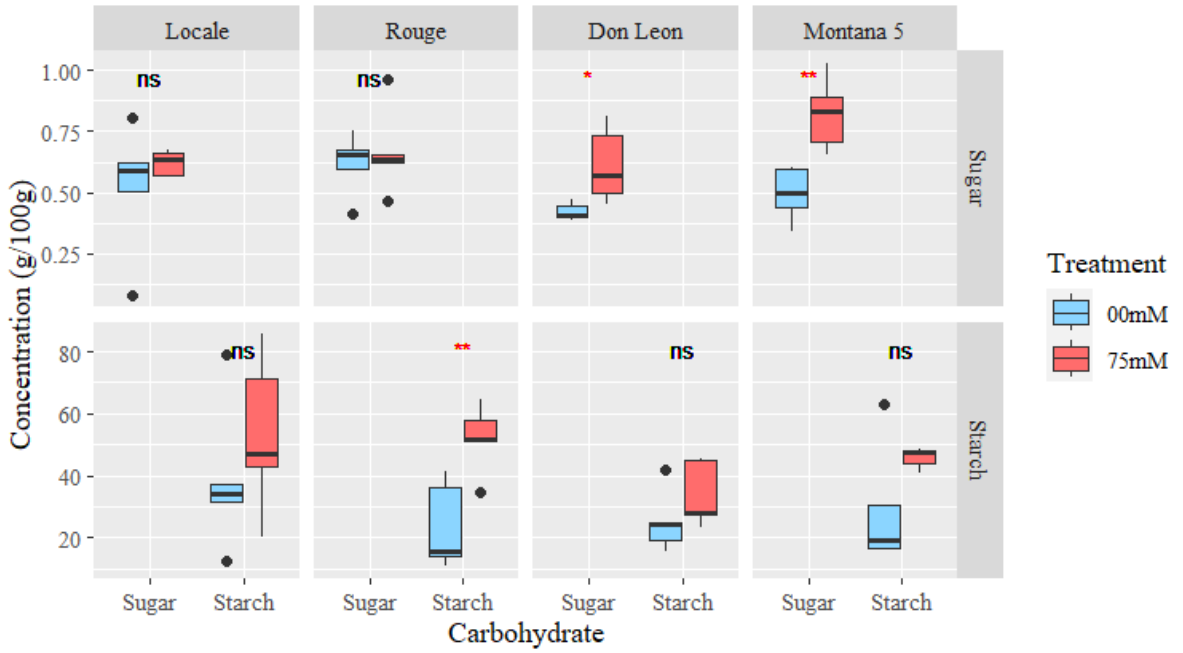

Figure 11: Effect of salinity (0 and 75 mM NaCl) on the total soluble sugar and starch concentration of four *A. cruentus* cultivars expressed in mg.100g^{-1} of dry powdered seeds. Significance is represented using Student's t-test.

Salt stress had an impact on total soluble sugars and starch levels (Figure 11; Appendix 8: p = 0.001 and p = 0.005, respectively). Plant variety and its interaction with salt concentration did not have an impact on sugar and starch concentrations (all p > 0.05).

Intertreatments analysis revealed differences in sugar and starch concentrations for Don Leon, Montana-5 and Rouge. For instance, grain cultivars DL and M5 had higher total soluble sugar concentration under 75 mM (Appendix 9: p = 0.049 and p = 0.005, respectively). On the other end, Rouge showed higher starch levels under salt stress (p = 0.009).

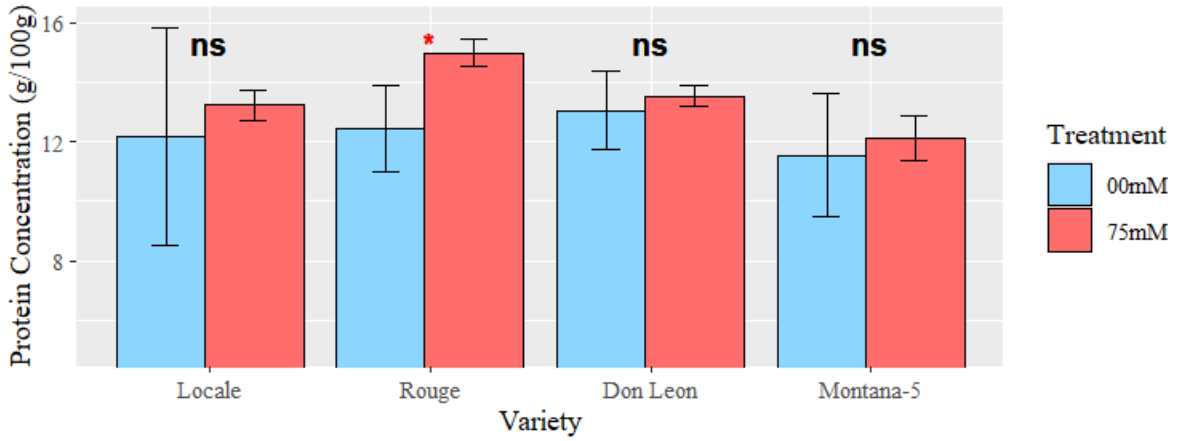

Figure 12: Effect of salinity (0 and 75 mM NaCl) on the total protein concentration of four *A. cruentus* cultivars expressed in $g.100g^{-1}$ of dry powdered seeds. Significance is represented using Student's t-test.

Salt concentration, variety, and the interaction between the two had no impact on total protein concentration (Figure 12; Appendix 8: all $p > 0.05$). However, further analysis between treatments showed a higher protein concentration in Rouge seeds (Appendix 9: $p = 0.017$) under 75 mM NaCl.

Discussion

1 • Impact of Salinity on the Vegetative Phase

Salt stress negatively impacted plant height, leaf production and biomass (Figure 3 and 4). The reduction of both plant growth and overall biomass is a recurring phenomenon observed across diverse plants when exposed to salt, encompassing beans (Delgado *et al.*, 1994), wheat (EL Sabagh *et al.*, 2021), rice (Haq *et al.*, 2009), and tomato (Parvin *et al.*, 2019) to name a few, as well as other *Amaranthus* species (Omamt *et al.*, 2006). However, it is noteworthy that the range of these effects varies upon the developmental stage of the plant (Katerji *et al.*, 2003). Salinity induces a reduction in the water potential (Ψ) of the apoplast, making it more negative than the symplast, resulting in decreased turgor (Ψ_p) and volume (Taiz *et al.*, 2015). Similar to water deficit stress, salinity leads to secondary effects such as the reduction of cellular and metabolic activities, altered carbon partitioning and even leaf abscission (Taiz *et al.*, 2015), inevitably leading to a biomass decrease.

The water content of organs (Figure 5) exhibited minimal impact and demonstrated a trend of slight elevation under salinity stress. Conversely, the water potential (Figure 6) decreased. As previously stated, the osmotic component of salt stress causes water deficits (Taiz *et al.*, 2015). This osmotic stress can manifest at the root level when higher external NaCl concentration reduces the water potential (Shabala and Cuin, 2008: Fig. 1), thereby impeding the absorption of water (AbdElgawad *et al.*, 2016). Water not only serves as a trigger for seed germination in certain plants (Blatt *et al.*, 2014), but is also essential for their growth and proper functioning. Approximately 97% of the water absorbed through the roots will pass through the plant (Xing *et al.*, 2022) to finish transpired from the leaves. The absorbed water serves various functions, including but not limited to photosynthesis, thermoregulation, and the transportation of molecules through osmosis (Taiz *et al.*, 2015). Another important mechanism is osmotic adjustment. This active buildup of cellular solutes (Morgan, 1984) plays a role in resisting osmotic stress (Chen and Jiang, 2010). These solutes can include inorganic ions such as Na^+ and K^+ (Taiz *et al.*, 2015).

While there was sodium accumulation in both leaves and roots, root sodium accumulation was lower compared to the salt-free treatment (Figure 7). Similar observations have been recorded on *A. cruentus* cultivars (Luyckx *et al.*, 2023).

Furthermore, the intracellular accumulation of Na^+ ions exerts an influence on cellular homeostasis, leading to changes in the Na^+/K^+ ratio within cells (Sudhir and Murthy, 2004). While potassium ions are essential for plants (Taiz *et al.*, 2015), sodium, on the other hand, can be harmful if present in high concentrations (Kader *et al.*, 2006). Hence, it is essential for plants to maintain a low

Na^+/K^+ ratio to prevent the interference of Na^+ with enzymatic functions activated by K^+ in cells (Kader *et al.*, 2006; Bhandal and Malik, 1988; Tester, 2003; Munns *et al.*, 2006). Plants exhibit a certain degree of resilience to this stress by reducing the Na^+/K^+ ratio through an increase in potassium content (Munns and Tester, 2008). This aligns with the findings of the present study, which demonstrate a lower Na^+/K^+ ratio in salt-resistant cultivars (Figure 10; Luyckx *et al.*, 2023).

A study on beans (Bayuelo-Jiménez *et al.*, 2003), and tomatoes (Pérez-Alfocea *et al.*, 1996), showed an increase in calcium ions located in the leaves under salt stress. However, another study on the C_4 halophyte *Haloxylon recurvum* (Khan *et al.*, 2000) showed a decrease in calcium ions in roots and shoots under salt stress. This observation aligns with the results of this study (Figure 10) despite being in seeds; however, this was not the case for grain cultivars. Calcium decrease in both leaves and roots for the Locale cultivar under 100-200 mM NaCl has already been observed in another study (Atou *et al.*, 2023). It is plausible that calcium transport mechanisms in grain cultivars are more selective, thus maintaining a normal amount of Ca^{2+} ions in their seeds. For instance, it has been observed that the detrimental effects of salt stress on amaranth seed germination, survival rate, and growth were mitigated through priming with calcium-containing compounds (Omami, 2005). Hence, these Ca^{2+} ions could influence physiological and biochemical mechanisms, mimicking priming effects (Lutts *et al.*, 2016). Calcium ions play a dual role in plant cells; binding between membrane lipids (via their acidic groups) and to pectins for structural purposes, while also serving as secondary messengers by initiating plant responses to surrounding stimuli (Taiz *et al.*, 2015).

Copper concentration in seeds, on the other hand, was reduced under 75 mM NaCl (Figure 10). A previous study (Loudari *et al.*, 2020) showed an increase in copper levels in the aerial parts of tomatoes when exposed to salt stress. These ions and their uptake could play a role in the protective response of amaranth to soil salinity stress, thus leading to reduced levels in seeds. In fact, copper has been shown to decrease lipid peroxidation through the accumulation of phenolic compounds (Hejazi-Mehrizi *et al.*, 2012) and mitigate damages caused by salt stress (Pérez-Labrada *et al.*, 2019; Hernández-Hernández *et al.*, 2018; Iqbal *et al.*, 2018). Nevertheless, a previous investigation into the copper concentration within Locale and Rouge leaves failed to discern any significant disparity across a range of salt stress concentrations ranging from 0 to 90 mM (Luyckx *et al.*, 2021). It is interesting to note that this absence of copper variation may be attributed solely to the limited stress duration, lasting a mere two weeks (Lichtenthaler, 1998). Nonetheless, copper ions play a role in redox reactions through the binding to enzymes, such as plastocyanin in this case, which is involved in the electron transfer chain of light-dependent reactions (Taiz *et al.*, 2015).

Photosynthesis-irradiance curves showed a decline in photosynthetic rate under salt treatment with an incremental increase in irradiance (Figure 9, **A**). Notably, the rate declined at lower irradiance for leafy cultivars and decreased after higher irradiance for grain cultivars. However, definitive

conclusions cannot be drawn due to the limited sample size (n=1). The minor response variations between different salt concentrations on the measured photosynthetic parameters may suggest that stressed plants can maintain an almost normal level of photosynthesis. Furthermore, this observation would provide additional confirmation of the robust resistance of amaranth to salt stress. Luyckx *et al*. (2023) reported sustained photosynthetic activity in two *A. cruentus* cultivars (K91 and Red Amaranth), notwithstanding the accumulation of foliar sodium; this sodium content was linked to salt stress ranging from 0 to 100 mM NaCl.

A/Ci curves revealed that stressed plants were not utilizing CO_2 for photosynthesis as efficiently as non-stressed plants (Figure 9, **B**). These photosynthetic observations could be linked to stomatal closure, as suggested by a study on the Locale cultivar (Gandonou *et al.*, 2018). They could also be attributed to photorespiration, which limits ROS production (Voss *et al.*, 2013). However, photorespiration is reduced by suppressed oxygenase activity in C_4 plants (Kanai and Edwards, 1999). Other factors such as damage to the photosynthetic machinery (Ball *et al.*, 1987), or nutrient limitations (Underwood and Kromkamp, 1999) could also account for these observations. For instance, iron is crucial for photosynthesis and respiration as it serves as cofactor for proteins involved in those metabolic processes (Walker and Connolly, 2008; Taiz *et al*., 2015).

Upon comparing leafy and grain cultivars, the latter exhibited less impact on their leaf production (Figure 3). Since *A. cruentus* grain cultivars did not exhibit axillary stem growth under both conditions, it is plausible that salt stress has a less pronounced impact on the main stem. Their measured dry mass, encompassing leaves, stems, and roots, consistently exceeded those of leafy cultivars despite varying treatments (Figure 4). Further analysis of the reproductive stage could help elucidate whether this biomass accumulation serves as a strategy to ensure a high rate of viable seed production. These observations do not align with findings from previous studies (Cardoso de Lima, 2023; Kudryk, 2022) on these four cultivars and may be attributed to the duration of the salinity stress (DiCara and Gedan, 2023). Water content and water potential (Figure 5 and 6) did not reveal discernible trends based on cultivar type. No consistent response trend to the treatment was observed in the mineral profiles between leafy and grain cultivars. Similar observations have already been made (Luyckx *et al.*, 2023).

Upon comparing salt-sensitive and salt-tolerant cultivars, no apparent trends were observed. Although Rouge height and leaf production (Figure 3) appeared to be as negatively impacted as both salt-sensitive cultivars (LO and M5) under salt stress, other measured parameters did not provide insights to suggest salt tolerance when compared to salt-sensitive cultivars. Don Leon, on the other hand, exhibited minimal impact on the morphological parameters, while showing minimal to no differences on the remaining parameters when compared to the other cultivars. One key determinant of salt tolerance is the capacity to uphold an elevated K^+/Na^+ ratio (Shabala and Cuin, 2008). This

ratio was found to be similar in all cultivars (Figure 7) at the leaf and root level, while being greater at the seed level of Rouge and Don Leon (Figure 10).

2 • Impact of Salinity on Seed Nutritional Quality

A concentration of 75 mM NaCl resulted in a decrease in calcium and copper concentrations in the seeds, while elevating sodium and potassium (Figure 10). The following table (Table 2) gives an overview of recommended dietary allowance for the nutrients that were measured in the seeds of *A. cruentus*.

		K	Na	Ca	Mg	Fe	Cu	Zn	Carbohydrates	Proteins
RDA (mg/d)	**Adult ♂**	3400	1500	1000 **a**	400 **a**	8	0.9	11	130 000	800 **c**
	Adult ♀	2600			310 **a**	18 **b** 8		8		
References		NASEM, 2019		IM, 2011	IM, 1997	IM, 2001			IM, 2005	
100g of *A. cruentus* seeds under 75 mM NaCl in % of the above RDA		2.26	0.29	1.96	8.35	7.00	12.22	2.00		
		2.95			10.78	3.11 7.00		2.75		

Table 2: Recommended dietary allowance (RDA) of nutrients for adults (>18 years old) expressed in mg per day. (**a**): changes with decades; (**b**): for women aged between 19 and 50 y.o.; (**c**): per kg of body mass. Carbohydrates include both sugar and starch. Alongside is the RDA percentage covered by 100g of *A. cruentus* seeds (all four cultivars mixed) from this study that underwent 75 mM NaCl, while mimicking the above layout.

When compared to whole flour of rye, spelt and wheat (Appendix 11), *A. cruentus* whole flour, from which the plants underwent 75 mM NaCl, exhibited near 50% lower calcium content and up to 94% lower zinc content; the remaining values falling within this range. However, sodium, despite a high standard deviation, exhibited a concentration approximately 8 times higher, on average, than that found in the other whole flours. Interestingly, the plants from which rye, spelt and wheat values come from were not exposed to any stress (Ertl and Goessler, 2018). As a matter of fact, increase in sodium, potassium and calcium was observed at the seed level in wheat that underwent soil salinity (Tareq *et al.*, 2011). Additionally, iron, magnesium and zinc contents were decreased (Nadeem *et al.*, 2020). These minerals are necessary in humans for the optimal functioning of both the innate and adaptive immune systems (Weyh *et al.*, 2022).

The concentrations of soluble sugars were notably higher in the seeds of grain cultivars subjected to 75 mM NaCl (Figure 11). Despite this observation, the study from which the seeds were harvested (Cardoso de Lima, 2023) did not reveal a discernible disparity in soluble sugar content within the leaves under conditions of 0 or 75 mM NaCl. However, comparable findings for which increase in sugar have been reported on sorghum seeds, where salt stress was directly applied to the seeds (Gill *et al.*, 2003). At the whole plant level, sugar accumulation is often a protective (Parvaiz and Satyawati, 2008) response to salinity (Ashraf and Harris, 2004; Ashraf and Tufail, 1995; Parida *et al.*, 2002). A discernible elevation in starch concentration was exclusively noted in Rouge cultivar under 75 mM

NaCl, in comparison to its respective control counterpart (Figure 11). A study on rice (Thitisaksakul *et al.*, 2015) also demonstrated an augmentation in grain starch, implying a stimulating effect of salt on the accumulation of starch. Starch is the main component in *Amaranthus* seeds (Malik *et al.*, 2023; Schnetzler, 2018; USDA, 2019). Its level is known to vary in response to abiotic stress and does not always result in lower concentration (Hasan *et al.*, 2023) under the influence of such stresses. This carbohydrate is essential for the plant and its offspring (Rolletschek *et al.*, 2004), mainly when abiotic stresses are present (Thalmann and Santelia, 2017).

Total protein content was solely slightly higher in Rouge seeds under salt stress when compared to 0 mM (Figure 12). A previous investigation on leaf protein content in Rouge and Locale cultivars (Luyckx *et al.*, 2021) consistently revealed a higher concentration of proteins in the Locale cultivar compared to the Rouge cultivar, irrespective of the salt stress levels ranging from 0 to 90 mM. The accumulation of proteins in plants cultivated under NaCl stress may serve as nitrogen reservoir and utilized upon stress cessation (Zhang *et al.*, 2013). Essential for growth, maintenance, and physiological processes, proteins are vital in the human body (Boye *et al.*, 2012). Amino acids contribute significantly to muscle and organ synthesis/functioning, alongside enzymes, and last but not least, the immune system (Wu, 2009). Despite plant based proteins being less digestible than animal based proteins (Berrazaga *et al.*, 2019), choosing plant-based foods that are rich in nutrients and reducing the consumption of animal products may promote better health (Thompson *et al.*, 2023). When comparing the protein content of amaranth seeds, it surpasses that of other crops such as corn and rice (Soriano-García and Saraid Aguirre-Díaz, 2020); these two being among the most consumed cereals on the planet (Muthayya *et al.*, 2014; Ranum *et al.*, 2014).

Conclusions and Perspectives

The decrease in biomass and photosynthetic efficiency under salt stress manifested beyond salt-sensitive cultivars. Ultimately, the nutritional profile of salt-resistant cultivars under 75 mM NaCl exhibited negative impacts comparable to those observed in salt-sensitive cultivars.

Despite showing various impacts on both the vegetative phase and the seed composition, *A. cruentus* remains a promising crop when exposed to salt stress. Indeed, every cultivar exhibited resilience by successfully enduring salt stress conditions, notwithstanding the discernible morphological and physiological alterations observed.

These physiological alterations in response to soil salinity encompass trade-offs. To ensure a successful reproduction, the genetically determined development of plants must be altered, ultimately resulting in a compromise manifested through reduced yield and/or smaller seeds (Taiz *et al*., 2015). Prolonged exposure to salt may induce heritability of acclimation through epigenetic mechanisms without modifying the genetic code (Taiz *et al*., 2015).

Therefore, assessing the nutritional composition of seeds subjected to a 75 mM NaCl treatment and their subsequent generations exposed to the same salt concentration holds the potential to provide valuable genetic and physiological insights.

Interestingly, priming of amaranth seeds in combination with sustained calcium supplementation (Omami, 2005) throughout the entire developmental stages of the plant may further contribute in understanding the key mechanisms involved in the high survival rate of this plant species when exposed to abiotic stresses, particularly salt stress.

The experiment was conducted under semi-controlled greenhouse conditions, characterized by a limited presence of insect life and a restricted diversity of microorganisms at the root level of the investigated plants. It is conceivable that plants subjected to analogous conditions in open-field settings may exhibit better suitability across successive generations. Furthermore, the potential presence of mutualistic endophytes (de la Rosa *et al.*, 2022; Uppala *et al.*, 2010; Parra-Cota *et al.*, 2014) and/or arbuscular mycorrhizal fungi (AMF) inoculations (Akhter *et al.*, 2011) could enhance both the survivability and yield of amaranth species when exposed to salt stress.

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The perfecting of this work involved employing advanced language techniques to enhance the overall linguistic structure through natural language processing. The potential for the inappropriate use of language exists.

Inclusion of errors and mistakes are conceivable occurrences; while y-axis values for carbohydrates and proteins may differ, their proportional representation is accurate.

The interpretation of any idea or sentence, irrespective of contextual considerations, resides exclusively within the purview of the reader. It is important to note that my original ideas may have been misexpressed.

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Appendices

Morphology of *A. cruentus* by the Flora of North America Editorial Committee	
Plants	Nearly hairless or exhibit slight pubescence toward their tips, particularly during their early stages of growth.
Stems	Stand upright, green or reddish-purple, branching toward the top, primarily in inflorescence to almost simple, ranging from 0.4 to 2m in height.
Leaves	Feature petioles roughly half the length of the blade or about equal in length; the blade takes on a rhombic-ovate or ovate to broadly lanceolate shape, measuring 3-15(-20) by 1.5-10(-15) cm, occasionally larger in vigorous plants, with a cuneate to broadly cuneate base, smooth, unbroken margins, and a pointed or slightly blunt, sometimes notched, apex, often with a tiny projection (mucro).
Inflorescences	Typically dark red, purple, or deep beet-red, though occasionally nearly green or greenish-red, are located at the tips and in the axils of the leaves, and they can be erect, reflexed, or nodding, characterized by their large and sturdy appearance, often devoid of leaves toward the upper part.
Bracts	Elongated and spoon-shaped, measuring 2-3mm, about the same length as or slightly longer than the tepals, and they have short, spine-like points at the tips.
Pistillate flowers	Typically feature 5 tepals that are oblong to lanceolate in shape, lacking claws and measuring between 1.5 to 3mm. These tepals are generally equal or nearly equal in size and terminate with a pointed apex. The style branches are typically upright or slightly bent backward, and the flowers possess 3 stigmas.
Staminate flowers	Located at the apex of the inflorescences, featuring 5 tepals and either 4 or 5 stamens.
Utricles	Shaped like obovoid to elongated obovoid structures and measure from 2 to 2.5mm. They exhibit a smooth texture or slight wrinkling towards the distal end, and their regular mode of dehiscence is circumscissile.
Seeds	Appear in shades of white to ivory, occasionally with a reddish or yellowish hue, and on occasion, may be dark brown to dark reddish-brown. They exhibit a broad lenticular to elliptic-lenticular shape, with a diameter ranging from 1.2 to 1.6mm. Their surface is either smooth or shows faint, indistinct punctuations.

Appendix 1: Morphological characteristics of *Amaranthus cruentus* based on the flora of North America editorial committee.

	Compound	Concentration ($g.L^{-1}$)
Hoagland A	NH_4NO_3	8
	$Ca(NO_3).4H_2O$	82.6
	KNO_3	35.7
Hoagland B	KNO_3	5
	KH_2PO_4	27.4
	$MgSO_4.7H_2O$	24.6
	$MnSO_4.5H_2O$	0.053
	H_3BO_3	0.14
	$CuSO_4.5H_2O$	0.015
	$(NH_4)Mo_7O_{24}.4H_2O$	0.008
	$ZnSO_4.7H_2O$	0.06
Fe Solution	Fe EDDHA	1.87

Appendix 2: Composition of Hoagland A, B and Fe Solution.
Hoagland: 125mL of Hoagland A, 125mL of Hoagland B, 125mL of Fe solution, 24.625L of distilled water;
Hoagland + NaCl: 125mL of Hoagland A, 125mL of Hoagland B, 125mL of Fe solution, 468mL of NaCl (75mM) solution and 24.157L of distilled water.

	Height						Leaf Production					
	term	df	sumsq	meansq	statistic	p.value	term	df	sumsq	meansq	statistic	p.value
Day 0	Var	3	10.62275	3.5409167	14.17075204	0.000005	Var	3	29.90	9.97	4.20E+01	0.000000
	Treatment	1	0.11025	0.11025	0.44122061	0.511292	Treatment	1	0.00	0.00	3.12E-29	1.000000
	Var:Treatment	3	1.27475	0.4249167	1.70051693	0.186596	Var:Treatment	3	0.40	0.13	5.61E-01	0.644386
	Residuals	32	7.996	0.249875			Residuals	32	7.60	0.24		
Day 7	Var	3	193.43475	64.47825	55.90397746	0.000000	Var	3	35.40	11.80	4.50E+01	0.000000
	Treatment	1	2.16225	2.16225	1.87471551	0.180470	Treatment	1	3.60	3.60	1.37E+01	0.000800
	Var:Treatment	3	7.32875	2.4429167	2.11805932	0.117386	Var:Treatment	3	0.20	0.07	2.54E-01	0.857922
	Residuals	32	36.908	1.153375			Residuals	32	8.40	0.26		
Day 14	Var	3	304.734	101.578	51.0506345	0.000000	Var	3	86.48	28.83	6.78E+01	0.000000
	Treatment	1	57.6	57.6	28.94836035	0.000007	Treatment	1	18.23	18.23	4.29E+01	0.000000
	Var:Treatment	3	0.174	0.058	0.02914939	0.993161	Var:Treatment	3	0.48	0.16	3.73E-01	0.773341
	Residuals	32	63.672	1.98975			Residuals	32	13.60	0.43		
Day 21	Var	3	596.66475	198.88825	71.52645538	0.000000	Var	3	59.08	19.69	8.20E+00	0.000343
	Treatment	1	242.55625	242.55625	87.23083839	0.000000	Treatment	1	65.03	65.03	2.71E+01	0.000011
	Var:Treatment	3	6.63875	2.2129167	0.79583427	0.505292	Var:Treatment	3	37.88	12.63	5.26E+00	0.004594
	Residuals	32	88.98	2.780625			Residuals	32	76.80	2.40		
Day 28	Var	3	768.79475	256.2649167	68.47883403	0.000000	Var	3	1,439.88	479.96	1.48E+02	0.000000
	Treatment	1	228.96225	228.96225	61.18304496	0.000000	Treatment	1	265.23	265.23	8.19E+01	0.000000
	Var:Treatment	3	7.23075	2.41025	0.6440644	0.592377	Var:Treatment	3	137.28	45.76	1.41E+01	0.000005
	Residuals	32	119.752	3.74225			Residuals	32	103.60	3.24		
Day 35	Var	3	368.07875	122.6929167	14.22155573	0.000005	Var	3	2,967.30	989.10	2.02E+02	0.000000
	Treatment	1	487.90225	487.90225	56.55362369	0.000000	Treatment	1	577.60	577.60	1.18E+02	0.000000
	Var:Treatment	3	6.14475	2.04825	0.23741633	0.869603	Var:Treatment	3	257.80	85.93	1.75E+01	0.000001
	Residuals	32	276.072	8.62725			Residuals	32	156.80	4.90		
Day 41	Var	3	313.30675	104.4355833	6.48617861	0.001486	Var	3	5,136.60	1,712.20	2.35E+02	0.000000
	Treatment	1	1213.30225	1213.30225	75.35453769	0.000000	Treatment	1	1,232.10	1,232.10	1.69E+02	0.000000
	Var:Treatment	3	58.10075	19.3669167	1.20282069	0.324493	Var:Treatment	3	790.10	263.37	3.62E+01	0.000000
	Residuals	32	515.24	16.10125			Residuals	32	232.80	7.28		

Appendix 3a: Anova results using height and leaf production as dependent variable, and using the interaction between treatment and variety as explanatory variables.

Height

Day	Variety	PValue[, 1]	Day	Variety	PValue[, 1]	Day	Variety	PValue[, 1]	Day	Variety	PValue[, 1]
0	Locale	0.0704839969	14	Locale	**0.0033960523**	28	Locale	**0.0047522076**	41	Locale	**0.0244820473**
	Rouge	0.7379975476		Rouge	0.1910681524		Rouge	**0.0120566821**		Rouge	**0.0009576779**
	Montana-5	0.2321973889		Montana-5	0.0600841888		Montana-5	**0.0077664508**		Montana-5	**0.0061189472**
	Don Leon	0.2357131366		Don Leon	0.0640480297		Don Leon	0.0761043956		Don Leon	**0.0132636406**
7	Locale	0.1368211693	21	Locale	**0.0326529053**	35	Locale	**0.0308403079**			
	Rouge	0.2652638898		Rouge	**0.0233905408**		Rouge	**0.0064257716**			
	Montana-5	0.2042330867		Montana-5	**0.0026734057**		Montana-5	**0.0021577357**			
	Don Leon	0.2977954937		Don Leon	**0.0159916724**		Don Leon	**0.0172273174**			

Leaf Production

Day	Variety	PValue[, 1]	Day	Variety	PValue[, 1]	Day	Variety	PValue[, 1]	Day	Variety	PValue[, 1]
0	Locale	0.373900966	14	Locale	**0.024896163**	28	Locale	**0.001733213**	41	Locale	**0.001016663**
	Rouge	0.373900966		Rouge	**0.004635839**		Rouge	**0.019301878**		Rouge	**0.004000954**
	Montana-5	0.621308295		Montana-5	**0.002837846**		Montana-5	**0.031229814**		Montana-5	**0.001348171**
	Don Leon	0.621308295		Don Leon	0.089009343		Don Leon	0.476620667		Don Leon	0.476620667
7	Locale	0.177807808	21	Locale	**0.025481481**	35	Locale	**0.004515429**			
	Rouge	0.070483997		Rouge	0.105463873		Rouge	**0.005129326**			
	Montana-5	**0.01613009**		Montana-5	0.077741641		Montana-5	**0.003939629**			
	Don Leon	0.070483997		Don Leon	1		Don Leon	0.467604755			

Appendix 3b: Student's t-test results for height and leaf production comparing the two treatment groups per day.

variable	term	df	sumsq	meansq	statistic	p.value
WDLT	Var	3	11.2082	3.7360667	15.5243603	**0.000008**
	Treatment	1	32.8455125	32.8455125	136.4819246	**0.000000**
	Var:Treatment	3	1.2136375	0.4045458	1.6809966	0.197653
	Residuals	24	5.7758	0.2406583		
WDS	Var	3	19.1410375	6.3803458	33.5234248	**0.000000**
	Treatment	1	30.0700125	30.0700125	157.9929725	**0.000000**
	Var:Treatment	3	0.7676375	0.2558792	1.3444328	0.283481
	Residuals	24	4.5678	0.190325		
WDR	Var	3	8.2580344	2.7526781	16.0909656	**0.000006**
	Treatment	1	17.3019031	17.3019031	101.1394411	**0.000000**
	Var:Treatment	3	0.4006094	0.1335365	0.7805964	0.516389
	Residuals	24	4.105675	0.1710698		
WaterContentLeaf	Var	3	6.0593035	2.0197678	2.2792487	0.105129
	Treatment	1	7.1221381	7.1221381	8.0371239	**0.009153**
	Var:Treatment	3	0.8281506	0.2760502	0.3115145	0.816848
	Residuals	24	21.2677218	0.8861551		
WaterContentStems	Var	3	1.3777366	0.4592455	0.2683842	0.847525
	Treatment	1	58.0838709	58.0838709	33.944355	**0.000005**
	Var:Treatment	3	4.8951224	1.6317075	0.9535738	0.430546
	Residuals	24	41.0675914	1.7111496		
WaterPot	Var	3	21.6642	7.2214	1.989747	0.130968
	Treatment	1	644.7468	644.7468	177.650211	**0.000000**
	Var:Treatment	3	16.8234	5.6078	1.545144	0.217732
	Residuals	40	145.1722	3.629305		

Appendix 4: Anova results using plant organs dry mass (Leaves: WDLT, Stems: WDS and Roots: WDR) as well as leaf and stem water content and water potential as dependent variable, and using the treatment and variety as well as their interaction as explanatory variables.

Seeds

variable	term	df	sumsq	meansq	statistic	p.value
Ca	Var	3	1.65E+03	5.50E+02	23.445722	0.000000
	Treatment	1	7.71E+02	7.71E+02	32.896304	0.000002
	Var:Treatment	3	3.14E+02	1.05E+02	4.462592	0.009969
	Residuals	32	7.50E+02	2.34E+01		
Cu	Var	3	6.51E-03	2.17E-03	1.5350626	0.224358
	Treatment	1	3.87E-02	3.87E-02	27.3428473	0.000010
	Var:Treatment	3	2.85E-03	9.50E-04	0.6723542	0.575305
	Residuals	32	4.52E-02	1.41E-03		
Fe	Var	3	5.38E-01	1.79E-01	4.8718635	0.006672
	Treatment	1	3.63E-02	3.63E-02	0.985719	0.328240
	Var:Treatment	3	2.08E-02	6.92E-03	0.188051	0.903751
	Residuals	32	1.18E+00	3.68E-02		
K	Var	3	8.35E+02	2.78E+02	3.267297	0.033870
	Treatment	1	2.39E+03	2.39E+03	27.9848077	0.000009
	Var:Treatment	3	3.34E+02	1.11E+02	1.305827	0.289511
	Residuals	32	2.73E+03	8.52E+01		
Mg	Var	3	1.24E+03	4.13E+02	14.6238185	0.000004
	Treatment	1	3.29E+02	3.29E+02	11.6415181	0.001765
	Var:Treatment	3	9.94E+01	3.31E+01	1.1738562	0.335040
	Residuals	32	9.04E+02	2.82E+01		
Na	Var	3	6.13E+01	2.04E+01	2.9413861	0.047888
	Treatment	1	1.71E+02	1.71E+02	24.5468987	0.000023
	Var:Treatment	3	6.08E+01	2.03E+01	2.9135231	0.049339
	Residuals	32	2.22E+02	6.95E+00		
Zn	Var	3	1.09E+00	3.65E-01	1.2168101	0.319513
	Treatment	1	8.06E-01	8.06E-01	2.6919874	0.110648
	Var:Treatment	3	1.12E+00	3.75E-01	1.2507776	0.307726
	Residuals	32	9.59E+00	3.00E-01		

Leaves

variable	term	df	sumsq	meansq	statistic	p.value
K	Var	3	1.73E+04	5.75E+03	0.300224	0.824889
	Treatment	1	4.85E+04	4.85E+04	2.528478	0.124896
	Var:Treatment	3	3.23E+04	1.08E+04	0.562419	0.645014
	Residuals	24	4.60E+05	1.92E+04		
Na	Var	3	6.79E+01	2.26E+01	6.4581133	0.002320
	Treatment	1	4.55E+02	4.55E+02	129.6501258	0.000000
	Var:Treatment	3	5.97E+01	1.99E+01	5.6799786	0.004369
	Residuals	24	8.41E+01	3.51E+00		

Roots

variable	term	df	sumsq	meansq	statistic	p.value
K	Var	3	8.25E+01	2.75E+01	0.01177953	0.998200
	Treatment	1	2.26E+05	2.26E+05	96.6346333	0.000000
	Var:Treatment	3	7.77E+03	2.59E+03	1.10983416	0.364543
	Residuals	24	5.60E+04	2.33E+03		
Na	Var	3	4.90E+04	1.63E+04	6.14158793	0.002990
	Treatment	1	7.35E+05	7.35E+05	276.309602	0.000000
	Var:Treatment	3	4.89E+04	1.63E+04	6.12242145	0.003036
	Residuals	24	6.39E+04	2.66E+03		

Appendix 5: Anova results using mineral concentration of plant organs (Seeds, Leaves and Roots) as dependent variable, and using the treatment and variety as well as their interaction as explanatory variables.

Seeds

Var	Mineral	PValue[, 1]	Var	Mineral	PValue[, 1]
LO	Ca	0.009816084	M5	Ca	0.3553217
	Cu	0.117219374		Cu	0.034973548
	Fe	0.220481405		Fe	0.760070868
	K	0.048185495		K	0.033161153
	Mg	0.190536216		Mg	0.513499242
	Na	0.045716182		Na	0.034179987
	Zn	0.28726599		Zn	0.764292658
RO	Ca	0.037143282	DL	Ca	0.074125072
	Cu	0.106570392		Cu	0.043136867
	Fe	0.900734264		Fe	0.744115052
	K	0.260960841		K	0.023853129
	Mg	0.063440923		Mg	0.147448114
	Na	0.14362203		Na	0.03265724
	Zn	0.218715918		Zn	0.108602173

Leaves

Var	Mineral	PValue[, 1]
LO	K	0.272738903
	Na	0.111714152
RO	K	0.607684914
	Na	0.00553764
M5	K	0.30188787
	Na	0.004257102
DL	K	0.784519503
	Na	0.013072291

Roots

Var	Mineral	PValue[, 1]
LO	K	0.0008457961
	Na	0.0001553024
RO	K	0.0113512422
	Na	0.0314117998
M5	K	0.0101262094
	Na	0.0008622233
DL	K	0.0297741105
	Na	0.0008373065

Appendix 6: Student's t-test results using mineral concentration of plant organs (Seeds, Leaves and Roots) comparing the two treatment groups as dependent variables.

Nitrogen Content

variable	term	df	sumsq	meansq	statistic	p.value	variable	term	df	sumsq	meansq	statistic	p.value
Day 7	Var	3	192.03675	64.01225	21.094826	0.000000	Day 28	Var	3	178.58475	59.52825	3.896976	0.017617
	Treatment	1	6.80625	6.80625	2.242956	0.144024		Treatment	1	89.70025	89.70025	5.872165	0.021216
	Var:Treatment	3	9.62075	3.206917	1.056819	0.381061		Var:Treatment	3	46.08875	15.362917	1.005723	0.402952
	Residuals	32	97.104	3.0345				Residuals	32	488.816	15.2755		
Day 14	Var	3	269.035	89.678333	5.705182	0.003025	Day 35	Var	3	258.20275	86.067583	5.160507	0.005053
	Treatment	1	46.656	46.656	2.968175	0.094570		Treatment	1	147.84025	147.84025	8.864321	0.005504
	Var:Treatment	3	60.18	20.06	1.276183	0.299186		Var:Treatment	3	72.00675	24.00225	1.439146	0.249643
	Residuals	32	503	15.71875				Residuals	32	533.7	16.678125		
Day 21	Var	3	196.226	65.408667	3.030617	0.043533	Day 41	Var	3	264.04875	88.01625	4.409936	0.010504
	Treatment	1	216.225	216.225	10.018476	0.003391		Treatment	1	289.98225	289.98225	14.529170	0.000593
	Var:Treatment	3	100.741	33.580333	1.555897	0.219211		Var:Treatment	3	117.93075	39.31025	1.969587	0.138356
	Residuals	32	690.644	21.582625				Residuals	32	638.676	19.958625		

Nitrogen Concentration

Day	Var	PValue[, 1]	Day	Var	PValue[, 1]	Day	Var	PValue[, 1]
7	LO	0.61297612	21	LO	0.06579227	35	LO	0.84958658
	RO	0.46563121		RO	0.47947501		RO	0.3937449
	M5	0.10298886		M5	0.69154635		M5	0.1032403
	DL	0.05125294		DL	0.01061327		DL	0.0166703
14	LO	0.07987872	28	LO	0.06568166	41	LO	0.29914875
	RO	0.72123372		RO	0.72805984		RO	0.06062817
	M5	0.51129029		M5	0.16892321		M5	0.78579927
	DL	0.43817558		DL	0.57648011		DL	0.01089977

Appendix 7: Anova results using plant leaf nitrogen concentration (top) as dependent variable, and using the treatment and variety as well as their interaction as explanatory variables. Student's t-test results (bottom).

Seeds anova Results

variable	term	df	sumsq	meansq	statistic	p.value
Sugar	Var	3	0.1324999	0.04416662	1.935582	0.1436755
	Treatment	1	0.2787532	0.27875318	12.21623	0.0014110
	Var:Treatment	3	0.1110283	0.03700945	1.621922	0.2036664
	Residuals	32	0.7301845	0.02281827		
Starch	Var	3	7.300289	2.4334297	1.624743	0.2044165
	Treatment	1	14.075031	14.0750309	9.397563	0.0045675
	Var:Treatment	3	2.563543	0.8545144	0.570539	0.6387569
	Residuals	30	44.931959	1.497732		
Proteins	Var	3	0.6115302	0.20384341	1.1292263	0.3570605
	Treatment	1	0.3701586	0.37015862	2.0505586	0.1650463
	Var:Treatment	3	0.2192854	0.07309512	0.4049232	0.7508003
	Residuals	24	4.3323839	0.18051599		

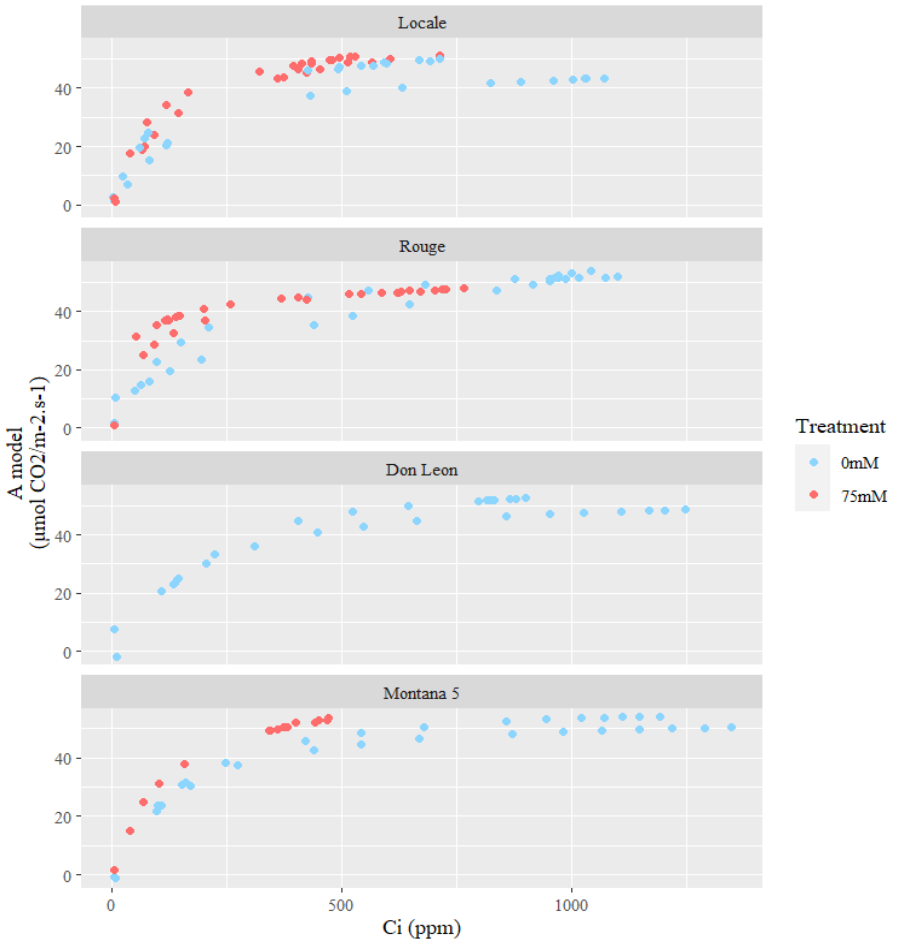
Appendix 8: Anova results using sugar, starch and protein concentration as dependent variable, and using the treatment and variety as well as their interaction as explanatory variables.

WDLT		WDS		WDR	
VARIETY	p-value	VARIETY	p-value	VARIETY	p-value
LO	0.0002	LO	0.0009	LO	0.0018
RO	0.0048	RO	0.0022	RO	0.0216
DL	0.0190	DL	0.0020	DL	0.0001
M5	0.0008	M5	0.0005	M5	0.0104

WaterContentLeaves		WaterContentStems		WaterPotential	
VARIETY	p-value	VARIETY	p-value	VARIETY	p-value
LO	0.5980	LO	0.1707	LO	0.0312
RO	0.3533	RO	0.1277	RO	0.0001
DL	0.0276	DL	0.0022	DL	0.0000
M5	0.0554	M5	0.0088	M5	0.0000

Sugar		Starch		Proteins	
VARIETY	p-value	VARIETY	p-value	VARIETY	p-value
LO	0.4513	LO	0.3849	LO	0.5668
RO	0.6381	RO	0.0085	RO	0.0170
DL	0.0485	DL	0.2120	DL	0.4798
M5	0.0050	M5	0.1353	M5	0.5970

Appendix 9: Student's t-test per variety of dry mass (Leaves: WDLT, Stems: WDS and Roots: WDR), water content (Leaves and Stems), water potential and nutrients concentration (Sugar, Starch and Proteins).



Appendix 10: Modeled [plantecophys::fitaci()] photosynthetic rate (A model) depending on intercellular CO_2 concentration (C_i) for each variety and for each treatment applied. Curves for which modelization failed were removed (19 removed out of 32).

	Rye whole flour (n = 3)	**Spelt whole flour (n = 3)**	**Wheat whole flour (n = 5)**	***A. cruentus* whole flour (n = 20)**
	$x \pm \sigma$	$x \pm \sigma$	$x \pm \sigma$	$x \pm \sigma$
Na (mg/100g)	0.19 ± 0.02	0.73 ± 0.18	0.66 ± 0.14	4.29 ± 4.26
Mg (mg/100g)	100 ± 10	130 ± 10	140 ± 20	33.41 ± 6.38
K (mg/100g)	440 ± 20	360 ± 30	360 ± 60	76.68 ± 12.51
Ca (mg/100g)	37 ± 4	40 ± 1	39 ± 8	19.59 ± 5.96
Fe (mg/100g)	2.3 ± 0.1	3.9 ± 0.2	3.4 ± 0.3	0.56 ± 0.22
Cu (mg/100g)	0.35 ± 0.02	0.49 ± 0.02	0.44 ± 0.06	0.11 ± 0.04
Zn (mg/100g)	3 ± 0.2	4.3 ± 0.3	3.9 ± 0.6	0.22 ± 0.13

Appendix 11: Table based on: 'Table 3: Elemental composition of different grain and flour types expressed on a dry mass basis' from Ertl and Goessler (2018), showing the different types of whole flour and their nutrient composition. *A. cruentus* seeds that underwent 75 mM NaCl in this study (all cultivars mixed) was added in red.

“It is the time you have wasted for your
rose that makes your rose so important”.

taken from

de Saint-Exupéry, A. (**2018**). The Little Prince, Ch. 21 (I. Testot-Ferry, Trans.).
Wordsworth Editions. (Original Work Published in 1943)