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MORPHOMETRIC AND MOLECULAR PROPERTIES OF *Aloe vera* (L.) UNDER WATER DEFICIT AND SALINITY CONDITIONS

PROPIEDADES MORFOMÉTRICAS Y MOLECULARES DE *Aloe vera* (L.) EN CONDICIONES DE DÉFICIT HÍDRICO Y SALINIDAD

THESIS

Submitted as a partial requirement to obtain the degree of:

DOCTOR EN CIENCIAS EN RECURSOS NATURALES Y MEDIO AMBIENTE EN ZONAS ÁRIDAS

By:

MARÍA DEL SOCORRO MOTA ITUARTE

Supervised by: Dr. Aurelio Pedroza Sandoval

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APROBADA

MORPHOMETRIC AND MOLECULAR PROPERTIES OF *Aloe vera* (L.) UNDER WATER DEFICIT AND SALINITY CONDITIONS

Thesis made by MARÍA DEL SOCORRO MOTA ITUARTE under the supervision of the indicated Advisory Committee, approved by the same and accepted as a partial requirement to obtain the degree of:

DOCTOR EN CIENCIAS EN RECURSOS NATURALES Y MEDIO AMBIENTE EN ZONAS ÁRIDAS

DIRECTOR: 

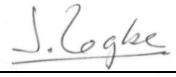
DR. AURELIO PEDROZA SANDOVAL

CO-DIRECTOR: 

DR. JOSÉ RAFAEL MINJARES FUENTES

ADVISOR: 

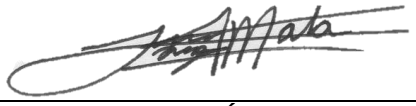
DR. RICARDO TREJO CALZADA

ADVISOR: 

DR. JORGE A. ZEGBE DOMÍNGUEZ

ADVISOR: 

DR. JESÚS JOSAFATH QUEZADA RIVERA

EXTERNAL READER: 

DR. JORGE SÁENZ MATA

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DEDICATED TO

To my parents, for their love, support and effort.

To my brothers and sister, with their presence, support and love encourage me to get ahead.

To my husband, because, as I always tell him, he is a blessing in my life.

To my daughters, because, as I always tell them, I don't know where I would be without them.

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BIOGRAPHICAL DATA

PERSONAL INFORMATION

Name: María del Socorro Mota Ituarte

Date of birth: June 27th, 1986

Place of birth: Ceballos, Durango, México.

CURP: MOIS860627MDGTTC08

ACADEMIC BACKGROUND

Bachelor degree: Ingeniero Industrial at the Instituto Tecnológico Superior de Lerdo. Class 2004-2008.

Master Degree: Ciencias Químicas at the Facultad de Ciencias Químicas of the Universidad Juárez del Estado de Durango. Class 2016-2018.

Scientific development: Author of a scientific article about abiotic stress in plants. Presentations of posters about morphometric and molecular characteristics of plants under stress conditions, in Congress on Biotic Resources of Arid Lands (Universidad Autónoma Chapingo) and Congress of Agrigenomics on engineering, science and industry (Instituto Tecnológico y de Estudios Superiores de Monterrey).

GENERAL ABSTRACT

Morphometric and molecular properties of *Aloe vera* (L.) under water deficit and salinity conditions

Aloe vera (L.) is a well-known plant with valuable properties attributed to the polysaccharides present in its gel, particularly acemannan. However, the structure and composition of these polysaccharides can be influenced by environmental factors prevailing in arid regions. This study aimed to compare the morphometric and molecular behavior of *A. vera* under semi-controlled conditions of shade netting in response to water deficit and salinity. A randomized block design with four replications was used, featuring large plots with two soil moisture contents (20.7% field capacity and 12.3% permanent wilting point) and small plots with five salinity levels (0, 20, 40, 60, and 80 mM NaCl). Results showed a reduction in leaf width and thickness, fresh biomass, and gel percentage, but an improvement in gel quality. Additionally, an RNA extraction method suitable for samples with high polysaccharide content is described. Finally, RT-qPCR (Reverse Transcription Quantitative Polymerase Chain Reaction) analysis revealed a positive regulation of a *glucomannan mannosyltransferase* (*GMMT*) gen of *A. vera* induced by salinity and salt-water stress combination.

Keywords: acemannan, RNA, abiotic stress combination, RT-qPCR.

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Author: M.C. María del Socorro Mota Ituarte
Thesis Director: Dr. Aurelio Pedroza Sandoval

RESUMEN GENERAL

Propiedades morfométricas y moleculares de *Aloe vera* (L.) en condiciones de déficit hídrico y salinidad

El *Aloe vera* (L.) es una de las plantas más renombradas debido a sus propiedades benéficas asociadas a los polisacáridos presentes en el gel, como el acemanano. Propiedades que dependen de la estructura y composición de estos polisacáridos, y que pueden ser afectadas por factores ambientales presentes en zonas áridas. El objetivo de este estudio fue realizar un análisis comparativo del comportamiento de los parámetros morfométricos y moleculares de *A. vera* en respuesta al déficit hídrico y salinidad en condiciones semicontroladas de malla sombra. Se utilizó un diseño de bloques al azar en un arreglo de parcelas subdivididas con cuatro repeticiones, donde las parcelas grandes fueron los contenidos de humedad del suelo: capacidad de campo (20.7 %) y punto de marchitez permanente (12.3 %). Las parcelas pequeñas fueron salinidad a cinco niveles: 0, 20, 40, 60 y 80 mM NaCl. Se encontró una reducción en el ancho y grosor de la hoja, biomasa fresca y porcentaje de gel, pero mejoró la calidad del gel. En cuanto a las propiedades moleculares de *A. vera*, se describe un método de extracción de RNA. Puede utilizarse en muestras con alto contenido en polisacáridos, ya que estos últimos dificultan la extracción de ácidos nucleicos. Finalmente, mediante RT-qPCR (Reverse Transcription - Quantitative Polymerase Chain Reaction) se encontró una regulación positiva de un gen de *glucomanano manosiltransferasa (GMMT)* de *A. vera*, inducida por salinidad, así como por una combinación de estrés hídrico-salino.

Palabras clave: acemanano, RNA, combinación de estrés abiótico, RT- qPCR,

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Director de Tesis: Dr. Aurelio Pedroza Sandoval

CHAPTER I: GENERAL INTRODUCTION

The use of plants in food, medicinal and industrial areas dates back several millennia. Today, strategies are being studied to identify the most effective plant compounds with the highest yields (Akaberi et al., 2016). Such is the case of *Aloe vera* (L.) or aloe vera (*Aloe barbadensis* M.), which is recognized for providing many of health benefits. Since, it is one of the most used herbal remedies in the treatment of different diseases, which has been mainly associated with polysaccharides and phenolic compounds, the main bioactive components present in this plant (Borges-Argáez et al., 2019; Guo & Mei, 2016). Most of the pharmacological properties attributed to *A. vera* can be explained by the storage polymer known as acemannan, which is its key component (Minjares-Fuentes et al., 2017). Its therapeutic uses include arthritis, asthma, candidiasis, intestinal digestive disorders, skin problems, and ulcers (Sahebnasagh et al., 2017). In addition to therapeutic applications, *A. vera* has applications in the cosmetic industry (Djiobie Tchienou et al., 2018), veterinary (Sharma et al., 2018), food industry (Khaliq et al., 2019) and tissue engineering (Liu et al., 2019).

Aloe vera plant has crassulacean acid metabolism (CAM), therefore it is naturally adapted to drought conditions and high temperatures (Delatorre-Herrera et al., 2010). These plant species usually develop in arid and semi-arid regions, which are characterized by frequent periods of drought and intensive use of water in agricultural irrigation areas, causing soil salinity problems (FAO, 2007), as well as heavy metal contamination. The latter is fundamentally related to human activities (Trejo-Calzada et al., 2015). *Aloe vera* or sábila, as it is also known, is being considered an alternative crop in marginal agricultural areas, due to its plasticity to resist various types of stress. For these conditions and their consequences on their properties, *A. vera* has been the subject of different studies (Delatorre-Herrera et al., 2010; Moghbeli et al., 2012; Xu et al., 2015). However, some causal processes that result in a better response in the quantity and quality of the gel production of *A. vera*, which is the main industrially used organic compound, still

need to be explored. Thus, the objective of the present study was to identify the impact of saline and water stress on the polysaccharide content, the morphometric properties of *A. vera*, as well as the genes expression induced by these external abiotic factors.

1.1 Justification

To understand the potential that dry lands offer, it is necessary to identify the specific practices that allow greater yields and benefits in the use of natural resources. In the study area of this project, known as the Comarca Lagunera in the states of Coahuila and Durango, the pressure on water resources has increased in recent decades due to a greater demand in the production of fodder for the dairy industry and other industrial uses. In addition to human consumption, having to satisfy the demand of a population of about 1.3 million people (Estrada-Ávalos et al., 2014). As a consequence, the groundwater withdrawal rate is approximately twice the total annual hydrological recharge. This excessive extraction has led to a reduction in the water table and the deterioration of water quality (CONAGUA, 2020). The use of alternative crops tolerant to these adverse factors, such as *A. vera*, may be a feasible choice for reconversion and productive profitability in arid lands with sustainable production systems.

1.2 General objective

To evaluate the response of growth and productivity of *A. vera* under conditions of water deficit and salinity; also analyze the gene expression involved in the synthesis of acemannan precursors of stressed plants. This to contribute to its positioning as an alternative crop with high potential in arid areas.

1.2.1 Specific objectives

• Identify the behavior of growth and development of the *A. vera* plants under conditions of water deficit in interaction with salinity.

• Optimize an effective RNA extraction procedure for isolation of high quality RNA from *A. vera* tissues that have been subjected to salinity and water deficit.

• Identify the molecular mechanisms involved in the synthesis of acemannan precursors from *A. vera* under water deficit and salinity.

1.3 Hypothesis

The water deficit in interaction with salinity negatively affects the productivity of *A. vera*, in terms of its growth, development, molecular properties of the mucilage and some bioactive compounds, such as acemannan.

1.4 References

Akaberi, M., Sobhani, Z., Javadi, B., Sahebkar, A., & Emami, S. A. (2016). Therapeutic effects of Aloe spp. in traditional and modern medicine: A review. *Biomedicine & Pharmacotherapy*, *84*, 759-772. https://doi.org/https://doi.org/10.1016/j.biopha.2016.09.096

Borges-Argáez, R., Chan-Balan, R., Cetina-Montejo, L., Ayora-Talavera, G., Sansores-Peraza, P., Gómez-Carballo, J., & Cáceres-Farfán, M. (2019). In vitro evaluation of anthraquinones from *Aloe vera* (Aloe barbadensis Miller) roots and several derivatives against strains of influenza virus. *Industrial crops and products*, *132*, 468-475.

CONAGUA. (2020). Comisión Nacional del Agua. Programa Nacional Hídrico 2020-2024. *Diario Oficial de la Federación, Mexico City.*

Delatorre-Herrera, J., Delfino, I., Salinas, C., Silva, H., & Cardemil, L. (2010). Irrigation restriction effects on water use efficiency and osmotic adjustment in *Aloe Vera* plants (*Aloe barbadensis* Miller). *Agricultural Water Management*, *97*(10), 1564-1570. https://doi.org/https://doi.org/10.1016/j.agwat.2010.05.008

Djiobie Tchienou, G. E., Tsatsop Tsague, R. K., Mbam Pega, T. F., Bama, V., Bamseck, A., Dongmo Sokeng, S., & Ngassoum, M. B. (2018). Multi-response optimization in the formulation of a topical cream from natural ingredients [Article]. *Cosmetics*, *5*(1), Article 7. https://doi.org/10.3390/cosmetics5010007

Estrada-Ávalos, J., Delgado-Ramírez, G., Palomo-Rodríguez, M., Rivera-González, M., Jacobo-Salcedo, M. d. R., & Vilanueva-Díaz, J. (2014). La cuenca del río Nazas en el norte de México: condición e iniciativas en la conservación de sus recursos naturales. *Agrofaz: publicación semestral de investigación científica*, *14*(2), 123-135.

FAO. (2007). Secuestro de carbono en tierras áridas. *Organización de las Naciones Unidas para la Agricultura y la Alimentación*.

Guo, X., & Mei, N. (2016). *Aloe vera*—a review of toxicity and adverse clinical effects. J. *Environ. Sci. Health C Environ. Carcinog. Ecotoxicol. Rev.*, 77–96.

Khaliq, G., Ramzan, M., & Baloch, A. H. (2019). Effect of *Aloe vera* gel coating enriched with Fagonia indica plant extract on physicochemical and antioxidant activity of sapodilla fruit during postharvest storage [Article]. *Food chemistry*, *286*, 346-353. https://doi.org/10.1016/j.foodchem.2019.01.135

Liu, X., You, L., Tarafder, S., Zou, L., Fang, Z., Chen, J., Zhang, Q. (2019). Curcumin-releasing chitosan/aloe membrane for skin regeneration. *Chemical Engineering Journal*, *359*, 1111-1119. https://doi.org/10.1016/j.cej.2018.11.073

Minjares-Fuentes, R., Rodríguez-González, V. M., González-Laredo, R. F., Eim, V., González-Centeno, M. R., & Femenia, A. (2017). Effect of different drying procedures on the bioactive polysaccharide acemannan from Aloe vera (Aloe barbadensis Miller). *Carbohydrate Polymers*, *168*, 327-336. https://doi.org/https://doi.org/10.1016/j.carbpol.2017.03.087

Moghbeli, E., Fathollahi, S., Salari, H., Ahmadi, G., Saliqehdar, F., Safari, A., & Hosseini Grouh, M. S. (2012). Effects of salinity stress on growth and yield of Aloe vera L. *Journal of Medicinal Plants Research*, *6(16)*, 3272-3277. https://doi.org/10.5897/JMPR11.1698

Sahebnasagh, A., Ghasemi, A., Akbari, J., Alipour, A., Lashkardoost, H., Ala, S., & Salehifar, E. (2017). Successful treatment of acute radiation proctitis with aloe vera: a preliminary randomized controlled clinical trial, . *J. Alternative Compl. Med. 23 (11) (2017 28618234*, 858–865. https://doi.org/https://doi.org/10.1089/acm.2017.0047

Sharma, S., Singh, D. k., Gurung, Y. B., Shrestha, S. P., & Pantha, C. (2018). Immunomodulatory effect of Stinging nettle (Urtica dioica) and Aloe vera (Aloe barbadensis) in broiler chickens. *Veterinary and Animal Science*, *6*, 56-63. https://doi.org/10.1016/j.vas.2018.07.002

Trejo-Calzada, R., Pedroza-Sandoval, A., Reveles-Hernández, M., Ruiz-Torres, J., & Arreola-Ávila, J. (2015). Especies vegetales de zonas áridas para la fitorremediación de suelos contaminados con metales pesados. In (pp. 87-104).

Xu, C. X., Ma, Y. P., & Liu, Y. L. (2015). Effects of silicon (Si) on growth, quality and ionic homeostasis of aloe under salt stress. *South African Journal of Botany*, *98*, 26-36. https://doi.org/https://doi.org/10.1016/j.sajb.2015.01.008

CHAPTER II: THEORETICAL-CONCEPTUAL FRAMEWORK

2.1 *Aloe vera* (*L.)*

Aloe vera (Aloe barbadensis M.) belongs to the group of plants classified as CAM (Crassulaceae acid metabolism), which are commonly known as "succulents" due to the ability to accumulate higher amounts of water inside their leaves and stems. This has been mainly attributed to the presence of different types of polysaccharides, such as glucomannans, and its photosynthetic metabolism that allows greater efficiency in the use of water (Nobel, 1997). This plant belongs to the *Liliaceae* family, and can grow in any soil, but it is best adapted to light, sandy soils and does not require a lot of water (Radha & Laxmipriya, 2015).

2.1.1 Chemical composition

According to Tyler (1993), the *A. vera* leaf is mainly composed of three layers. The cortex, within which the vascular bundles occur as the xylem and phloem. The xylem helps in the transport of water while the phloem is involved in the transport of sucrose and other small organic molecules. The middle layer contains bitter yellow sap with anthraquinones and C-glycosides as the main compounds. *Aloe vera* is composed by more than 70 active components, including anthraquinones, vitamins, minerals, enzymes, lignin, phytosterols, non-essential amino acids, inorganic compounds, and salicylic acids (Minjares-Fuentes et al., 2018; Vogler & Ernst, 1999). Aloin, aloesin, aloenin, aloeresin, aloe-emodin, and chrysophanol are some examples of such bioactive compounds (Amoo et al., 2014). In *A. vera* mucilage there is approximately 98.5 - 99.5% water, and more than 200 different components have been identified in the remaining solids fraction, polysaccharides being the most abundant compound (Femenia et al., 1999).

2.1.2 Beneficial properties

Among the properties of *A. vera*, it has been displayed that chemical compounds, such as aloin, emodin, barbaloin, isobarbaloin, and chrysophanic acid, have antibacterial, fungicidal, diuretic, laxative, antiviral, hepatoprotective, and vasorelaxing activities (Lindsey et al., 2002; Salah et al., 2017). The mucilage is rich in polysaccharides, which are responsible for immunostimulating and anti-inflammatory properties which have been attributed to the occurrence of an acetylated mannose-rich polysaccharide, commonly named as acemannan, followed by pectins present in smaller amounts (Alvarado-Morales et al., 2019; Campestrini et al., 2013; Minjares-Fuentes & Femenia, 2019). It is important to point out that acemannan has been considered as the storage polysaccharide of *A. vera,* located within the protoplast of the parenchymatous tissue (Femenia et al., 1999).

2.1.3 Applications

From the industrial point of view, the use of *A. vera* mucilage is very important in the manufacture of topical ointments, tablets and capsules. In the cosmetics industry, *A. vera* mucilage has been used as a base material for the preparation of creams, lotions, soaps, shampoos, face creams, and a wide variety of toiletries (Domínguez-Fernandez et al., 2011; Pedroza-Sandoval et al., 2015).

One of the uses of *A. vera* mucilage is in the food industry, mainly as a functional ingredient, commonly used for the preparation of healthy food drinks among others (Alvarado-Morales et al., 2019).

Perhaps the most important use of *A. vera* is in the area of medicine. Among the reported medicinal plants, *A. vera* is used as folk medicine throughout the world (Arunkumar & Muthuselvam, 2009). It is traditionally used as a laxative agent and to relieve pain. It is frequently used to treat wounds, sores, rashes, and burns (Wyk et al., 1997). Also, it is used in veterinary medicine with good results, like a study done by Drudi et al. (2018) where they verified the efficiency and speed with which *A. vera* helps to heal wounds in dogs and cats. As well as, the *A. vera* plant has been used successfully in tissue engineering. Liu et al. (2019) found that this

plant promotes the intrinsic regeneration of skin tissues, as it led to accelerated healing and regeneration of skin tissue, in Wistar rats.

2.2 Glucomannans

Glucomannans (GM) are neutral polysaccharides that act as a source of soluble dietary fiber (Tester & Al-Ghazzewi, 2017). They are characterized by having an exceptionally high viscosity, unlike other soluble fibers (McCarty, 2002). These hydrophilic polysaccharides are found in different parts of some plants and have been studied for their desirable quality and health characteristics (Shi et al., 2020). The GM structure shows a backbone mainly composed by glucose (60%) and mannose linked by β–1-4 glycosidic linkage with some galactose units forming side chains. In some cases, mannose from GM is often acetylated. Usually, GM has a high molecular weight, generally of 200-2000 kDa (Khanna, 2003). The most studied GM is that of *Amorphophallus konjac*, its isolation, structural properties, as well as its beneficial effects on health (Cao et al., 2022; Chua et al., 2010; Gille et al., 2011). Acemannan, the storage glucomannan of *A. vera*, has also been the subject of numerous studies due to its potential health benefits. It is a polysaccharide known to be biodegradable, biocompatible, and could also induce a beneficial immune response, making it attractive for numerous biomedical applications (Kumar & Tiku, 2016).

Acemannan contains a unique acetylated mannose as the main sugar residue (Chow et al., 2005; Femenia et al., 1999). The occurrence of acetylated mannose has been considered as a fingerprint from *Aloe* polysaccharides (Minjares-Fuentes et al., 2018). The acetyl groups from acemannan play key role for most of the properties associated with this polymer, enhancing its potential ability to interact with other biomacromolecules (Chokboribal et al., 2015; Kumar & Kumar, 2019).

Studies carried out in this regard indicate that acemannan has a molecular weight in the range of around 40 to 190 kDa (Chokboribal et al., 2015; Femenia et al., 2003; Minjares-Fuentes, Medina-Torres, et al., 2017; Thunyakitpisal et al., 2017). It is composed primarily of a backbone of partially acetylated mannose (>60%)

and glucose (~20%) units with side chains consisting of galactose units (<10%) attached to C-6 mannose (Chokboribal et al., 2015; Femenia et al., 1999; Rodríguez-González et al., 2011; Tai-Nin Chow et al., 2005) as shown in figure 1.

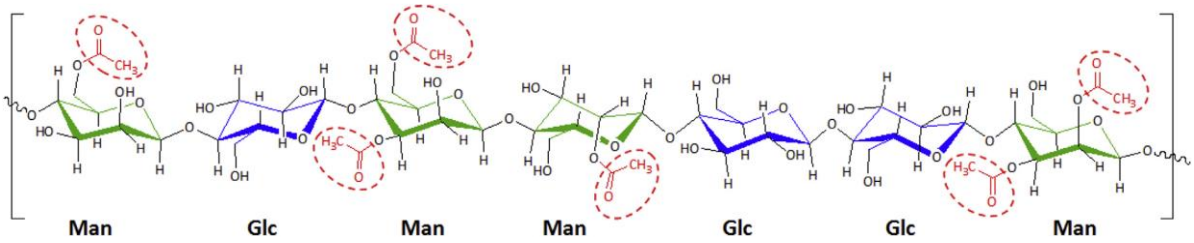

Figure 1. Structure of acemannan (Salinas et al., 2019)

On the other hand, the acemannan content in *A. vera* plants can be affected by various factors, both internal and external. There are few studies that document changes in the architecture and functionality that abiotic stress causes in the acemannan polysaccharide. Minjares-Fuentes, Medina-Torres, et al. (2017) carried out an investigation on the influence of water deficit on the main polysaccharides and rheological properties of *A. vera* mucilages. Their results show that the content and chemical characteristics of the acemannan storage polymer were modified due to water deficit, mannose decreased by 41% and acemannan did not undergo deacetylation, only up to 60% water deficit. They found that the high-water deficit (60%) promoted a significant increase in the dry matter content of *A. vera* mucilage. In accordance with Femenia et al. (2003), most of the dry matter of *A. vera* is made up of polysaccharides. Likewise, the water deficit promoted an apparent increase in the molecular weight of acemannan. Salinas et al. (2019) carried out a study with *A. vera* plants subjected to water stress, their results found an insignificant amount of galactose in the acemannan. This is contrary to what was reported by other authors who refer to the important presence of galactose in the structure of this polysaccharide (Femenia et al., 2003; Minjares-Fuentes, Medina-Torres, et al., 2017; Tai-Nin Chow et al., 2005). The possible explanation for the absence of galactose in acemannan given in the study is that drying methods may affect the degree of

acetylation and the loss of galactose side branches (Minjares-Fuentes, Rodríguez-González, et al., 2017), since the samples were lyophilized. Other variables that can explain the presence or lack of galactose are the age of the plants, the plant tissue from which the gel is obtained and, mainly, the environmental conditions of plant cultivation, including abiotic stress.

Regarding abiotic stress due to salinity, Jiang et al. (2014) found that when *A. vera* is irrigated with seawater, the 42% seawater stress treatment only reduces the concentration of polysaccharides in the base leaves, without decreasing the concentration of polysaccharides in the upper and middle parts. Other factors, in addition to water stress and salinity, that influence the polysaccharide content in *A. vera* are the harvest season and light stress (Lucini et al., 2013; Ray et al., 2015).

2.3 Abiotic stress

Stress in plants is a physiological condition induced by an alteration in the environment or when normal environments are aggressive, altering the physiological and adaptive pattern of plants. In particular, the variation temperature, humidity, aqueous saline solution, soil pH, radiation, and pollutants, such as heavy metals, and mechanical damage are propably the most important factors responsible for the abiotic stress in plants (Ahuja et al., 2010). Plants respond to adverse environmental stressors through physiological, morphologic, biochemical, and molecular variations. For instance, they use different types of phytochemical compounds to overcome biotic stresses (Chinnusamy et al., 2007). Short-term changes in environmental factors can lead to cumulative reactions, while gradual changes can lead to plant adaptation. The response to environmental stressors depends on many factors, such as the type and duration of the stress, as well as the plant species (Bahuguna & Jagadish, 2015).

According to Jaspers and Kangasjärvi (2010), plants usually respond to abiotic stress by producing reactive oxygen species (ROS). The synthesis of ROS alters cellular redox homeostasis and leads to oxidative stress (Asada, 2006). Likewise, the overproduction of ROS can damage cells and cause necrosis in plant species

(Petrov et al., 2015). Nevertheless, plants have developed different defense mechanisms, such as the activation of antioxidant systems which can be mediated by several enzymes such as superoxide dismutase, peroxidase, glutathione peroxidase, catalase, ascorbate peroxidase among others, or by non-enzymatic antioxidants as ascorbate, glutathione and phenolic compounds. Simultaneously, plants accumulate water by synthesizing osmolytes such as oligosaccharides and proline to adjust the osmotic balance (Ma et al., 2016).

Individually, stress conditions such as drought, salinity, heat or cold have been the subject of intense research. However, few studies have been conducted investigating plant responses to environmental stress applied in combination. This research is particularly important since, in nature, simultaneous abiotic stresses are common, such as, heat stress is often accompanied by water deficiency and drought by salinity (Ahuja et al., 2010).

2.3.1 Response to salt stress

Approximately, 19.5% of irrigated agricultural land is considered saline (Dos Reis et al., 2012). Plants under salt stress have less dry matter, leaf area and yield (Amirjani, 2011). It has also been reported by Zadeh (2007) that salinity changes the morphological characteristics of plants. In accordance with FAO (2013), increasing soil salinity, aquifer depletion and land degradation reduce achievable yields, jeopardizing farmers' ability to bridge production gaps and improve food security.

High soil salinity is one of the environmental factors that limits the distribution and productivity of crops, causing yield losses. This is because it reduces the ability of plants to absorb water, leading to a reduction in the growth rate, due to a hormonal signal generated by the roots (Munns, 2002). To combat this problem, an important strategy for the use of salinity-affected lands is salt-tolerant crops. This strategy is considered the most efficient, economical and permanent to the problem (Dos Reis et al., 2012).

Salt stress results in a wide variety of physiological and biochemical changes in plants, such as the activation of salt-inducible genes and the large-scale

production and accumulation of osmolytes. Plants accumulate derivatives of these low molecular weight solutes to mitigate the detrimental effects of salt stress by reducing the water potential of cells (Jha et al., 2011; Kishor et al., 2005). In addition, plant species can reduce the harmful effects of this type of stress through the compartmentalization of toxic ions such as Na^+ in the vacuole and the adjustment of photosynthetic processes (DeFreitas et al., 2018)

In *A. vera,* some studies have been done subjecting it to saline stress (Moghbeli et al., 2012; Murillo-Amador et al., 2015; Xu et al., 2015), finding affectations in the morphometric properties of the plant. However, it is necessary to know how this type of stress affects the structure and composition of its polysaccharides, which are responsible for its medicinal properties.

2.3.2 Response to water deficit

Stress caused by water scarcity has a negative impact on plant products and medicinal plants. Evidently, this stress occurs in arid and semi-arid environments, currently more common due to the phenomenon of global warming. Drought stress can induce biochemical, physiological and genetic responses, depending on the plant species (Zhou et al., 2017).

Drought has several important effects on plants, such as poor seed germination (Farooq et al., 2009), reduced seedling growth (Okçu et al., 2005) and a decrease in nutrient availability and photosynthesis (Manickavelu et al., 2006). In addition, drought can also affect the number of leaves and the size of each leaf, as well as the fresh and dry weight of the plant (Zhao et al., 2006), among other effects.

Water stress increases the synthesis of bioactive molecules to face water scarcity (Khan et al., 2011). Furthermore, this type of stress creates a secondary metabolism, called oxidative stress, which reduces the rate of photosynthesis and produces some phenolic compounds. These compounds help the plant in its defensive mechanism (Jaafar et al., 2012). In accord with Van Den Ende et al., (2004) plants stressed by water deficiency synthesize protective molecules, such as fructans, which mitigate the detrimental effects induced by water deficit. These polysaccharide molecules are synthesized in plants exposed to very dry and/or

very cold environments, increasing cellular osmotic pressure by rapidly releasing oligofructans from polyfructans (Van den Ende et al., 2004).

Particularly, the CAM, crassulacean acid metabolism of *A. vera* optimizes water use efficiency and allows the plant to tolerate water deficit. The changes in fresh weight and leaf thickness are probably due to a decrease in the water content of the leaves, specifically the water stored in the leaf mucilage (Silva et al., 2010).

Acemannan is the main water-retaining molecule in the leaf mesophyll. Since the length of the leaves is affected by water stress, it is very likely that the photosynthetic area will also be reduced. Silva et al., (2014) found that photosynthetic cells decrease in length and mesophyll thickness is reduced with water stress in *A. vera* leaves; therefore, the amount of mucilage is also reduced. Hence, it is known that *A. vera* plants can suffer water stress despite being drought tolerant.

2.3.3 Molecular response to abiotic stress

When environmental stimuli act on a plant, signals are produced that trigger a biological response. These responses are mainly mediated by genes and transcription factors, which are activated in response to stimuli. In addition, environmental factors can also generate reactive molecules, which activate signaling cascades in the organism, leading to the activation of genes that encode useful products, such as proteins and metabolites. These products can help the plant to survive or resist stress conditions (Imran et al., 2021).

Plant responses to abiotic stresses occur at all levels of organization. Cellular responses include adjustments of the membrane system, changes in cell wall architecture, changes in the cell cycle, and cell division. In addition to the synthesis of specific endogenous molecules of low molecular weight that mainly regulate the protective responses of plants, such as salicylic acid, jasmonic acid, ethylene and abscisic acid (Fujita et al., 2006). At the molecular level, this response also includes the expression of stress-inducible genes involved in the direct protection of plants against stress (Shinozaki & Yamaguchi-Shinozaki, 2007).

Different types of environmental stress generate a similar response in plants. For instance, it has been observed that many genes that are activated in response to drought are also activated when the organism is exposed to salt and low temperatures (Chinnusamy et al., 2007). Likewise, these genes may also be involved in multiple responses to different abiotic conditions. For instance, genes that are activated during drought stress not only protect cells from dehydration, but also activate the production of metabolic proteins that regulate signal transduction after drought stress (Rudrabhatla & Rajasekharan, 2002; Shinozaki et al., 2003).

In *A. vera*, Jaiswal et al. (2021) reported that among the abiotic stress response genes, there are four categories: a) water-related stress response genes, b) DNA damage response genes involved in the reactive oxidative species (ROS) stress response, c) of the nuclear pore complex involved in the stress response by regulating cytoplasmic trafficking and d) genes related to the biosynthesis of secondary metabolites dealing with different types of biotic and abiotic stresses.

In *A. vera*, the *G6PD5* gene showed positive selection protecting plants against different types of stress such as salinity stress by producing a nitric oxide (NO) molecule, which leads to the expression of defense genes (Agarwal & Jha, 2010). Also, *BASS6*, a sodium cotransporter/metabolite gene, was found to be positively selected in *A. vera*. This gene is differentially expressed under drought and other abiotic stress and alters the expression of the specific target gene by binding to *ABRE* (abscisic acid response element), the characteristic element of ABA-inducible genes (Feng et al., 2019). ABA also regulate stomatal closure and solute transport and therefore have implications for tolerance to drought and water stress (Yamaguchi-Shinozaki & Shinozaki, 2006). The trehalase 1 (*TRE1*) gene was also positively selected, and overexpression of this gene causes better drought tolerance through ABA-guided stomatal closure (Van Houtte et al., 2013). The closure of stomas is a direct response also in the water deficit typical of CAM metabolism in plants such as *A. vera* (Habibi, 2020).

In general, the sensitivity of a plant to salt stress is based on the modulation of gene expression belonging to the families of functional genes (*HKT* (high-affinity

K^+ transporter), *SOS* (global response to deoxyribonucleic acid damage), *NHX* (sodium/hydrogen antiporter) and *HAK* (high-affinity K+)). In addition, in halophytes, both ion exclusion strategies and tissue tolerance strategies play an important role, since these plants can use Na^+ as an osmoregulator, as is the case with *A. vera* (Chakraborty et al., 2022b).

2.3.4 Response to a combination of abiotic stress

In nature, plants often experience a combination of two or more types of abiotic stress, such as salinity and water stress, which can activate specific molecular stress responses in the plant. During exposure to a combination of stresses, plants can integrate two different systemic signals generated at the same time. The way plants perceive different stresses and the plant parts affected can influence how quickly and effectively systemic signals such as reactive oxygen species (ROS), transcriptomic, hormonal and stomatal responses, as well as plant acclimatization (Zandalinas et al., 2020).

Although the interaction of different types of abiotic stresses in plants is becoming more common due to climate change and its effects on the environment, studies analyzing the effects of these combinations are rare (Xue et al., 2021). An illustration of this interaction is the coincidence between water and saline stress, which can occur in irrigated agricultural areas in arid regions of northern Mexico, due to irrigation with brackish water and the accumulation of salt after water evaporation.

When plants are faced with combinations of stresses, they activate specific physiological and molecular responses and adjust their metabolic pathways to counteract the negative effects on their growth, development, and reproduction. To achieve this, plants produce various metabolites that regulate different aspects of their growth and development, as well as their response to stress. (Zandalinas et al., 2022). On the other hand, it has been shown in several studies that photosynthesis is very sensitive to the combination of different abiotic stresses, such as lack of water, salinity and heat together. As a result of these combined

stress conditions, the efficiency of photosynthesis decreases, as do transpiration rates (Chakraborty et al., 2022a).

2.4 References

Agarwal, P., & Jha, B. (2010). Transcription factors in plants and ABA dependent and independent abiotic stress signalling. *Biologia Plantarum*, *54*(2), 201-212. https://doi.org/10.1007/s10535-010-0038-7

Ahuja, I., de Vos, R. C., Bones, A. M., & Hall, R. D. (2010). Plant molecular stress responses face climate change. *Trends in plant science*, *15*(12), 664-674.

Alvarado-Morales, G., Minjares-Fuentes, R., Contreras-Esquivel, J. C., Montañez, J., Meza-Velázquez, J. A., & Femenia, A. (2019). Application of thermosonication for Aloe vera (Aloe barbadensis Miller) juice processing: Impact on the functional properties and the main bioactive polysaccharides. *Ultrasonics Sonochemistry*, *56*, 125-133. https://doi.org/https://doi.org/10.1016/j.ultsonch.2019.03.030

Amirjani, M. R. (2011). Effect of salinity stress on growth, sugar content, pigments and enzyme activity of rice. *International Journal of Botany*, *7*(1), 73-81.

Amoo, S. O., Aremu, A. O., & Van Staden, J. (2014). Unraveling the medicinal potential of South African Aloe species. *Journal of Ethnopharmacology*, *153*(1), 19-41. https://doi.org/https://doi.org/10.1016/j.jep.2014.01.036

Arunkumar, S., & Muthuselvam, M. (2009). Analysis of phytochemical constituents and antimicrobial activities of Aloe vera L. against clinical pathogens. *World J Agric Sci*, *5*(5), 572-576.

Asada, K. (2006). Production and scavenging of reactive oxygen species in chloroplasts and their functions. *Plant physiology*, *141*(2), 391-396.

Bahuguna, R. N., & Jagadish, K. S. (2015). Temperature regulation of plant phenological development. *Environmental and Experimental Botany*, *111*, 83-90.

Campestrini, L. H., Silveira, J. L. M., Duarte, M. E. R., Koop, H. S., & Noseda, M. D. (2013). NMR and rheological study of Aloe barbadensis partially acetylated glucomannan. *Carbohydrate Polymers*, *94*(1), 511-519. https://doi.org/https://doi.org/10.1016/j.carbpol.2013.01.020

Cao, X., Zhang, Y., Peng, Y., Wang, Y., Li, B., & Tian, J. (2022). Impacts of konjac glucomannan with different degree of degradation or deacetylation on the stress resistance and fitness in Caenorhabditis elegans. *International Journal of Biological Macromolecules*, *204*, 397-409. https://doi.org/https://doi.org/10.1016/j.ijbiomac.2022.01.161

Chakraborty, K., Mondal, S., Bhaduri, D., Mohanty, A., & Paul, A. (2022a). Chapter 11 - Interplay between sodium and chloride decides the plant's

fate under salt and drought stress conditions. In V. Kumar, A. K. Srivastava, & P. Suprasanna (Eds.), *Plant Nutrition and Food Security in the Era of Climate Change* (pp. 271-314). Academic Press. https://doi.org/10.1016/B978-0-12-822916-3.00020-2

Chakraborty, K., Mondal, S., Bhaduri, D., Mohanty, A., & Paul, A. (2022b). Interplay between sodium and chloride decides the plant's fate under salt and drought stress conditions. In *Plant Nutrition and Food Security in the Era of Climate Change* (pp. 271-314). Elsevier.

Chinnusamy, Zhu, & Zhu. (2007). Cold stress regulation of gene expression in plants. *Trends Plant Sci*, *12*(10), 444-451. https://doi.org/10.1016/j.tplants.2007.07.002

Chokboribal, J., Tachaboonyakiat, W., Sangvanich, P., Ruangpornvisuti, V., Jettanacheawchankit, S., & Thunyakitpisal, P. (2015). Deacetylation affects the physical properties and bioactivity of acemannan, an extracted polysaccharide from Aloe vera. *Carbohydrate Polymers*, *133*, 556-566. https://doi.org/https://doi.org/10.1016/j.carbpol.2015.07.039

Chow, J. T.-N., Williamson, D. A., Yates, K. M., & Goux, W. J. (2005). Chemical characterization of the immunomodulating polysaccharide of Aloe vera L. *Carbohydrate Research*, *340*(6), 1131-1142.

Chua, M., Baldwin, T. C., Hocking, T. J., & Chan, K. (2010). Traditional uses and potential health benefits of Amorphophallus konjac K. Koch ex N.E.Br. *Journal of Ethnopharmacology*, *128*(2), 268-278. https://doi.org/https://doi.org/10.1016/j.jep.2010.01.021

DeFreitas, P. A. F., DeSouza Miranda, R., Marques, E. C., Prisco, J. T., & Gomes-Filho, E. (2018). Salt tolerance induced by exogenous proline in maize is related to low oxidative damage and favorable ionic homeostasis. *Journal of plant growth regulation*, *37*(3), 911-924.

Domínguez-Fernandez, R. N., Arzate-Vazquez, *I.*, Chanona-Perez, J. J., Welti-Chanes, J. S., Alvarado-Gonzalez , J. S., Calderon-Domínguez, G., . . . Gutierrez-López, G. F. (2011). El gel de aloe vera: Estructura, composicion química, procesamiento, actividad biologica e importancia en la industria farmaceutica y alimentaria *Revista mexicana de ingeniería química*, *11*, 23-43.

Dos Reis, S. P., Lima, A. M., & De Souza, C. R. B. (2012). Recent Molecular Advances on Downstream Plant Responses to Abiotic Stress. *International Journal of Molecular Sciences*, *13*(7), 8628-8647. http://www.mdpi.com/1422-0067/13/7/8628

Drudi, D., Tinto, D., Ferranti, D., Fiorelli, F., Pozzo, M. D., & Capitani, O. (2018). Aloe barbadensis miller versus silver sulfadiazine creams for wound healing by secondary intention in dogs and cats: A randomized controlled

study. *Research in Veterinary Science*, *117*, 1-9. https://doi.org/https://doi.org/10.1016/j.rvsc.2017.10.010

FAO. (2013). World Food and Agriculture. *Rome, Italy*. http://www.fao.org/docrep/018/i3107e/i3107e.PDF

Farooq, M., Wahid, A., Kobayashi, N., Fujita, D., & Basra, S. M. A. (2009). Plant drought stress: effects, mechanisms and management. *Agronomy for Sustainable Development*, *29*(1), 185-212. https://doi.org/10.1051/agro:2008021

Femenia, A., García-Pascual, P., Simal, S., & Rosselló, C. (2003). Effects of heat treatment and dehydration on bioactive polysaccharide acemannan and cell wall polymers from Aloe barbadensis Miller. *Carbohydrate Polymers*, *51*(4), 397-405. https://doi.org/https://doi.org/10.1016/S0144-8617(02)00209-6

Femenia, A., Sánchez, E. S., Simal, S., & Rosselló, C. (1999). Compositional features of polysaccharides from Aloe vera (Aloe barbadensis Miller) plant tissues. *Carbohydrate Polymers*, *39*(2), 109-117. https://doi.org/https://doi.org/10.1016/S0144-8617(98)00163-5

Feng, R.-J., Ren, M.-Y., Lu, L.-F., Peng, M., Guan, X., Zhou, D.-B., . . . Xie, J.-H. (2019). Involvement of abscisic acid-responsive element-binding factors in cassava (Manihot esculenta) dehydration stress response. *Scientific Reports*, *9*(1), 12661. https://doi.org/10.1038/s41598-019-49083-3

Fujita, M., Fujita, Y., Noutoshi, Y., Takahashi, F., Narusaka, Y., Yamaguchi-Shinozaki, K., & Shinozaki, K. (2006). Crosstalk between abiotic and biotic stress responses: a current view from the points of convergence in the stress signaling networks. *Current opinion in plant biology*, *9*(4), 436-442.

Gille, S., Cheng, K., Skinner, M. E., Liepman, A. H., Wilkerson, C. G., & Pauly, M. (2011). Deep sequencing of voodoo lily (Amorphophallus konjac): an approach to identify relevant genes involved in the synthesis of the hemicellulose glucomannan. *Planta*, *234*(3), 515-526.

Imran, Q. M., Falak, N., Hussain, A., Mun, B.-G., & Yun, B.-W. (2021). Abiotic Stress in Plants; Stress Perception to Molecular Response and Role of Biotechnological Tools in Stress Resistance. *Agronomy*, *11*(8), 1579. https://www.mdpi.com/2073-4395/11/8/1579

Jaafar, H. Z., Ibrahim, M. H., & Mohamad Fakri, N. F. (2012). Impact of soil field water capacity on secondary metabolites, phenylalanine ammonia-lyase (PAL), maliondialdehyde (MDA) and photosynthetic responses of Malaysian Kacip Fatimah (Labisia pumila Benth). *Molecules*, *17*(6), 7305-7322.

Jaiswal, S. K., Mahajan, S., Chakraborty, A., Kumar, S., & Sharma, V. K. (2021). The genome sequence of Aloe vera reveals adaptive evolution of drought

tolerance mechanisms. *iScience*, *24*(2), 102079. https://doi.org/https://doi.org/10.1016/j.isci.2021.102079

Jaspers, P., & Kangasjärvi, J. (2010). Reactive oxygen species in abiotic stress signaling. *Physiologia Plantarum*, *138*(4), 405-413.

Jha, Y., Subramanian, R., & Patel, S. (2011). Combination of endophytic and rhizospheric plant growth promoting rhizobacteria in Oryza sativa shows higher accumulation of osmoprotectant against saline stress. *Acta Physiologiae Plantarum*, *33*(3), 797-802.

Jiang, C.-Q., Quan, L.-T., Shi, F., Yang, N., Wang, C.-H., Yin, X.-M., & Zheng, Q.-S. (2014). Distribution of Mineral Nutrients and Active Ingredients in Aloe vera Irrigated with Diluted Seawater. *Pedosphere*, *24*(6), 722-730. https://doi.org/https://doi.org/10.1016/S1002-0160(14)60059-X

Khan, M. A. M., Ulrichs, C., & Mewis, I. (2011). Water stress alters aphid-induced glucosinolate response in Brassica oleracea var. italica differently. *Chemoecology*, *21*(4), 235-242.

Khanna, S. (2003). *The chemical, physical and nutritional properties of the plant polysaccaride konjac glucomannan* Glasgow Caledonian University].

Kishor, P. K., Sangam, S., Amrutha, R., Laxmi, P. S., Naidu, K., Rao, K. S., . . . Sreenivasulu, N. (2005). Regulation of proline biosynthesis, degradation, uptake and transport in higher plants: its implications in plant growth and abiotic stress tolerance. *Current science*, 424-438.

Kumar, S., & Kumar, R. (2019). Role of acemannan O-acetyl group in murine radioprotection. *Carbohydrate Polymers*, *207*, 460-470. https://doi.org/https://doi.org/10.1016/j.carbpol.2018.12.003

Kumar, S., & Tiku, A. B. (2016). Immunomodulatory potential of acemannan (polysaccharide from Aloe vera) against radiation induced mortality in Swiss albino mice. *Food and Agricultural Immunology*, *27*(1), 72-86. https://doi.org/10.1080/09540105.2015.1079594

Lindsey, K. L., Jäger, A. K., Viljoen, A. M., & van Wyk, B. E. (2002). Cyclooxygenase inhibitory activity of Aloe species. *South African Journal of Botany*, *68*(1), 47-50. https://doi.org/https://doi.org/10.1016/S0254-6299(16)30453-7

Liu, X., You, L., Tarafder, S., Zou, L., Fang, Z., Chen, J., . . . Zhang, Q. (2019). Curcumin-releasing chitosan/aloe membrane for skin regeneration. *Chemical Engineering Journal*, *359*, 1111-1119. https://doi.org/10.1016/j.cej.2018.11.073

Lucini, L., Pellizzoni, M., & Molinari, G. P. (2013). Anthraquinones and β-polysaccharides content and distribution in Aloe plants grown under different light intensities. *Biochemical Systematics and Ecology*, *51*, 264-268. https://doi.org/https://doi.org/10.1016/j.bse.2013.09.007

Ma, X., Ou, Y.-B., Gao, Y.-F., Lutts, S., Li, T.-T., Wang, Y., . . . Yao, Y.-A. (2016). Moderate salt treatment alleviates ultraviolet-B radiation caused impairment in poplar plants. *Scientific reports*, *6*, 32890.

Manickavelu, A., Nadarajan, N., Ganesh, S. K., Gnanamalar, R. P., & Chandra Babu, R. (2006). Drought tolerance in rice: morphological and molecular genetic consideration. *Plant Growth Regulation*, *50*(2), 121-138. https://doi.org/10.1007/s10725-006-9109-3

McCarty, M. F. (2002). Glucomannan minimizes the postprandial insulin surge: a potential adjuvant for hepatothermic therapy. *Medical Hypotheses*, *58*(6), 487-490. https://doi.org/https://doi.org/10.1054/mehy.2001.1457

Minjares-Fuentes, R., & Femenia, A. (2019). Aloe vera. 145-152. https://doi.org/10.1016/b978-0-12-812491-8.00020-5

Minjares-Fuentes, R., Femenia, A., Comas-Serra, F., & Rodríguez-González, V. M. (2018). Compositional and Structural Features of the Main Bioactive Polysaccharides Present in the Aloe vera Plant. *Journal of AOAC International*, *101*(6), 1711-1719. https://doi.org/10.5740/jaoacint.18-0119

Minjares-Fuentes, R., Medina-Torres, L., González-Laredo, R. F., Rodríguez-González, V. M., Eim, V., & Femenia, A. (2017). Influence of water deficit on the main polysaccharides and the rheological properties of Aloe vera (Aloe barbadensis Miller) mucilage. *Industrial crops and products*, *109*, 644-653. https://doi.org/https://doi.org/10.1016/j.indcrop.2017.09.016

Minjares-Fuentes, R., Rodríguez-González, V. M., González-Laredo, R. F., Eim, V., González-Centeno, M. R., & Femenia, A. (2017). Effect of different drying procedures on the bioactive polysaccharide acemannan from Aloe vera (Aloe barbadensis Miller). *Carbohydrate Polymers*, *168*, 327-336. https://doi.org/https://doi.org/10.1016/j.carbpol.2017.03.087

Moghbeli, E., Fathollahi, S., Salari, H., Ahmadi, G., Saliqehdar, F., Safari, A., & Hosseini Grouh, M. S. (2012). Effects of salinity stress on growth and yield of Aloe vera L. *Journal of Medicinal Plants Research*, *6(16)*, 3272-3277. https://doi.org/10.5897/JMPR11.1698

Munns, R. (2002). Comparative physiology of salt and water stress. *Plant, cell & environment*, *25*(2), 239-250.

Murillo-Amador, B., Nieto-Garibay, A., Troyo-Diéguez, E., García-Hernández, J. L., Hernández-Montiel, Luis, & Valdez-Cepeda, R. D. (2015). Moderate salt stress on the physiological and morphometric traits of aloe vera l. . *Botanical Sciences*, *93 (3)*, 639-648. https://doi.org/10.17129/botsci.73

Nobel, P. S. (1997). Root distribution and seasonal production in the northwestern Sonoran Desert for a C3 subshrub, a C4 bunchgrass, and a CAM leaf succulent. *American Journal of Botany*, *84(8)*, 949–955. https://doi.org/https://doi.org/10.2307/2446285

Okçu, G., Kaya, M. D., & Atak, M. (2005). Effects of salt and drought stresses on germination and seedling growth of pea (Pisum sativum L.). *Turkish journal of agriculture and forestry*, *29*(4), 237-242. https://journals.tubitak.gov.tr/agriculture/vol29/iss4/2

Pedroza-Sandoval, A., Aba-Guevara, C. G., Samaniego-Gaxiola, J. A., Trejo-Calzada, R., Sánchez-Cohen, I., & Chávez-Rivero, J. A. (2015). Características morfométricas y calidad de gel en sábila (Aloe barbadensis M.) aplicando algaenzimas y composta*. *Revista mexicana de ciencias agrícolas*, *6*, 7-18.

Petrov, V., Hille, J., Mueller-Roeber, B., & Gechev, T. S. (2015). ROS-mediated abiotic stress-induced programmed cell death in plants. *Frontiers in plant science*, *6*, 69.

Radha, M. H., & Laxmipriya, N. P. (2015). Evaluation of biological properties and clinical effectiveness of Aloe vera: A systematic review. *Journal of Traditional and Complementary Medicine*, *5*(1), 21-26. https://doi.org/https://doi.org/10.1016/j.jtcme.2014.10.006

Ray, A., Ghosh, S., Ray, A., & Aswatha, S. M. (2015). An analysis of the influence of growth periods on potential functional and biochemical properties and thermal analysis of freeze-dried Aloe vera L. gel. *Industrial crops and products*, *76*, 298-305. https://doi.org/https://doi.org/10.1016/j.indcrop.2015.05.015

Rodríguez-González, V. M., Femenia, A., González-Laredo, R. F., Rocha-Guzmán, N. E., Gallegos-Infante, J. A., Candelas-Cadillo, M. G., . . . Rosselló, C. (2011). Effects of pasteurization on bioactive polysaccharide acemannan and cell wall polymers from Aloe barbadensis Miller. *Carbohydrate Polymers*, *86*(4), 1675-1683. https://doi.org/https://doi.org/10.1016/j.carbpol.2011.06.084

Rudrabhatla, P., & Rajasekharan, R. (2002). Developmentally Regulated Dual-Specificity Kinase from Peanut That Is Induced by Abiotic Stresses. *Plant Physiology*, *130*(1), 380-390. https://doi.org/10.1104/pp.005173

Salah, F., Ghoul, Y. E., Mahdhi, A., Majdoub, H., Jarroux, N., & Sakli, F. (2017). Effect of the deacetylation degree on the antibacterial and antibiofilm activity of acemannan from Aloe vera. *Industrial Crops and Products*, *103*, 13-18. https://doi.org/https://doi.org/10.1016/j.indcrop.2017.03.031

Salinas, P., Salinas, C., Contreras, R. A., Zuniga, G. E., Dupree, P., & Cardemil, L. (2019). Water deficit and abscisic acid treatments increase the expression of a glucomannan mannosyltransferase gene (GMMT) in Aloe vera Burm. F. *Phytochemistry*, *159*, 90-101. https://doi.org/10.1016/j.phytochem.2018.12.009

Shi, X.-D., Yin, J.-Y., Cui, S. W., Wang, Q., Wang, S.-Y., & Nie, S.-P. (2020). Plant-derived glucomannans: Sources, preparation methods, structural

features, and biological properties. *Trends in Food Science & Technology*, *99*, 101-116. https://doi.org/https://doi.org/10.1016/j.tifs.2020.02.016

Shinozaki, K., & Yamaguchi-Shinozaki, K. (2007). Gene networks involved in drought stress response and tolerance. *Journal of experimental botany*, *58*(2), 221-227.

Shinozaki, K., Yamaguchi-Shinozaki, K., & Seki, M. (2003). Regulatory network of gene expression in the drought and cold stress responses. *Current Opinion in Plant Biology*, *6*(5), 410-417. https://doi.org/https://doi.org/10.1016/S1369-5266(03)00092-X

Silva, H., Sagardia, S., Seguel, O., Torres, C., Tapia, C., Franck, N., & Cardemil, L. (2010). Effect of water availability on growth and water use efficiency for biomass and gel production in Aloe Vera (Aloe barbadensis M.). *Industrial Crops and Products*, *31*(1), 20-27. https://doi.org/https://doi.org/10.1016/j.indcrop.2009.08.001

Silva, H. E., Verhoef, E. T., & Van Den Berg, V. A. (2014). Airlines' strategic interactions and airport pricing in a dynamic bottleneck model of congestion. *Journal of Urban Economics*, *80*, 13-27. https://doi.org/https://doi.org/10.1016/j.jue.2013.08.002

Tai-Nin Chow, J., Williamson, D. A., Yates, K. M., & Goux, W. J. (2005). Chemical characterization of the immunomodulating polysaccharide of Aloe vera L. *Carbohydrate Research*, *340*(6), 1131-1142. https://doi.org/https://doi.org/10.1016/j.carres.2005.02.016

Tester, R., & Al-Ghazzewi, F. (2017). Glucomannans and nutrition. *Food Hydrocolloids*, *68*, 246-254. https://doi.org/https://doi.org/10.1016/j.foodhyd.2016.05.017

Thunyakitpisal, P., Ruangpornvisuti, V., Kengkwasing, P., Chokboribal, J., & Sangvanich, P. (2017). Acemannan increases NF-κB/DNA binding and IL-6/-8 expression by selectively binding Toll-like receptor-5 in human gingival fibroblasts. *Carbohydrate Polymers*, *161*, 149-157. https://doi.org/https://doi.org/10.1016/j.carbpol.2016.12.034

Tyler, V. (1993). The honest herbal: a sensible guide to the use of herbs and relatedremedies. *Binghamton, New York.*, *3*.

Van den Ende, W., De Coninck, B., & Van Laere, A. (2004). Plant fructan exohydrolases: a role in signaling and defense? *Trends Plant Sci*, *9*(11), 523-528.

Van Houtte, H., Vandesteene, L., López-Galvis, L., Lemmens, L., Kissel, E., Carpentier, S., . . . Van Dijck, P. (2013). Overexpression of the Trehalase Gene AtTRE1 Leads to Increased Drought Stress Tolerance in Arabidopsis and Is Involved in Abscisic Acid-Induced Stomatal Closure *Plant Physiology*, *161*(3), 1158-1171. https://doi.org/10.1104/pp.112.211391

Vogler, B. K., & Ernst, E. (1999). Aloe vera: A systematic review of its clinical effectiveness [Article]. *British Journal of General Practice*, *49*(447), 823-828. https://www.scopus.com/inward/record.uri?eid=2-s2.0-0032854467&partnerID=40&md5=f5b3e4449cec9823eed759e406eb77d3 https://bjgp.org/content/bjgp/49/447/823.full.pdf

Wyk, B. E. v., Oudtshoorn, B. v., & Gericke, N. (1997). *Medicinal plants of South Africa*. Briza.

Xu, C. X., Ma, Y. P., & Liu, Y. L. (2015). Effects of silicon (Si) on growth, quality and ionic homeostasis of aloe under salt stress. *South African Journal of Botany*, *98*, 26-36. https://doi.org/https://doi.org/10.1016/j.sajb.2015.01.008

Xue, F., Liu, W., Cao, H., Song, L., Ji, S., Tong, L., & Ding, R. (2021). Stomatal conductance of tomato leaves is regulated by both abscisic acid and leaf water potential under combined water and salt stress. *Physiologia Plantarum*, *172*(4), 2070-2078. https://doi.org/doi.org/10.1111/ppl.13441

Yamaguchi-Shinozaki, K., & Shinozaki, K. (2006). Transcriptional regulatory networks in cellular responses and tolerance to dehydration and cold stresses. *Annual Review of Plant Biology*, *57*(1), 781-803. https://doi.org/10.1146/annurev.arplant.57.032905.105444

Zadeh, H. M. (2007). Effects of salinity stress on the morphology and yield of two cultivars of canola (Brassica napus L.). *Journal of Agronomy*.

Zandalinas, S. I., Balfagón, D., Gómez-Cadenas, A., & Mittler, R. (2022). Responses of plants to climate change: Metabolic changes during abiotic stress combination in plants. *Journal of Experimental Botany*.

Zandalinas, S. I., Fichman, Y., Devireddy, A. R., Sengupta, S., Azad, R. K., & Mittler, R. (2020). Systemic signaling during abiotic stress combination in plants. *Proceedings of the National Academy of Sciences*, *117*(24), 13810-13820.

Zhao, T.-J., Sun, S., Liu, Y., Liu, J.-M., Liu, Q., Yan, Y.-B., & Zhou, H.-M. (2006). Regulating the drought-responsive element (DRE)-mediated signaling pathway by synergic functions of trans-active and trans-inactive DRE binding factors in Brassica napus. *Journal of Biological Chemistry*, *281*(16), 10752-10759. https://doi.org/10.1074/jbc.M510535200

Zhou, R., Yu, X., Ottosen, C.-O., Rosenqvist, E., Zhao, L., Wang, Y., . . . Wu, Z. (2017). Drought stress had a predominant effect over heat stress on three tomato cultivars subjected to combined stress. *BMC plant biology*, *17*(1), 1-13.

CHAPTER III. Water deficit and salinity modify some morphometric, physiological and productive attributes of *Aloe vera* (L.)

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Water deficit and salinity modify some morphometric, physiological, and productive attributes of *Aloe vera* (L.)

El déficit hídrico y salinidad modifican algunos atributos morfométricos, fisiológicos y productivos de *Aloe vera* (L.)

María Mota-Ituarte[1], Aurelio Pedroza-Sandoval[1]*, Rafael Minjares-Fuentes[2], Ricardo Trejo-Calzada[1], Jorge A. Zegbe[3], Jesús Josafath Quezada-Rivera[4]

[1] Universidad Autónoma Chapingo, Unidad Regional Universitaria de Zonas Áridas, Durango, Mexico.
[2] Universidad Juárez del Estado de Durango, Facultad de Ciencias Químicas, Durango, Mexico.
[3] Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias, Campo Experimental Zacatecas, Zacatecas, Mexico
[4] Universidad Juárez del Estado de Durango, Facultad de Ciencias Biológicas, Durango, Mexico.
*Corresponding author: apedroza@chapingo.uruza.edu.mx

Abstract

Background: Water deficit and salinity are common conditions in arid agro-ecosystems.
Hypothesis: Salinty increases the hydric stress effect on the yield and gel quality of *Aloe vera*.
Study species and data description: In *A. vera* plants the variables meassured were: plant height, leaf length, width, and thickness, fresh biomass, the relative content of water, total solids, gel percentage, and harvest index.
Study site and dates: The study was conducted from spring to summer 2020 in Bermejillo, Durango, Mexico.
Methods: A split-block experimental design with four replications was used. The large plots were two soil moisture contents: field capacity (18.5 ± 2.5 %) and permanent wilting point (12.5 ± 2.5 %). The small plot was the salinity, with levels: 0, 20, 40, 60, and 80 mM of NaCl.
Results: Water deficit and salinity reduced the width and thickness of leaves and, consequently, the fresh biomass and gel percentage were reduced, but gel quality increased.
Conclusions: Water deficit and salinity, mainly the latter, reduced plant growth and leaves, and therefore, the yield of fresh biomass and gel. Although gel yield was reduced, its quality was enhanced, in terms of high total solids content.
Keywords: abiotic stress, fresh biomass, plant growth, gel quality.

Resumen

Antecedentes: El déficit hídrico y la salinidad son condiciones comunes en los agroecosistemas de zonas áridas.
Hipótesis: El estrés salino incrementa los efectos del estrés hídrico sobre el rendimiento y calidad de gel de *Aloe vera*.
Especies de estudio y descripción de datos: En *A. vera* se midieron las variables: altura de planta, longitud, ancho y grosor de hoja, el contenido relativo de agua, sólidos totales, biomasa fresca, porcentaje de gel e índice de cosecha.
Sitio de estudio y fechas: El estudio se llevó a cabo durante el periodo primavera-verano de 2020 en Bermejillo, Durango, México.
Métodos: Se usó un diseño experimental en parcelas divididas con arreglo en bloques completos al azar con cuatro repeticiones. La parcela grande fue el contenido de humedad en el suelo: capacidad de campo (18.5 ± 2.5 %) y punto de marchitez permanente (12.5 ± 2.5 %). La parcela chica fue la salinidad con cinco niveles: 0, 20, 40, 60 y 80 mM de NaCl.
Resultados: El déficit hídrico y la salinidad del agua, redujeron el ancho y grosor de hojas, biomasa fresca y porcentaje de gel, pero mejoró la calidad de este último.
Conclusiones: El déficit hídrico y la salinidad, principalmente este último factor, redujeron el crecimiento de la planta y hojas y, por ende, la biomasa fresca. Aun cuando, el rendimiento de gel fue reducido, la calidad de éste mejoró, en términos de alto contenido de sólidos totales.
Palabras clave: estrés abiótico, biomasa fresca, crecimiento de planta, calidad de gel.

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Arid zones are regions of low rainfall, high evaporation rate, and extreme temperatures (Mazuela 2013). In irrigated agricultural lands, water is an increasingly scarce resource due to drought, aquifers overexploitation, soil salinity, and contamination of water with heavy metals (Azpilcueta-Pérez *et al*. 2018). Furthermore, climate change is exacerbating these situations, with low water availability for agriculture production (Zárate & Miranda 2016).

In the last decades, the problem of soil salinization in agricultural areas by irrigation using water with a high salt content has become more acute (Kopittke *et al*. 2019). In arid and semi-arid zones, the salinization of agricultural soils due to intensive use of chemically contaminated water is caused by the accumulation of soluble salts that are deposited on the soil surface by capillary action and high evaporation rates. The problem of soil salinity in these regions is exacerbated by inappropriate water management practices, such as the inefficient application of irrigation water and inadequate soil drainage systems (İbrahimova *et al*. 2021).

Degradation of resources due to extreme natural and anthropic processes, and the need to maintain agri-food production make relevant the application of more sustainable production strategies (Balvanera *et al*. 2017). An alternative to this production approach is productive reconversion through the performance of profitable alternative crops (Mattas *et al*. 2015), which mitigate the environmental problems in marginal lands of arid zones.

Aloe vera is a crop of high acclimatization capacity to different environments and tolerance to extreme biotic and abiotic conditions. So, it is a productive alternative for marginal areas where traditional crops are no longer economically competitive (Davis *et al*. 2019). In addition, aloe leaves products, such as gel, juice as well as powdery are in high demand in the international market due to multiple benefits in the food, medical, and cosmetic industries (Sifuentes-Rodríguez *et al*. 2020).

More than 200 active chemical compounds have been reported in *Aloe vera* gel, such as vitamins, minerals, enzymes, simple and complex polysaccharides, phenolic compounds, and organic acids (Boudreau *et al*. 2013). Most of these chemical compounds are produced by secondary metabolism, which is activated when the plant is stressed (Isah 2019) .

Aloe vera responses to water deficit (Delatorre-*Herrera et al*. 2010, Silva *et al*. 2010, Espinoza-Garrido 2011, Hazrati *et al*. 2017, Minjares-Fuentes *et al*. 2017) and salinity (Moghbeli *et al*. 2012, Murillo-Amador *et al*. 2015, Xu *et al*. 2015) have been studied at length. Plant responses to water deficit and salinity are diverse and complex. Some of them are, for instance, cell membrane and cell wall adjustments, changes in the metabolic cycle, and cell division. In addition, the latter involves the synthesis of specific endogenous compounds of low molecular mass such as salicylic acid, jasmonic acid, ethylene, and abscisic acid (Fujita *et al*. 2006). Also, salt and water stresses cause an increase in reactive oxygen species (ROS) levels which may produce oxidative stress (Ahanger *et al*. 2017).

Some studies have shown that *A. vera* can tolerate moderate salt and water stresses. Murillo-Amador *et al*. (2015) found that *A. vera* maintains dry mass production under moderate concentrations of NaCl. Sifuentes-Rodríguez *et al*. (2020) found that *A. vera* is tolerant to certain levels of salinity by keeping biomass and gel production. Habibi (2018) reported that leaf thickness, biomass, and gel production of *A. vera* are not affected by moderate water stress, which may even improve plant growth. However, most studies have examined individual stress factors. The joint action of these stressors could trigger more complex response effects that need to be analyzed and, where appropriate, used for the knowledge and technology generated (Mittler 2006).

The joint occurrence of water deficit and salinity is frequent in agricultural regions where intensive water use, soil, and inputs are made, which presents a high risk in agri-food production (Mittler *et al*. 2001). Simultaneous study of both abiotic factors is imperative to deepen knowledge of the response to growth, development, and production of *A. vera* plants. This will make it feasible to design technological mitigation strategies in marginal agricultural lands. The objective of this study was to evaluate the effect of water deficit and soil salinity on some indicators of plant and leaf growth, physiology, and productivity of *A. vera.*

Materials and methods

Geographic location of the experimental area. The study was conducted at the University Regional Unit for Arid Zones of the Autonomous University of Chapingo in Bermejillo, Durango, Mexico. The region is located at lat. 23º

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54'N and long. 103º 37'W and elevation 1,130 m. It has a dry climate with summer rains, an average annual rainfall of 239 mm, a winter rainfall percentage < 5 %, and a thermal oscillation from 7 to 17 ºC (Cano-Villegas *et al*. 2021).

Experimental design and treatments. The experiment was carried out under controlled shade-mesh conditions, during the spring to summer of 2020. A split-block experimental design with four replications was used. The large plots were the soil moisture contents at field capacity (FC) and permanent wilting point (PWP), corresponding to 20.7 and 12.3 % of soil water content, respectively. Soil moisture constants (FC and PWP) were determined using the membrane pot (Richards 1948) (Table 1). To maintain moisture content, irrigation was applied in ranges of 16 to 21 % and 10 to 15 %, respectively. The upper level of the first range corresponded to FC, while the lower level of the second range corresponded to a moisture content around PWP, to induce a water stress condition, due to the high drought tolerance of *A. vera* (Pedroza & *Gómez 2006).* Only two treatments of water content in the soil were carried out to study the response to extreme ranges (optimal and suboptimal) of water availability for the plant combined with five NaCl concentrations.

The subplots varied in NaCl concentration: 0, 20, 40, 60, and 80 mM dissolved in the irrigation water to induce soil salinity. After four months of standardized irrigation at FC, irrigation was cut off until the soil moisture reached the lower limit of 16 or 10 %. Recovery irrigation brought each plot up to the corresponding upper limits of 21 and 15 %, respectively. Irrigation was made with a manual sprinkler and the water application to each treatment was based on daily monitoring of soil moisture using a digital sensor with a real-time readout (Soil Moisture Meter Mod. MO750, manufacturer Extech, Taiwan, China).

Six-month-old *A. vera* plants of sizes from 25 to 30 cm were used, which were obtained in the form of suckers from the mother plant. The experimental unit was one plant per pot containing 10 kg of soil. The soil was collected in the study region and according to the physical and chemical analysis is a soil with a slightly alkaline pH and an electrical conductivity (EC) of 3.48 dS m^{-1} equivalent to a non-saline soil (Mahdy 2011), among other features (Table 1). *Variables of plant growth and leaves*. Plant height and leaf length were measured with a flexometer, the first variable

Table 1. Initial physical and chemical characteristics of the soil used in the study.

Physical characteristics*		**Chemical characteristics***	
Texture	sandy loam	Cation exchange capacity (ECC)	18.2
Field capacity (%)	20.7	pH (paste)	7.92
Permanent wilting point (%)	12.3	Electrical conductivity (Paste, dS m^{-1})	3.48
% Saturation	30.57	Extractable sodium (mg kg^{-1})	297.3
Apparent density (g cm^{-3})	1.56	Sodium adsorption ratio (SAR)	1.47
		Exchangeable sodium percentage (ESP)	6.1
		Percentage of total carbonates ($CaCO_3$)	21.6

* Determinations carried out by the Centro Nacional de Investigación Disciplinaria en Relaciones Agua Suelo Planta Atmósfera (CENID-RASPA) of the Instituto Nacional de Investigaciones Forestales Agrícolas y Pecuarias (INIFAP), Gomez Palacio, Durango, Mexico.

was measured from ground level to the end of the largest leaf, and the second variable was measured at the outermost leaf of the bud; while the width and thickness of the leaf were measured with a vernier (Truper, model 14388, China). All variables were measured at intervals of 22 days during 110 days after starting the treatments (DAST).

Physiological attributes. Plant water status was determined monthly, in terms of relative water content (RWC) (Yamasaki & Dillenburg 1999). At the end of the experiment, total solids (TS), which are defined as the organic and inorganic matter that remains as a residue after evaporation and dried at 103 - 105 °C to constant mass, were

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determined (Calzada & Pedroza 2005). This variable was determined by weighting five g gel with an analytical balance (model SHIMADZU AY220) and then each sample was placed in a porcelain crucible to achieve the constant mass. These variables were determined at 14, 42, and 80 DAST. The dates of sampling were based on finding possible differences in response due to a change in the sunshine and seasonal temperature during the experimental period.

Productivity and performance attributes. The fresh biomass (FB) production, including the aerial part and roots, was determined using a gravimetric balance, while the percentage of gel was estimated using the following equation:

$$\% \, gel = \left[\frac{Gel \, mass}{Total \, leaf \, mass}\right] \times (100) \qquad (1)$$

For this determination, the leaves of the plant were cut from their base and filleted with a knife, cutting the tip and the lateral parts to subsequently detach the cuticle from the beam and the underside with the use of a spatula and obtain the crystalline gel separated from the cuticle.

The harvest index (HI) was defined as the ratio of the weight of gel and the weight of fresh biomass, and was calculated following the equation:

$$HI = \left[\frac{Gel \, mass}{Fresh \, biomass}\right] \qquad (2)$$

Data Analysis. The information was analyzed with a split-block experimental model with a factorial arrangement in treatments with the GLM procedure of the statistical analysis system (SAS Institute v. 9.0, Cary, NC, USA). In the separation of means, the minimum significant difference of Tukey's test was used ($P \leq 0.05$). The regression models for gel production and harvest index versus salinity levels were fitted in Microsoft Excel.

Results

Plant growth and leaves. Growth of the aloe plant and leaves was slightly affected by the soil moisture content, mainly in the width and thickness of the leaf and, less consistently, in the height of the plant, without any measurable effect on leaf length. The effect of soil moisture content and levels of salinity on plant height was not consistent, with a trend to decrease at 60 mM NaCl and above at either soil moisture content. Salt stress affected leaf width only at the highest NaCl concentrations (80 mM) in plants under FC. However, there were significant effects at 60 mM NaCl in plants under PWP. Control plants had leaf width values of 3.1 cm and 3.0 cm for FC and PWP, respectively. Leaf thickness was more sensitive to salinity, a decrease in this trait was observed since 40 mM NaCl in plants under FC and PWP. The higher leaf thickness was shown by plants under 0 mM NaCl with values of 1.26 cm and 1.02 cm under FC and PWP, respectively (Table 2).

Relative water content and total solids. The relative water content (RWC), an indicator of the water status of the leaf tissue, was not affected by either soil moisture or salinity ($P > 0.05$) (Table 3). Total solids (TS) showed a significant effect ($P \leq 0.05$), which depended on soil moisture content, salinity level, and monitoring sampling. There was a non-consistent response at the first two monitoring dates, corresponding to 14 and 42 DAST at both soil moisture contents. There was a trend toward a significantly lower concentration of TS in the control, compared to salinity treatments from 20 to 80 mM, mainly under PWP conditions, where the greatest concentrations of TS were obtained at 60 and 80 mM. The TS values for 80 mM NaCl were 5.2 and 5.3 % compared to 3.1 and 3.8 % for the control, respectively. There was a consistent treatment effect of significantly higher values ($P \leq 0$ .05) at 80 DAST at both soil moisture contents with a TS concentration of 8.9 % for plants under PWP and 40 mM NaCl and 8.8 % plants under FC plus 80 mM NaCl, while control plants showed 3.8 % at both soil moisture contents (Table 4).

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Table 2. Effect of soil moisture content and salt concentrations on different growth variables of the aloe (*Aloe vera* L.) plant and leaf.

Salinity (mM)	FC	PWP	FC	PWP	FC	PWP	FC	PWP
	plant height (cm)		leaf length (cm)		leaf width (cm)		leaf thickness (cm)	
0 (Control)	46.2 ± 3.10 abc*	44.2 ± 3.99 b	31.3 ± 8.80 a	30.1 ± 7.55 a	3.1 ± 0.70 a	3.0 ± 0.41 a	1.26 ± 0.08 a	1.02 ± 0.12 a
20	46.8 ±2.99 ab	46.5 ± 2.90 a	29.0 ± 8.36 a	29.8 ± 7.00 a	2.8 ± 0.55 ab	2.9 ± 0.48 ab	1.15 ± 0.16 ab	0.91 ± 0.11 a
40	45.3 ±2.60 bc	44.6 ± 1.82 ab	29.8 ± 7.64 a	27.8 ± 7.45 a	2.9 ± 0.62 ab	2.5 ± 0.39 b	1.13 ± 0.14 bc	0.87 ± 0.17 b
60	48.4 ± 2.94 a	44.0 ± 2.25 b	30.4 ± 7.85 a	28.5 ± 7.14 a	2.8 ± 0.54 ab	2.9 ± 0.66 a	1.01 ± 0.21 c	0.91 ± 0.19 a
80	44.2 ± 1.93 c	44.1 ± 0.19 b	25.8 ± 6.94 a	29.0 ± 6.59 a	2.4 ± 0.40 b	2.7 ± 0.41ab	0.85 ± 0.11 c	0.85 ± 0.17 b

*Mean values (± standard deviation) with the same letters within columns, are statistically equal according to the minimum significant difference of Tukey's test ($P \leq 0.05$). FC: field capacity (20.7 %); and PWP: permanent wilting point (12.3 %).

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Table 3. Effect of salinity and two soil moisture contents on different monitoring dates on the relative water content (RWC) in *Aloe vera* leaves.

Salinity (mM)	Relative water content (%)					
	14 DAST		42 DAST		80 DAST	
	FC	PWP	FC	PWP	FC	PWP
0	94.3± 0.46 a*	94.6±0.38 a	96.4±2.17 a	95.8±0.15 a	92.7±0.90 a	92.8±0.25 a
20	95.6±0.10 a	94.8±0.53 a	94.1±0.27 a	93.3± 1.45 a	90.3±0.75 a	92.3±2.61 a
40	97.0±0.14 a	96.7±2.02 a	93.5±0.79 a	93.5±0.79 a	89.4±0.001 a	91.3±1.49 a
60	93.6±0.22 a	94.8±1.26 a	94.2±0.003 a	91.8±0.11 a	88.5± 1.28 a	86.6± 1.81 a
80	95.6±0.55 a	92.0±0.71 a	92.9± 1.64 a	91.9±0.10 a	89.1±1.10 a	89.1±0.71 a

*Mean values (± standard deviation) with the same letters within columns, are statistically equal according to the minimum significant difference of Tukey's test ($P \leq 0.05$). DAST: days after starting treatments; FC: field capacity (20.7%); and PWP: permanent wilting point (12.3%).

Table 4. Effect of salinity at two soil moisture contents on different monitoring dates on the concentration of total solids (TS) in *Aloe vera* leaf.

Salinity (mM)	Total solids (%)					
	14 DAST		42 DAST		80 DAST	
	FC	PWP	FC	PWP	FC	PWP
0	2.7±0.33 c*	3.1± 0.27 ab	4.9± 1.53 a	3.8±0.11 c	3.8±0.63 b	3.8±0.18 b
20	3.8±0.07 b	2.8± 0.37 ab	4.4±0.19 a	5.9±1.02 a	7.3±0.53 a	6.8± 1.85 ab
40	4.6±0.1 a	2.6±1.42 b	4.1±0.55 a	4.5±0.56 bc	8.5±0.0008 a	8.9± 1.05 a
60	4.6±0.15 a	4.8± 0.89 ab	5.5±0.002 a	5.7±0.08 ab	8.1±0.90 a	7.5± 1.28 a
80	3.7±0.39b	5.2±0.50 a	5.5± 1.16 a	5.3±0.07 ab	8.8±0.78 a	7.8±0.50 a

*Mean values (± standard deviation) with the same letters within columns are statistically equal according to the minimum significant difference of Tukey's test ($P \leq 0.05$). DAST: days after starting treatments; FC: field capacity (20.7 %); and PWP: permanent wilting point (12.3 %).

Fresh biomass (FB), gel percentage (GP), and harvest index (HI). FB was affected by both water deficit and salinity, but to a lesser extent in FC than in PWP. In both irrigation treatments, the negative impact on productivity was above 20 mM salinity (40, 60, and 80 mM). The latter effect was more severe in PWP at any salinity level (Figure 1).

Percentage of gel showed a negative relationship with salinity, the effect was slightly greater in PWP (β_1 = -4.41; R^2 = 0.98) than in FC (β_1 = -4.63; R^2 = 0.95), respectively (Figure 2). The maximum gel production (73.1 %) was

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found in the salinity control at FC. The plants under PWP and 0 NaCl produced 63 % gel . The harvest index (HI) behaved similar to gel production. The values for FC and PWP were β_1 = -1.40 and R^2 = 0.75 and β_1 = -1.50 and R^2 = 0.71, respectively (Figure 3).

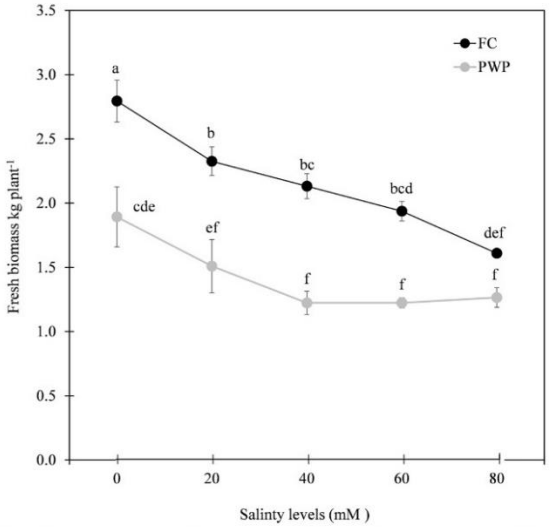

Figure 1. Effect of treatment on fresh biomass production in *Aloe vera* grown with two soil moisture contents and different salinity levels. Values with the same letters on each line are statistically equal according to the minimum significant difference of Tukey's test ($P \leq 0.05$). FC: field capacity (20.7 %); and PWP: permanent wilting point (12.3 %).

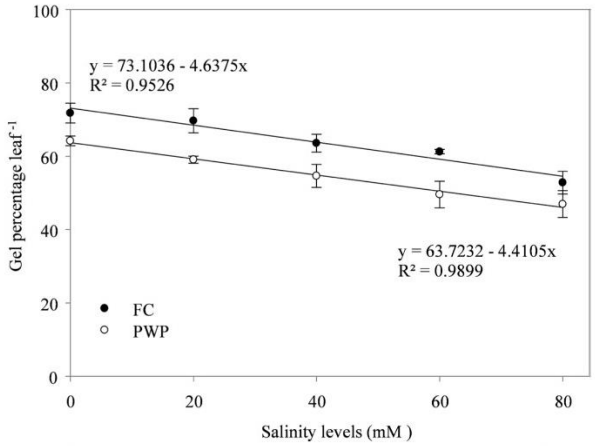

Figure 2. Aloe leaf gel production in *Aloe vera* grown with two moisture contents and different salinity levels. FC: field capacity (20.7 %); and PWP: permanent wilting point (12.3 %).

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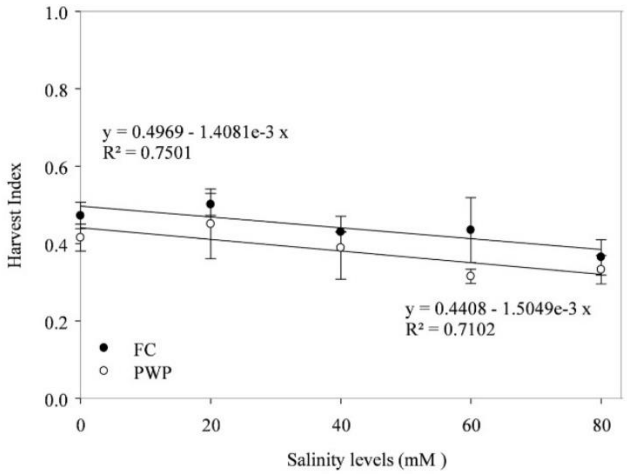

Figure 3. Relationship of the harvest index in *Aloe vera* plants grown with two soil moisture conditions and different salinity levels. FC: field capacity (20.7 %); and PWP: permanent wilting point (12.3 %).

Discussion

Plant growth and leaves. The minimum soil moisture content in this study was 10 %, corresponding to moisture below the PWP (12.3 %), but this was not enough to induce a stress condition within the *A. vera* plant tissues and reduce growth. Tolerance to water stress in *A. vera* is widely recognized and is associated with tissue succulence and the photosynthetic pathway of crassulacean acid metabolism (CAM) (Pedroza & Gómez 2006).

Salinity affected the width and thickness of the leaf, with a tendency to reduce both at 40 mM salinity on average. This suggests a moderate tolerance to this stress factor at both soil moisture contents, without drastically reducing leaf width and thickness, which are directly related to fresh biomass production. This confirms the high tolerance of *A. vera* to water stress conditions in the growth of the plant and leaves, but there was a negative impact of salinity, in the thickness and width of the leaves and, inconsistently, in plant height. This is supported by Asghari & Ahmadvand (2018), who found that salinity did not affect *A. vera* plant height consistently; however, other authors reported that salinity does inhibit plant growth (Aziz *et al*. 2008, Said-Al & Omer 2011).

This negative effect of salinity is recognized as a product of stress, which triggers physiological and biochemical changes in plant metabolism that determine their survival and productivity (Samadi *et al*. 2019). Salinity damage is associated with a toxic effect of ions such as sodium (Na^+), chlorides (Cl^-), and sulfates (SO_4^-), although sodium is an essential micronutrient in plants with C_4 and CAM photosynthetic pathways; hence, its intermediate-range of tolerance to salinity (Lamz & Gonzalez 2013). Another effect of salinity in irrigated soils with saline water is a nutritional imbalance in the plant from reduced assimilation of other mineral nutrients, such as K, Ca, and Mn (Karimi *et al*. 2009). To soil salinity must be added poor management, which reduces water permeability and soil aeration and compacts the soil structure (Singh *et al*. 2012).

The width and thickness of the leaf suggest that salinity has a negative effect, somewhat independent of the soil moisture content, due to the parallel but differentiated response, with an inhibition effect on both variables from 20 mM, but a greater impact on leaf thickness in FC and 40 mM salinity. This is important since these variables are directly related to gel production, the main commercial product in the international market (Sanchez 2016). The moderating effect of salinity on these morphometric variables may be related to an acclimatization process due to

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the gradual accumulation of NaCl in the soil. Therefore, a moderate initial exposition to a stress factor can induce protection mechanisms that allow plants to tolerate a subsequent drastic stress factor (Janda *et al*. 2016).

Plants previously treated with a low concentration of NaCl, turned out to be effective in inducing greater tolerance to salinity, throughout an acquired tolerance process (Pandolfi *et al*. 2016). Plant height and leaf length were not affected by either soil moisture content or salinity, suggesting that the total plant water potential remained high enough to allow cell division and growth of aloe plants (Rodríguez *et al*. 2019).

Relative water content (RWC) and total solids (TS). The RWC is the most commonly measured indicator of plant water status and is directly related to the water potential since its components, turgor potential and osmotic potential, are a function of the volume of water in the protoplasm (Argentel *et al*. 2006). The null effect of water deficit and salinity in the RWC shows high aloe plants' tolerance to these stress factors, as levels applied in this study were insufficient to modify tissue water status, which is determinant to the survival, the growth, and productivity of plants (Bacarrillo-López *et al*. 2021). These results are supported by Moghbeli *et al*. (2012) and Murillo-Amador *et al*. (2015), who found no effect on RWC under saline stress conditions. Although there were no significant differences in this variable under the soil moisture and NaCl conditions evaluated in this study, some authors reported that average RWC values > 90 % are acceptable to maintain the growth and productivity of aloe plants (Minjares-Fuentes *et al*. 2017).

TS increased in a more defined way after 80 DAST, somewhat independent of the soil moisture content, but strongly, starting at 20 mM salinity. This is of great relevance for gel quality, which requires a higher concentration of Total solids that leads to increased high and low-molecular-mass polysaccharides, and other products, which are greatly valued in the international market according to the International Aloe Science Council (IASC 2008). These results matched with other studies where an increase of TS concentration in *A. vera* gel was obtained by an increase in soil salinity. According to Sifuentes-Rodríguez *et al*. (2020), the concentration of sugars and percentage of total soluble solids was the highest as so did the soil salinity, even when aloe leaf production was reduced.

Fresh biomass (FB), gel percentage (GP), and harvest index (HI). The variable most affected by both water deficit and salinity was the FB, mainly in PWP at high salinity levels; while at FC, the plants maintained adequate productivity of FB. This suggests that, although there was a moderate effect on leaf width and thickness at low or no salinity, regardless of the moisture ranges evaluated in this study, the overall FB is reduced. This could be probably due to less water availability for metabolic activity and its final products and great concentration of solutes (osmotic pressure), where water in the plant's tissues plays a role more for survival than physiological availability for productivity (Sifuentes-Rodríguez *et al*. 2020). This could also be associated with the effect of osmotic adjustment in extreme environments and reduction in the cytosolic and vacuolar volumes of the cells (Flower *et al*. 2015). These results disagree with those of the Delatorre-Herrera *et al*. (2010) report, who indicates that, at a moderate water deficit, the production of FB increases. However, the results of our study were consistent with those reported by Choudhury *et al*. (2017), who found that a combination of two or more abiotic stresses produces greater yield loss than single stress.

The responses of gel reduction were directly related to FB production, most notably when going from FC to PWP, but with similar rates of decrease in both soil water treatments as salinity increased. The stress-induced by the combination of salinity and water deficit increased the severity of productivity reduction in *Aloe vera*. Rodríguez-Dorantes *et al*. (2003) mentioned that the first and most sensitive response to water deficit is a decrease in turgor and, consequently, the FB and gel production. Silva *et al*. (2013) found that plants usually react to extreme water stress by favoring the photosynthesis maintenance and reducing gel yield. Plants in general trend to reduce growth and productivity under conditions of biotic and/or abiotic stress (Cramer *et al*. 2011).

The HI of a crop determines yield under drought conditions (Deguchi *et al*. 2010). The decreased HI in this study was lesser than that of gel production and FB, which confirms the tolerance to stress that *Aloe vera* has compared to other crops that are more drastically affected by water deficit (Schafleitner *et al*. 2007, Ruttanaprasert *et al*. 2016).

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Finally, water deficit and salinity, mainly the latter, moderately affected the width and thickness of the *Aloe vera* leaf, which reduced FB, gel content, and HI, but increased gel quality through increased TS, an integral quality attribute demanded in the international market since reach high values of organic and inorganic matters as minerals as well as diverse types of polysaccharides of high and low molecular weight, among other types of residues. *Aloe vera* showed a high tolerance to water stress and moderate tolerance to salinity in the width and thickness of leaves, FB, and gel production. This allows focusing on managing low soil moisture content close to PWP, without negatively affecting FB, while using irrigation water with moderate salinity to maintain a balance between quantity and quality in the gel production of *A. vera.*

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Literature Cited

Ahanger MA, Tomar NS, Tittal SA, Argal S, Agarwal RM. 2017. Plant growth under water/salt stress: ROS production: antioxidant and significance of added potassium under such conditions. *Physiology and Molecular Biology of Plants* **23**: 731-744. DOI: https://doi.org/10.1007/s12298-017-0462-7

Argentel L, González LM, Ávila C, Aguilera R. 2006. comportamiento del contenido relativo de agua y la concentración de pigmentos fotosintéticos de variedades de trigo cultivadas en condiciones de salinidad. *Cultivos Tropicales* **27**: 49-53.

Asghari R, Ahmadvand R. 2018. Salinity stress and its impact on morpho physiological characteristics of *Aloe vera*. *Pertanika Journal of Tropical Agricultural Science* **41**: 411-422.

Aziz EE, Al-Amier H, Craker LE. 2008. Influence of salt stress on growth and essential oil production in peppermint, pennyroyal, and apple mint. *Journal of Herbs, Spices & Medicinal Plants* **14**: 77-87. DOI: https://doi.org/10.1080/10496470802341375

Azpilcueta-Pérez M, Pedroza-Sandoval A, Trejo-Calzada R, Sánchez-Cohen I, Jacobo-Salcedo MR. 2018. Chemical residuality in maize (*Zea mays* L.) fields irrigated with deep well water. *Ecosystems and Agricultural Resources* **5**: 111-17. DOI: https://doi.org/10.19136/era.a5n13.1311

Bacarrillo-López R, Pedroza-Sandoval A, Inzunza-Ibarra MA, Flores-Hernández A. Macías-Rodríguez FJ. 2021. Forage productivity of cactus varieties (*Opuntia* spp.) under different soil moisture regimes. *Ecosistemas y Recursos Agropecuarios* **8**: e2878, 2021. DOI: https://doi.org/10.19136/era.a8n3.2878

Balvanera PM, D Gurri AF, Zermeño-Hernández I. 2017. Resiliencia, vulnerabilidad y sustentabilidad de sistemas socioecológicos en México. *Revista Mexicana de Biodiversidad* **88**: 141-149. https://doi.org/10.1016/j.rmb.2017.10.005

Boudreau MD, Beland FA, Nichols JA, Pogribna M. 2013. Toxicology and carcinogenesis studies of a noncolorized whole leaf extract of *Aloe barbadensis* Miller (*Aloe vera*) in F344/N rats and B6C3F1 mice (drinking water study). *National Toxicology Program Technical Report Series* **577**: 1-266.

Calzada RAM, Pedroza SA. 2005. Evaluación físico-química del gel y jugo de la hoja de sábila (*A. barbadensis* M.) en diferentes prácticas de manejo. *Revista Chapingo Serie Zonas Áridas* **4**: 93-101.

Cano-Villegas O, Muro-Pérez G, Jurado E, Flores J, Castañeda-Gaytán JG, Aguirre O, Sánchez 0. 2021. Trends of climate change at the mid-low Nazas-Aguanaval inland basin based on a geographical approach. *Ecosistemas y Recurcursos Agropecuarios* **8**: e2704. https://doi.org/10.19136/era.a8n3.2704

Choudhury FK, Rivero RM, Blumwald E, Mittler R. 2017. Reactive oxygen species, abiotic stress and stress combination. *The Plant Journal* **90**: 856-67. DOI: https://doi.org/10.1111/tpj.13299

Cramer GR, Urano K, Delrot S, Pezzotti M, Shinozaki K. 2011. Effects of abiotic stress on plants: a systems biology perspective. *BMC Plant Biology* **11**: 1-14. https://doi.org/10.1186/1471-2229-11-163

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Davis SC, Simpson J, Gil-Vega KC, Niechayev NA, Tongerlo E van, Hurtado CN, Dever LV, Búrquez A. 2019. Undervalued potential of crassulacean acid metabolism for current and future agricultural production. *Journal of Experimental Botany* **70**: 6521-6537. DOI: https://doi.org/10.1093/jxb/erz223

Deguchi TTN, Wangchuk P, Itoh E, Matsumoto M, Zheng X, Gopa J, Iwama K. 2010. Aboveground characteristics, yield potential and drought tolerance in "Konyu" potato cultivars with large root mass. *Potato Research* **53**: 331-340. DOI: https://doi.org/10.1007/s11540-010-97

Delatorre-Herrera J, Delfino I, Salinas C, Silva H, Cardemil L. 2010. Irrigation restriction effects on water use efficiency and osmotic adjustment in *Aloe vera* plants (*Aloe barbadensis* Miller). *Agricultural Water Management* **97**: 1564-1570. DOI: https://doi.org/10.1016/j.agwat.2010.05.008

Espinoza-Garrido RM. 2011. Efecto de la disponibilidad de agua en el crecimiento y en la eficiencia en el uso del agua de aloe vera (*Aloe barbadensis* M.). BSc Thesis. Universidad de Chile.

Flower TJ, Munns R, Colmer TD. 2015. Sodium chloride toxicity and the cellular basis of salt tolerance in halophytes. *Annals of Botany* **115**: 419-431. DOI: https://doi.org/10.1093/aob/mcu217

Fujita M, Fujita Y, Noutoshi Y, Takahashi F, Narusaka Y, Yamaguchi-Shinozaki K, Shinozaki K. 2006. Crosstalk between abiotic and biotic stress responses: a current view from the points of convergence in the stress signaling networks. *Current Opinion in Plant Biology* **9**: 436-442. DOI: https://doi.org/10.1016/j.pbi.2006.05.014

Habibi G. 2018. Effects of severe and mild drought stress on the biomass, phenolic compounds production and photochemical activity of *Aloe vera* (L.) Burm.f. *Acta Agriculturae Slovenica* **111**: 463-476. DOI: https://doi.org/10.14720/aas.2018.111.2.19

Hazrati S, Tahmasebi-Sarvestani Z, Mokhtassi-Bidgoli A, Modarres-Sanavy SAM, Mohammadi H, Nicola S. 2017. Effects of zeolite and water stress on growth, yield and chemical compositions of *Aloe vera* L. *Agricultural Water Management* **181**: 66-72. DOI: https://doi.org/10.1016/j.agwat.2016.11.026

IASC [International Aloe Science Council]. 2008. Aloe Scientific Primer. Maryland, USA: International Aloe Science Council. Silver Spring,. 17 p. http://www.allaboutaloe.org/resources/Aloe%20Scientific%20Primer.pdf (accessed November 22, 2020)

İbrahimova U, Kumari P, Yadav S, Rastogi A, Antala M, Suleymanova Z, Zivcak M, Tahjib-Ul-Arif M, Hussain S, Abdelhamid M. 2021. Progress in understanding salt stress response in plants using biotechnological tools. *Biotechnology* **329**: 180-191 DOI: https://doi.org/10.1016/j.jbiotec.2021.02.007

Isah T. 2019. Stress and defense responses in plant secondary metabolites production. *Biological Research* **28**: 52-39. http://dx.doi.org/10.1186/s40659-019-0246-3

Janda T, Darko É, Shehata S, Kovács V, Pál M, Szalai G. 2016. Salt acclimation processes in wheat. *Plant Physiology and Biochemistry* **101**: 68-75. DOI: https://doi.org/10.17221/429/2020-PSE

Karimi S, Rahemi M, Maftoun ME, Tavallali V. 2009. Effects of long-term salinity on growth and performance of two pistachio (*Pistacia* L.) Rootstocks. *Australian Journal of Basic and Applied Sciences* **3**: 1630-1639.

Kopittke PM, Neal WM, Wang P, McKenna BA, Lombi E. 2019. Soil and the intensification of agriculture for global food security. *Environment International* **132**: 105078. https://doi.org/10.1016/j.envint.2019.105078

Lamz PA, Gonzalez CMC. 2013. Salinity as a problem in agriculture: plant breeding an immediate solution. *Tropical Crops* **34**: 31-42.

Mahdy AM. 2011. Soil properties and wheat growth and nutrients as affected by compost amendment under saline water irrigation. *Pedosphere* **21**: 773-781. https://doi.org/10.1016/S1002-0160(11)60181-1

Mattas K, Tsakiridou E, Michailidis A, Karelakis C. 2015. Sunflower: A high value crop? Thessaloniki: Working Papers from Aristotle University of Thessaloniki, Department of Agricultural Economics. DOI: https://doi.org/10.22004/ag.econ.232466

Mazuela APC. 2013. Agricultura en zonas áridas y semiáridas. *Idesia (Arica)* **31**: 3-4. https://doi.org/10.4067/S0718-34292013000200001

Minjares-Fuentes R, Medina-Torres L, González-Laredo RF, Rodríguez-González VM, Eim V, Femenia A. 2017. Influence of water deficit on the main polysaccharides and the rheological properties of *Aloe vera* (*Aloe*

This chapter was published as Mota-Ituarte, M., Pedroza-Sandoval, A., Minjares-Fuentes, R., Trejo-Calzada, R., Zegbe, J. A., & Quezada-Rivera, J. J. (2023). Water deficit and salinity modify some morphometric, physiological, and productive attributes of *Aloe vera* (L.). *Botanical Sciences*, *101*(2), 463-475. https://doi.org/10.17129/botsci.3175

barbadensis Miller) mucilage. *Industrial Crops and Products* **109**: 644-653. DOI: https://doi.org/10.1016/j.indcrop.2017.09.016

Mittler R. 2006. Abiotic stress, the field environment and stress combination. *Trends in Plant Science* **11**: 15-19. DOI: https://doi.org/10.1016/j.tplants.2005.11.002

Mittler R, Merquiol E, Hallak-Herr E, Rachmilevitch S, Kaplan A, Cohen M. 2001. Living under a 'dormant' canopy: a molecular acclimation mechanism of the desert plant Retama raetam. *The Plant Journal* **25**: 407-416. DOI: https://doi.org/10.1046/j.1365-313x.2001.00975.x

Moghbeli E, Fathollahi S, Salari H, Ahmadi G, Saliqehdar F, Safari A, Grouh MSH. 2012. Effects of salinity stress on growth and yield of *Aloe vera* L. *Journal of Medicinal Plants Research* **6**: 3272-77. DOI: https://doi.org/10.5897/JMPR11.1698

Murillo-Amador B. Nieto-Garibay A, Troyo-Diéguez E, García-Hernández JL, Hernández-Montiel L, Valdez-Cepeda RD. 2015. Moderate salt stress on the physiological and morphometric traits of *Aloe vera* L. *Botanical Sciences* **93**: 639-648. DOI: https://doi.org/10.17129/botsci.73

Pandolfi C, Azzarello E, Mancuso S, Shabala S. 2016. Acclimation improves salt stress tolerance in *Zea mays* plants. *Journal of Plant Physiology* **201**: 1-8. DOI: https://doi.org/10.1016/j.jplph.2016.06.010

Pedroza SA, Gómez LF. 2006. La Sábila (*Aloe* spp). Propiedades, Manejo Agronómico, Proceso Agroindustrial y de Mercado. Universidad Autónoma Chapingo. DR © Universidad Autónoma Chapingo. Departamento de Publicaciones de la Dirección General de Difusión Cultural y Servicio. Chapingo, México. ISBN: 968-02.0242.9

Richards LA. 1948. Porous plate apparatus for measuring moisture retention and transmission by soil. *Soil Science* **66**: 105-10. DOI: https://doi.org/10.1097/00010694-194808000-00003

Rodríguez LND, Torres SCN, Hidalgo RJE. 2019. Efecto del estrés salino en el crecimiento y contenido en las variedades IR-43 y amazonas de *Oryza sativa*. *Arnaldoa* **26**: 931-942.

Rodríguez-Dorantes AM, Gracía-Castañeda MT, Muñoz-Sánchez JL. 2003. Análisis bioquímico y fisiológico de *Fouquieria splendens* ssp. *breviflora* bajo la acción de agentes de estrés hídrico. *Polibotánica* **16**: 49-62.

Ruttanaprasert R, Jogloy S, Vorasoot N, Kesmala T, Rameshwar S, Kanwar C. Holbrook C, Patanothai A. 2016. Effects of water stress on total biomass, tuber yield, harvest index and water use efficiency in Jerusalem artichoke. *Agricultural Water Management* **166**: 130-138. DOI: https://doi.org/10.1016/j.agwat.2015.12.022

Said-Al AHAH, Omer EA. 2011. Medicinal and aromatic plants production under salt stress. A review. *Herba Polonica* **57**: 72-87.

Samadi S, Habibi G, Vaziri A. 2019. Exogenous trehalose alleviates the inhibitory effects of salt stress in strawberry plants. *Acta Physiologiae Plantarum* **41**: 1-11. DOI: https://doi.org/10.1007/s11738-019-2905-y

Sanchez ADL. 2016. *Analysis of commercial opportunities for Aloe vera gel in the Asian market, case: Japan and South Korea*. Bsc Thesis. La Salle University.

Schafleitner R, Gutierrez RRO, Gaudin A, Alvarado CAA, Nomberto MG, Tincopa MLR, Bolivar LAF, Delgado M, Reinhard S, Bonierbale M. 2007. Capturing candidate drought tolerance traits in two native Andean potato clones by transcription profiling of field grown plants under water stress. *Plant Physiology and Biochemistry* **45**: 673-690. DOI: https://doi.org/10.1016/j.plaphy.2007.06.003

Sifuentes-Rodríguez NS, Pedroza-Sandoval A, Zegbe JÁ, Trejo-Calzada R. 2020. Indicadores de productividad y calidad de gel de sábila en condiciones de estrés salino. *Revista Fitotecnia Mexicana* **43**: 181-187. https://doi.org/10.35196/rfm.2020.2.181

Silva H, Sagardia S, Ortiz M, Franck N, Opazo M, Quiroz M, Baginsky C, Tapia C. 2013. Relationships between leaf anatomy, morphology, and water use efficiency in *Aloe vera* (L) Burm f. as a function of water availability. *Chilean Journal of Natural History* **87**: 1-10. DOI: https://doi.org/10.1186/S40693-014-0013-3

Silva H, Sagardia S, Seguel O, Torres C, Tapia C, Franck N, Cardemil L. 2010. Effect of water availability on growth and water use efficiency for biomass and gel production in *Aloe Vera* (*Aloe barbadensis* M.). *Industrial Crops and Products* **31**: 20-27. DOI: https://doi.org/10.1016/j.indcrop.2009.08.001

This chapter was published as Mota-Ituarte, M., Pedroza-Sandoval, A., Minjares-Fuentes, R., Trejo-Calzada, R., Zegbe, J. A., & Quezada-Rivera, J. J. (2023). Water deficit and salinity modify some morphometric, physiological, and productive attributes of *Aloe vera* (L.). *Botanical Sciences*, *101*(2), 463-475. https://doi.org/10.17129/botsci.3175

Singh K, Singh B, Singh RR. 2012. Changes in physico-chemical, microbial and enzymatic activities during restoration of degraded sodic land: Ecological suitability of mixed forest over monoculture plantation. *CATE* **96**: 57-67. DOI: https://doi.org/10.1016/j.catena.2012.04.007

Xu CX, Ma, YP, Liu YL. 2015. Effects of silicon (Si) on growth, quality and ionic homeostasis of aloe under salt stress. *South African Journal of Botany* **98**: 26-36. https://doi.org/10.1016/j.sajb.2015.01.008

Yamasaki S, Dillenburg LR 1999. Measurements of leaf relative water content in *Araucaria angustifolia*. *Brazilian Journal of Plant Physiology* **11**: 69-75. https://www.agris.fao.org/agris-search/search.do?recordID=BR1999000685 (accessed May 26, 2019)

Zárate MAH, Miranda ZGA. 2016. Impact of climate change on food security in vulnerable peasant areas of the Andes of Peru. *Mexican Journal of Agricultural Sciences* **7**: 71-82. https://www.redalyc.org/articulo.oa?id=263144153006 (accessed March 17, 2020).

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CHAPTER IV. An improved method for RNA isolation from *Aloe vera* (L.) under abiotic stress and validation with RT-qPCR

Abstract

The initial process to analyze gene expression is to extract high-quality RNA. However, RNA extraction protocols are limited in their efficiency by the physicochemical characteristics of plant tissues that grow under environmental stress. Under these conditions, plants respond by producing secondary metabolites, such as polysaccharides and polyphenols, which can bind or co-precipitate with nucleic acids, acting as inhibitors. *Aloe vera*, a drought-resistant plant, produces polysaccharides and phenolic compounds as part of its secondary metabolism in response to stress. This study aimed to compare and evaluate five total RNA extraction methods from *Aloe vera* tissues, which have been subjected to salinity and water deficit. The final aim is to use the extracted RNA to analyze gene expression in future experiments. *Aloe vera* plants were treated with 20 and 80 mM NaCl solutions to induce salinity. Simultaneously, two soil moisture levels were maintained, field capacity (FC) and permanent wilting point (PWP), corresponding to 20.7 and 12.3 % of soil water content, respectively. A total of four treatments were obtained: M1 (FC and 20 mM), M2 (FC and 80 mM), M3 (PWP and 20 mM), and M4 (PWP and 80 mM). Five extraction methods evaluated: Plant RNeasy QIAGEN® Kit (hereinafter Kit); protocol proposed by Li et al. (2014) with modifications, using Trizol (hereinafter Trizol); protocol proposed by Salinas et al. (2019) with modifications, using CTAB (hereinafter CTAB2); protocol proposed by Rubio-Piña and Zapata-Pérez (2011) with modifications, using CTAB (hereinafter CTAB3). Finally, the proposed CTAB3a method is optimized for the characteristics of the study sample. The quality of the extracted RNA was evaluated through RT-qPCR. *Arabidopsis thaliana* was used as a control. Thus, the RNA concentration (ng μL^{-1}) showed significant differences among treatments and protocols ($P \leq 0.05$). Low RNA concentrations and purity

indicators below the expected value were obtained in the Kit. Trizol, CTAB2, and CTAB3 protocols. In the RT-qPCR analysis, the threshold cycle (C_T) values were taken as a measure of the efficiency of the extracted RNA, finding higher efficiency in the CTAB3a protocol. With the optimization of the CTAB3a method was possible to increase the quality and efficiency of the extracted RNA, for use in gene expression analysis.

Keywords: RNA extraction, recalcitrant tissues, real-time PCR, plant stress.

Resumen

El proceso inicial para analizar la expresión génica es extraer RNA de alta calidad. Sin embargo, los protocolos de extracción de RNA están limitados en su eficiencia por las características fisicoquímicas de los tejidos vegetales que crecen bajo estrés ambiental. En estas condiciones, las plantas responden produciendo metabolitos secundarios, como polisacáridos y polifenoles, que pueden unirse o co-precipitar con los ácidos nucleicos, actuando como inhibidores. El *Aloe vera*, una planta resistente a la sequía, produce polisacáridos y compuestos fenólicos como parte de su metabolismo secundario en respuesta al estrés. El propósito del estudio fue comparar y evaluar cinco métodos de extracción de RNA total de tejidos de *Aloe vera*, que han sido sometidos a salinidad y déficit hídrico. El objetivo final es utilizar el RNA extraído para analizar la expresión génica en futuros experimentos. Plantas de *Aloe vera* fueron tratadas con soluciones de NaCl 20 y 80 mM para inducir salinidad. Simultáneamente, se mantuvieron dos niveles de humedad del suelo, 20.7 % (capacidad de campo, FC) y 12.3 % (punto de marchitez permanente, PMP). Se obtuvieron un total de cuatro tratamientos: M1 (FC y 20 mM), M2 (FC y 80 mM), M3 (PWP y 20 mM) y M4 (PWP y 80 mM). Se evaluaron cinco métodos de extracción: Plant RNeasy QIAGEN® Kit (en adelante Kit); protocolo propuesto por Li et al. (2014) con modificaciones, utilizando Trizol (en adelante Trizol); protocolo propuesto por Salinas et al. (2019) con modificaciones, utilizando CTAB (en adelante CTAB2); protocolo propuesto por Rubio-Piña y Zapata-Pérez (2011) con modificaciones, utilizando CTAB (en adelante CTAB3). Finalmente, se propone el método

CTAB3a, optimizado para las características de la muestra de estudio. La calidad del RNA extraído se evaluó mediante RT-qPCR. *Arabidopsis thaliana* se utilizó como control. La concentración de RNA (ng μL^{-1}) mostró diferencias significativas entre tratamientos y entre protocolos ($P \leq 0.05$). En los protocolos Kit, Trizol, CTAB2 y CETAB3, se obtuvieron bajas concentraciones de RNA e indicadores de pureza por debajo de los valores esperados. En el análisis de RT-qPCR, los valores de C_T (threshold cycle o umbral de ciclo) se tomaron como medida de la eficiencia del RNA extraído, encontrando una mayor eficiencia en el protocolo CTAB3a. Con la optimización del método CTAB3a fue posible incrementar la calidad y eficiencia del RNA extraído para su uso en análisis de expresión génica.

Palabras clave: Extracción de RNA, tejidos recalcitrantes, PCR en tiempo real, estrés en plantas.

Introduction

The physicochemical characteristics of plant tissues grown under environmental stress conditions, such as salinity and water stress, limit the efficiency of total RNA extraction protocols.

This is mainly due to the increased accumulation of high-molecular-weight polysaccharides and secondary metabolites that interfere with RNA isolation (Vennapusa et al., 2020). Secondary metabolites in oxidized form act as inhibitors for downstream applications, as they irreversibly bind to nucleic acids (Rezadoost et al., 2016). In addition, residues of some reagents used during extraction, such as phenol, guanidinium thiocyanate, salts used to catalyze the deposition of nucleic acid molecules during precipitation, and residues of organic compounds, decrease the quality of the isolated RNA (Afifah et al., 2021). The importance of RNA extraction lies on its wide use in several molecular biology assays, including gene expression analysis via quantitative reverse transcription polymerase chain reaction (RT-qPCR) and next-generation (Li et al., 2021). However, accurate and reliable gene expression data relies on the proper extraction of high-quality RNA.

CTAB-based nucleic acid extraction methods have been used in many plant species, especially those containing a high level of phenolic compounds and polysaccharides (Gehrig et al., 2000; Mushtaq et al., 2022; Prasad et al., 2022; Ramachandran et al., 2022).

Some of the most popular RNA isolation protocols are those that contain guanidine salts, such as commercial kits such as RNeasy (QIAGEN, Germany) and Trizol reagent (Invitrogen, USA). These have been used successfully on a variety of plants (Blanca et al., 2011; Sundell et al., 2017). However, for some species with high polysaccharide content, these protocols have resulted in poor quality and low yield RNA, compared to other methods (Liu et al., 2018; Vennapusa et al., 2020).

The main objective of the present study was to optimize an efficient RNA extraction method to obtain high-quality RNA from *Aloe vera* tissues exposed to salinity and water deficit (for details on culture management and stress imposition, see Mota-Ituarte et al. (2023)). The protocol proposed in this paper was the result of the individual evaluation of four RNA extraction protocols, where the quantity and quality of the extracted RNA were compared.

Two commercial methods were evaluated, the RNeasy Kit (QIAGEN, Hilden–Germany), with the manufacturer's protocol, and Trizol (Invitrogen, USA), with the Li et al. (2014) protocol with modifications. Additionally, three methods based on CTAB were evaluated: the protocol proposed by Salinas et al. (2019) with modifications (CTAB2); the protocol proposed by Rubio-Piña and Zapata-Pérez (2011) with modifications (CTAB3) and finally the proposed method CTAB3a. The latter optimized for the characteristics of the study sample. The modifications made to these extraction protocols were mainly in the reduction of the volume of the reagents used. RNA isolated by these methods was evaluated by RT-qPCR.

Materials and methods

Stress conditions applied to A. vera

The *A. vera* plants were cultivated in the University Regional Unit for Arid Zones of the Autonomous University of Chapingo (Durango, Mexico), during the

spring and summer of 2020. The plants were treated with solutions of 20 and 80 mM NaCl to induce salt stress. In addition, together with the saline stress conditions, water stress was maintained, which was established with two soil moisture levels, field capacity (FC) and permanent wilting point (PWP), corresponding to 20.7 and 12.3 % of soil water content, respectively. The following treatments were obtained: M1 (FC and 20 mM), M2 (FC and 80 mM), M3 (PWP and 20 mM), and M4 (PWP and 80 mM). After the treatments, the plants were thoroughly washed with distilled water and the leaves were sectioned separately. Samples were immediately frozen in liquid nitrogen and stored at -70 °C until analysis. The tissue used for the RNA extraction assays was the leaf bark, for which it was separated, avoiding contamination with plant mucilage. The tissue was ground to an opaque green fine powder using a mortar and pestle, and liquid nitrogen. For each protocol, four samples were evaluated. *Arabidopsis thaliana*, grown under normal conditions, was used as a control.

RNA extraction

In all protocols, the same amount of sample was used, 200 mg of *A. vera* bark, in 1.5 mL Eppendorf-type microtubes. Additionally, all extraction buffers and materials were treated with DEPC distilled water and sterilized in an autoclave. In addition, the buffers were passed through sterile filters with a 0.22 um pore and 25 mm diameter. Table 5 shows the differences among the extraction methods used in this study.

Kit method. Protocol of Plant RNeasy mini QIAGEN® kit. The supplier-recommended protocol was used for samples with a high content of polyphenols and polysaccharides. At the end of the procedure, the pellet was resuspended in 25 µL of DEPC water. The method uses a guanidium thiocyanate-based lysis buffer and RNA purification was performed using a silica membrane column.

Table 5. Comparison of total RNA extraction methods. Four published methods (Kit, Trizol, CTAB2, CTAB3) versus the improved protocol (CTAB3a).

	Kit	Trizol	CTAB2	CTAB3	CTAB3a
Lysis	Homogenize in MBL solution, β-mercaptoethanol and PSS solution	Homogenize in Trizol, β-mercaptoethanol and KAc. Incubate at room temperature and centrifuge.	Homogenize in CTAB and β-mercaptoethanol Vortex and incubate at 65 °C.	Homogenize in CTAB and β-mercaptoethanol Vortex and incubate at 65 °C.	Homogenize in CTAB and β-mercaptoethanol Vortex and incubate at 65 °C.
RNA isolation	Vortex and centrifuge. Transfer the upper aqueous phase. Add IRS solution Vortex, incubate and centrifuge. Transfer the upper aqueous phase. Add PM3 and PM4 solution. Column and centrifuge.	Transfer the upper aqueous phase. **Add KAc and chloroform: isoamyl alcohol** Vortex, incubate and centrifuge. Transfer the upper aqueous phase. **Add chloroform: isoamyl alcohol.** Vortex, incubate and centrifuge. Transfer the upper aqueous phase.	**Add chloroform: isoamyl alcohol** Vortex and centrifuge. Transfer the upper aqueous phase and put on ice To the lower phase: **Add chloroform: isoamyl alcohol** Vortex and centrifuge. Mix the two previous supernatants	**Add chloroform** Vortex and centrifuge. Transfer the upper aqueous phase. **Add phenol: chloroform** Vortex and centrifuge. Transfer the upper aqueous phase. **Add chloroform: isoamyl alcohol** Vortex and centrifuge. Transfer the upper aqueous phase.	**Add chloroform** Vortex and centrifuge. Transfer the upper aqueous phase. **Add phenol: chloroform: isoamyl alcohol** Vortex and centrifuge. Transfer the upper aqueous phase. **Add chloroform: isoamyl alcohol** Vortex and centrifuge. Transfer the upper aqueous phase.
RNA precipitation	Add PM5 solution to the column. Centrifuge and put column in new tube.	Precipitate in isopropanol at -20°C for 45 min. Centrifuge.	Precipitate in LiCl at 4 °C overnight Centrifuge.	Precipitate in LiCl at 4 °C overnight Centrifuge.	Precipitate in LiCl at 4 °C overnight Centrifuge.
Washing	Add PM4 solution. Centrifuge.	Wash with 70% ethanol	Wash with 96% ethanol Wash with 70% ethanol	Wash with 96% ethanol Wash with 70% ethanol	Wash with 96% ethanol Wash with 70% ethanol
RNA solubilization	Add RNAse free water, incubate, centrifuge and discard column.	Dry at room temperature and resuspend in DEPC water.	Dry at room temperature and resuspend in DEPC water.	Dry at room temperature and resuspend in DEPC water.	Dry at room temperature and resuspend in DEPC water.

Trizol method. Li et al. (2014) with modifications. The modifications made to this protocol were mainly in the volumes of the reagents. A total of 500 µL of Trizol (Sigma-Aldrich), 100 µL of β-mercaptoethanol (Sigma-Aldrich), and 100 µL of 2.5 M KAc pH 5.2 were added to the sample. It was homogenized using vortexing. It was incubated at room temperature for 10 min, then centrifuged at 10,000 rpm for 5 min, at 4 ºC. The aqueous phase was transferred to a new tube and 300 µL of 2.5 M KAc pH 5.2 and an equal volume of the recovered supernatant of chloroform: isoamyl alcohol (24:1) were added. The mixture was vortexed at maximum speed. Subsequently, it was incubated for 10 min at room temperature and centrifuged at 10,000 rpm for 10 min at 4 ºC. The supernatant was carefully transferred to another tube, and again an equal volume of chloroform: isoamyl alcohol (24:1) was added. Again, the upper aqueous phase was recovered and two-thirds of the volume of isopropanol was added to the tube. The tube was kept at -20 °C for 45 min and centrifuged at 10,000 rpm for 10 min. The supernatant was discarded and the pellet was washed with 70% ethanol. Subsequently, it was allowed to dry at room temperature and the pellet was resuspended in 25 µL of DEPC water.

CTAB2 method. Salinas et al. (2019) with modifications. Extraction buffer CTAB 800 µL (2% w/v CTAB, 2% w/v PVP, 100 mM Tris-HCl pH 8, 25 mM EDTA, 2 M NaCl, 0.05% spermidine) and β-mercaptoethanol 100 µL were added to the sample. The mixture was shaken for 30 seconds and then incubated at 65 °C for 10 minutes, inverting the tube four times during the incubation, avoiding the formation of lumps. Subsequently, 500 µL of chloroform: isoamyl alcohol (24:1) was added and vortexed at maximum speed. Then, it was centrifuged at 10,000 rpm for 15 min at 4 °C. The supernatant was transferred to a new 1.5 mL Eppendorf-type microtube, placed on ice, and set aside. One volume of chloroform: isoamyl alcohol (24:1), equivalent to the recovered supernatant, was added to the pellet and vortexed again at full speed for 30 s. Then, it was centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatant was combined with the supernatant from the first centrifugation. Finally, to precipitate the RNA, 10 M LiCl was added in a volume

equivalent to a quarter volume of the total supernatant obtained, gently shaken, and incubated overnight at 4 °C. Subsequently, it was centrifuged at 10,000 rpm for 15 min at 4 °C. Finally, the pellet was resuspended in 20 µL of DEPC water.

CTAB3 method. Rubio-Piña and Zapata-Pérez (2011) with modifications. Extraction buffer CTAB 700 µL (2% w/v CTAB, 2% w/v PVP, 100 mM Tris-HCl pH 8, 20 mM EDTA, 1.4 M NaCl) and 100 µL of β-mercaptoethanol were added to the sample. The mixture was shaken for 30 seconds and then incubated at 65 °C for 10 minutes, inverting the tube four times during the incubation. Then, 500 µL of chloroform was added and the mixture was shaken for 30 seconds. Subsequently, the mixture was centrifuged at 10,000 rpm for 10 min at 4 °C. Then, the supernatant was transferred to a new tube, and 500 µL of phenol: chloroform (1:1) was added and shaken for 30 seconds. The mixture was centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatant was transferred to a new tube and an equal volume of chloroform: isoamyl alcohol (24:1) was added. Samples were shaken for 30 seconds and centrifuged at 10,000 rpm for 10 minutes at 4 °C. The final supernatant was transferred to a new tube and 1/3 volume of 10 M LiCl was added and kept at 4 °C overnight. Next, the samples were centrifuged at 10,000 rpm for 20 min at 4 °C. The pellet was washed with 96% and 70% ethanol. After drying at room temperature, the pellet was resuspended in 25 µL of DEPC water.

CTAB3a method. Extraction Buffer 700 µL (2% w/v CTAB, 2% w/v PVP, 100 mM Tris-HCl pH 8, 25 mM EDTA, 2 M NaCl, 0.05% spermidine) and 100 µL of β-mercaptoethanol were added to the sample. Then, it was shaken for 30 s in a vortex at maximum speed. It was incubated for 10 min at 65 ºC, inverting the tube 4 times during the incubation. 500 µL of chloroform was added and vortexed again for 30 s at maximum speed. Afterward, it was centrifuged for 10 min, at 4 ºC at 10,000 rpm. The supernatant was transferred to a new tube and 350 µL of phenol: chloroform: isoamyl alcohol (25:24:1) was added to it. It was vortexed for 30 s at maximum speed and centrifuged for 10 min at 4 ºC at 10,000 rpm. The supernatant was transferred to a new tube and an equal volume of the recovered supernatant of chloroform: isoamyl alcohol (24:1) was

added to it. It was shaken for 30 s in a vortex at maximum speed and centrifuged for 10 min, at 4 ºC and 10,000 rpm. Again, the supernatant was transferred to a new tube and 1/3 volume of 10 M LiCl was added. It was then allowed to precipitate overnight at 4 °C. Afterward, it was centrifuged for 20 min at 4 ºC at 10,000 rpm, decanted, and the pellet was washed with 800 µL of 96% ethanol. It was shaken gently and centrifuged for 5 min, at 4 ºC at 10,000 rpm. Additionally, the pellet was washed with 800 µL of 70% ethanol and gently agitated. It was centrifuged for 5 min at 4 ºC at 10,000 rpm and decanted. Finally, the pellet was dried at room temperature and resuspended in 20 µL of DEPC water.

Evaluation and quantification of the extracted RNA

RNA integrity was verified by electrophoresis on a 1% agarose gel, composed of DEPC water, MOPS, and 37% formaldehyde. Electrophoresis was performed at 50 V for 45 min. The gel was visualized under ultraviolet light on a UVP® MultiDoc It Imaging System photo documenter. Degraded band and lack of 28S and 18S RNA intensity indicate low RNA quality. RNA was quantified in a NanoDrop® 2000 Thermo Scientific spectrophotometer. RNA concentration (ng μL^{-1}) was determined and $A_{260/280}$ and $A_{260/230}$ ratios were used to assess RNA quality, where low $A_{260/280}$ (<1.8) and $A_{260/230}$ (< 2.0) indicate contamination by the presence of proteins or phenolic compounds. Subsequently, a DNase treatment was carried out using DNase I RNase free (Ambion Life Technologies) with the supplier protocol.

RT-qPCR

A total of 40 ng of pure RNA per reaction, obtained by each of the protocols described above, were used to estimate the expression levels of the actin gene in plants, using specific primers:

qActin-F: 5′- AGCCGTCGATGATTGGGATG -3′ with a melting temperature of 60 °C.

qActin-R: 5′- CCACTGAGCACAATGTTGCC -3′ with a melting temperature of 60 °C, designed based on conserved regions of sequences previously reported for other species (Salinas et al., 2019). The primers were synthesized by the Sigma-Aldrich company.

The RT-qPCR was carried out in triplicate using the Power SYBR® Green RNA-to-CT™ 1-Step kit, following the manufacturer's instructions, which were performed on the Applied Biosystems Step One™ with real-time PCR equipment. A negative control without RNA was used for RT-qPCR. The thermocycling program was as follows: reverse transcription at 48 °C for 30 min, followed by Taq polymerase activation at 95 °C for 10 min. Subsequently, an initial denaturation at 95 °C for 15 s, followed by alignment at 60 °C for 1 min. This for 40 cycles, followed by an analysis of the melting curve: 95 °C for 15 s, 60 °C for 1,5 s and 95 °C for 15 s. The value of C_T (Cycle Threshold) was taken as an indirect measure of efficiency. After RT-qPCR, melting curves were generated to verify the specificity of the amplification.

Statistical analysis

Statistical analyzes were performed using SAS 9.0 software (SAS Institute Inc., Cary, NC, USA.). Comparisons among groups were analyzed by one-way ANOVA, with the Least Significant Difference (LSD) test as a post hoc test. Differences were considered significant at $P \leq 0.05$. All data are reported as means ± standard deviation (SD).

Results and discussion

RNA integrity

RNA integrity was validated using the formaldehyde gel electrophoresis technique with MOPS-based agarose gels. A single band or two faint bands indicate a low RNA quality of the extracted samples. All the samples (100 %) from RNA isolated with the optimized method CTAB3a had better integrity characterized by sharp bands for 28S and 18S rRNA (Fig 1 Protocol: CTAB3a). In contrast, other methods produced faint RNA bands, with higher impurities in

the gel and a blurred background during the run, even with the control used in the extraction (Fig. 1 Protocol: Kit, Trizol, CTAB2, and CTAB3).

In accordance with Schoor et al. (2003), gene expression assays obtained from partially degraded RNA samples, with ribosomal bands still visible, exhibit a high degree of similarity compared to intact samples. So, this could still lead to significant results if used carefully.

One of the most important challenges in the use of total RNA from plants with a high polysaccharide content, such as *A. vera*, is the low amount of RNA obtained and its degradation. Therefore, high-quality extraction is essential. In this study, it was observed that the storage conditions of the samples, the temperature during the extraction, the processing and precipitation time, the centrifugation speed, the repetitions of the extractions and the elution volume are parameters that can be optimized in the RNA extraction processes from plants grown under abiotic stress conditions.

RNA integrity is directly related to RNA performance (Fleige & Pfaffl, 2006). That is, establishing an efficient RNA isolation protocol and introducing a method with minor modifications may maximize the amount of RNA recovered for downstream applications.

Afifah et al. (2021) found similar results to those of the present study, in the electrophoresis bands of plant RNA, with samples of *Solanum lycopersicum,* using a method based on Trizol. On the contrary, the bright bands indicate the presence of RNA of good quality, which could be seen in the gel of the CTAB3a method (Fig. 1).

These results are consistent with what was done by Deepa et al. (2014), who found that methods based on guanidine salts, such as Trizol and commercial kits, were not effective for extracting RNA from species of plants rich in secondary metabolites.

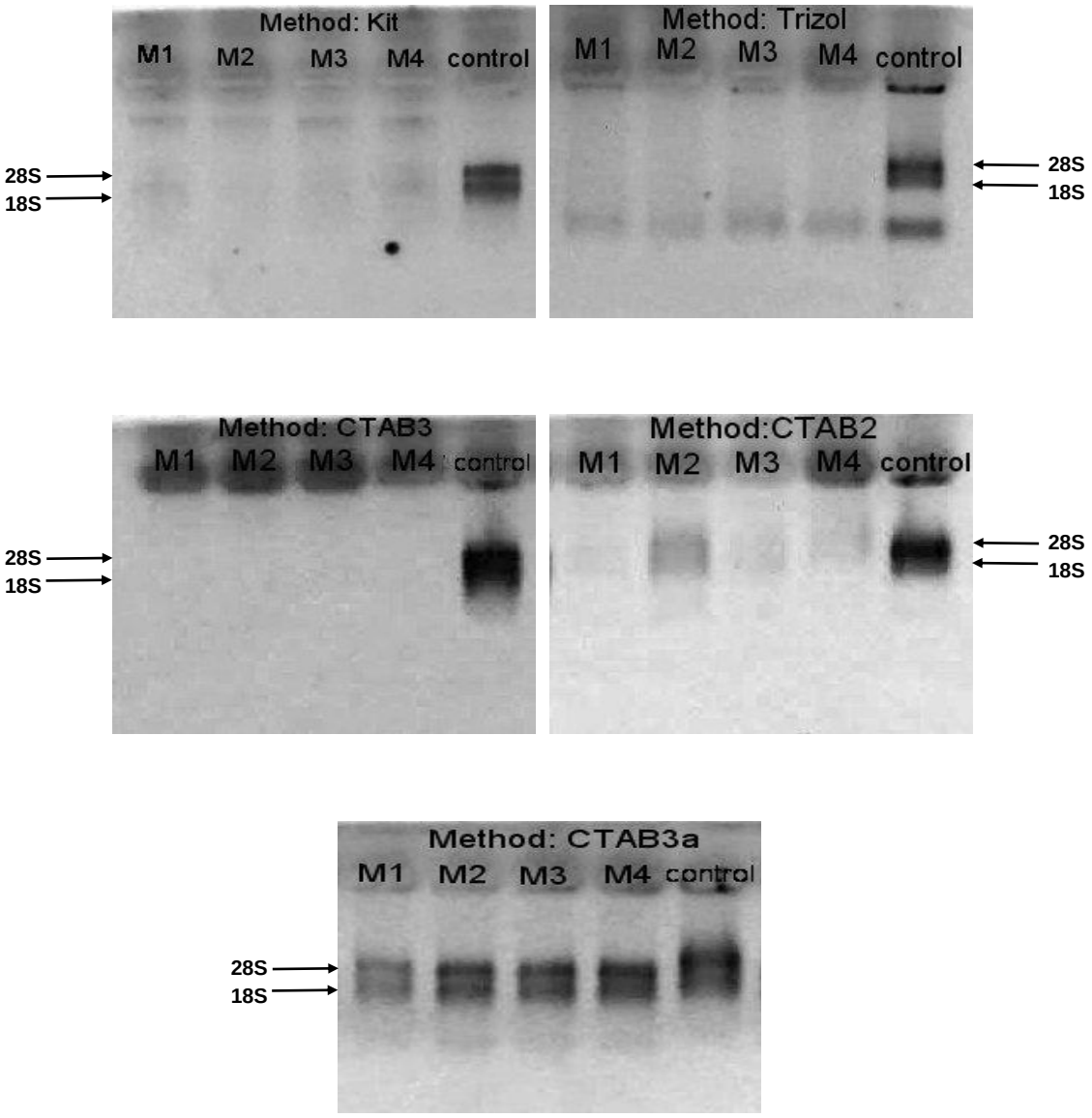

Figure 1. Agarose gel electrophoresis of *A. vera* RNA extracted by five extraction methods. The plants were grown under abiotic stress conditions. The agarose gel electrophoresis images used in this figure were autocorrected for the same brightness and contrast, using ImageJ software. Samples: M1 (FC and 20 mM), M2 (FC and 80 mM), M3 (PWP and 20 mM) and M4 (PWP and 80 mM), control *A. thaliana.*

RNA concentration

The RNA concentration (ng μL^{-1}) showed significant differences among samples and protocols ($P \leq 0.05$) (Table 6). Total RNA isolated with the CTAB3a method produced a yield statistically equal to the obtained with the Kit method. The other methods, Trizol, CTAB2, and CTAB3, were on average 68.64% higher compared to the CTAB3a method. However, during the extraction with these three methods, the handling of the sample was difficult since mucilage residues of the sample were still present in the washing and solubilization stages. The highest concentration values were found in the *A. thaliana* control sample for most of the protocols, except for the Trizol method. Previous studies reported a low RNA concentration in samples subjected to abiotic stress (Sajeevan et al., 2014; Shahrokhabadi et al., 2008). In this study, we observed a lower RNA concentration in *A. vera* samples subjected to water deficit and salinity stress, compared to the control *A. thaliana*. In accordance with Chang et al. (1993) and Shahrokhabadi et al. (2008), the reduced concentration of RNA and the interference of secondary metabolites in its extraction depend on the severity of the stress and the type of tissue.

Table 6. Comparative evaluation of the RNA concentration (ng μL^{-1}) extracted, using five different isolation methods, from *Aloe vera* grown under abiotic stress conditions.

Extraction Method	Sample M1				M2				M3				M4				Control*			
Kit	19.37	±	5.58	bcd	9.43	±	3.94	cd	21.57	±	9.88	bcd	20.53	±	3.31	bcd	107.60	±	0	b
Trizol	72.93	±	11.76	bcd	56.60	±	14.80	bcd	91.83	±	20.95	bcd	104.13	±	11.51	bc	78.40	±	0	bcd
CTAB2	49.80	±	12.56	bcd	108.80	±	12.16	b	31.63	±	9.54	bcd	35.73	±	6.69	bcd	243.30	±	0	a
CTAB3	11.67	±	3.50	cd	51.10	±	9.72	bcd	23.17	±	8.48	bcd	72.63	±	12.52	bcd	275.40	±	0	a
CTAB3a	15.73	±	3.80	bcd	10.33	±	2.58	d	10.37	±	3.48	cd	20.30	±	7.81	bcd	79.40	±	0	bcd

Values presented are the mean ± SD of three replicate technical samples. Values with the same literal, are statistically equal according to the LSD test ($P \leq 0.05$). Samples: M1 (FC and 20 mM), M2 (FC and 80 mM), M3 (PWP and 20 mM), and M4 (PWP and 80 mM). * *A. thaliana*

In accordance with Roy et al. (2020), there is a correlation between RNA recovery and the initial mass of a sample; in this study, the same amount of sample was used for all extraction methods to avoid discrepancy. Farrell Jr (2009) considers that changes in precipitation conditions affect performance, so overnight precipitation was recommended for samples containing high secondary metabolites to ensure RNA recovery. This coincides with what was done by Vasanthaiah et al. (2008) in grape tissue rich in polyphenols and polysaccharides. In the present study, the CTAB-based methods were carried out with precipitation in LiCl, overnight at 4 ºC, which is reflected in a higher recovery of nucleic acids.

The Kit method tested in this study has a procedure that requires little time to carry out, however, it demands a considerable investment. The latter can be a limitation when it is intended to process many samples.

On the other hand, the Trizol method is one of the most common methods for RNA purification due to its simplicity and economic feasibility. However, the drawback of this method is often the low quality of the extracted RNA due to phenol residue and guanidinium thiocyanate contaminants from the reagents (Afifah et al., 2021).

The modified CTAB3a protocol was developed for the extraction of RNA from plant tissue with high polysaccharide and polyphenol content, as it is one of the main obstacles challenging functional studies involving plants under abiotic stress conditions (Šamec et al., 2021). This protocol is cost-effective compared to commercially available kits and was successful in obtaining quality RNA from *A. vera*, whereas Trizol and other kits generally failed. This was demonstrated by the RT-qPCR assay. The protocol standardized here has been modified to be reliable, using basic laboratory chemicals that researchers can access without relying on expensive reagent kits.

RNA quality

The proportions $A_{260/280}$ showed significant differences among samples and protocols ($P \leq 0.05$) (Table 7). For the Trizol and Kit protocols, the mean $A_{260/280}$ ratios were <1.8 (1.69 and 1.79, respectively). While for the CTAB2, CTAB3, and CTAB3a protocols, $A_{260/280}$ were >1.8 (1.84, 2.13, and 1.93, on average, respectively). The $A_{260/230}$ ratios also showed significant differences among samples and protocols ($P \leq 0.05$) (Table 7).

In accordance with Imbeaud et al. (2005), a ratio $A_{260/280}$ greater than 1.8 indicates minimal protein contamination. With the CTAB2 and CTAB3 methods were obtained quality indicators on average, $A_{260/280} > 1.8$ (Manchester, 1996). However, visualizing the integrity of the RNA in the gel, the presence of swept bands was verified, indicating degraded RNA for CTAB2. For CTAB3, the presence of bands was not observed. Unlike the CTAB3a method, where the two typical bands, 28S and 18S, of a well-defined RNA sample can be seen. This makes it evident that the assessment of the quality and purity of the isolate must be evaluated by means of spectrophotometry and electrophoresis together. Nucleic acid quality and purity assessments are more reliable with direct visualization on an electrophoresis gel, according to Kasajima et al. (2013).

Most of the samples extracted in all the extraction methodologies, used in this study, had a ratio $A_{260/230}$ below 1.8, which indicates the presence of contamination with salts, polysaccharides, and residues of extraction reagents (Table 7).

Other studies have reported this same problem, with values of $A_{260/230}$ of 0.51 and 0.94, with samples of seeds from *Triticum aestivum* (Vennapusa et al., 2020) and leaf samples of *Glycine max* (Bilgin et al., 2009).

Table 7. Comparative evaluation of proportions $A_{260/280}$ y $A_{260/230}$ of RNA extracted, using five different isolation methods, from *Aloe vera* grown under abiotic stress conditions.

Extraction Method	Sample	$A_{260/280}$				$A_{260/230}$			
	M1	1.63	±	0.2	bcd	0.79	±	0.1	bcd
	M2	1.76	±	0.2	bcd	0.66	±	0.1	bcd
Kit	M3	1.67	±	0.1	bcd	0.71	±	0.2	bcd
	M4	1.61	±	0.3	bcd	0.69	±	0.3	bcd
	Control*	1.80	±	.	bcd	0.98	±	0.0	bcd
	M1	1.92	±	0.2	abcd	0.34	±	0.3	d
	M2	1.49	±	0.3	d	0.16	±	0.0	d
Trizol	M3	1.72	±	0.2	bcd	0.33	±	0.1	d
	M4	1.81	±	0.1	abcd	0.46	±	0.2	d
	Control*	2.00	±	.	abcd	0.16	±	0.0	d
	M1	1.76	±	0.4	bcd	0.34	±	0.1	d
	M2	1.54	±	0.4	cd	0.41	±	0.1	d
CTAB2	M3	1.89	±	0.5	abcd	0.32	±	0.1	d
	M4	1.99	±	0.4	abcd	0.29	±	0.1	d
	Control*	2.04	±	.	abcd	1.51	±	0.0	abc
	M1	2.03	±	0.1	abcd	0.16	±	0.1	d
	M2	2.39	±	0.1	a	0.19	±	0.1	d
CTAB3	M3	2.09	±	0.2	abc	0.33	±	0.1	d
	M4	1.96	±	0.2	abcd	0.48	±	0.3	dc
	Control*	2.18	±	.	ab	2.23	±	0.0	a
	M1	2.05	±	0.3	abcd	0.61	±	0.1	d
	M2	1.91	±	0.1	abcd	0.58	±	0.2	ab
CTAB3a	M3	1.80	±	0.4	bcd	0.55	±	0.1	d
	M4	1.84	±	0.2	abcd	0.64	±	0.3	bcd
	Control*	2.04	±	0.0	abcd	2.52	±	0.0	a

Values presented are the mean ± SD of three replicate technical samples. Values in the same column with the same literal are statistically equal according to the LSD test ($P \leq 0.05$). * *A. thaliana.* Samples: M1 (FC and 20 mM), M2 (FC and 80 mM), M3 (PWP and 20 mM), and M4 (PWP and 80 mM).

Alike, some authors have reported low-quality of RNA isolated using Trizol, due to contamination with polysaccharides in sunflower seeds (Ma & Yang, 2011) and fungi (Schumann et al., 2013). Sánchez-Rodríguez et al. (2008) reported contamination by the presence of residual sugars at the end of the RNA extraction from fungi, explaining that it can occur when extraction kits are used. These ones kits contain a glass fiber matrix with which the carbohydrates in the sample can interact since it has oligo dT groups necessary for the capture of the terminal sequences polyA+ of RNA.

The use of the CTAB3a protocol showed better performance, decreasing the presence of polysaccharides ($A_{260/230}$ = 0.98 on average), compared to the other protocols used in this study. However, the low $A_{260/230}$ ratio is due to contamination of the RNA with polyphenols contained in the samples. Since, according to Pavoković et al. (2012), polyphenols absorb light in the spectrum at 240-290 and 340-380 nm.

Regarding the use of CTAB and LiCl in RNA extraction, it has been reported that they are ideal for increasing the quality of samples with a high content of contaminants (Sánchez-Rodríguez et al., 2008; Vasanthaiah et al., 2008). Clarke (2009) explains that this is because CTAB, under conditions of high salt content, binds to polysaccharides, removing them from the solution. While the function of LiCl is to facilitate the precipitation of RNA, free of DNA, proteins, or carbohydrates (Barlow et al., 1963).

The differences between the CTAB3 and CTAB3a protocol is the spermidine in the lysis buffer and the phenol-chloroform isoamyl alcohol in the extraction step, used in the CTAB3a method, which may have contributed to the improvement in the CTAB3a protocol in the parameter $A_{260/230}$. Ludyga et al. (2012) reported nucleic acid extraction protocols using phenol chloroform isoamyl alcohol, where higher purity was obtained in the isolated RNA and yielded the longest amplicons for downstream applications, compared to commercial extraction kits. A mixture of phenol and chloroform has also been shown to increase RNA yield and stability (Farrell Jr, 2009). On the contrary, Gallagher (2000) reported that the bass $A_{260/230}$ is caused by polysaccharides and residual contamination with the phenol reagent used in the extraction. In

accordance with Rodrigues et al. (2009), phenol extraction is based on the separation of macromolecules into organic and aqueous phases. The aqueous phase dissolves nucleic acids, carbohydrates, and cellular debris, while the phenolic phase transports proteins and lipids. At the end, the proteins in the phenolic phase are precipitated with methanol.

Given the difficulty in the RNA extraction due to the physical and chemical characteristics of the samples used in this study, it was necessary to add polyvinylpyrrolidone (PVP) reagents (Deepa et al., 2014) and spermidine (Nashimoto, 1992), to optimize RNA recovery. In addition, β-mercaptoethanol was used to prevent oxidation of the sample (Gonzalez-Mendoza et al., 2008). Regarding the differences between the CTAB2 and CTAB3a protocols, they are mainly the addition of mechanical steps in the CTAB3a method. The repetition of mechanical steps with the use of chloroform in the extraction stage and the washing of the RNA with ethanol increase its recovery both in quantity and quality (Roy et al., 2020). The repetition was also carried out in the final washing step, to remove residues of extraction reagents and salts used during precipitation (Afifah et al., 2021). A CTAB-based methodology has also been used successfully by Favreau et al. (2019) for high-quality RNA extraction and gene expression analysis of *Eucalyptus grandis* cultivated under water deficit.

RT - qPCR

The quality of the total RNA was further examined by RT- qPCR. All samples of total RNA extracted from *A. vera* were successfully amplified using an actin gene. Threshold Cycle values (C_T) were taken as an indirect measure of the efficiency of the extracted RNA, and statistical differences were found among protocols ($P \leq 0.05$). The absolute values of C_T obtained individually for each sample are graphically represented in a box plot (Fig. 2), where the median of C_T raw is represented by lines. The values of C_T were lower (higher sensitivity) as expected for RNA samples isolated with the CTAB3a method (23.14 ± 4.91), which corresponds to quality RNA. Extraction methods Kit (26.29 ± 3.21), Trizol (29.11 ± 3.80), CTAB2 (27.98 ± 2.35) and CTAB3 (24.40 ± 4.98) had values of C_T 13.47% higher than the CTAB3a method, on average.

Assessing the integrity and quality of RNA is a critical factor before validation of gene expression data (Fleige & Pfaffl, 2006). In this sense, RT-qPCR is very sensitive to impurities, so it is convenient to evaluate the functional integrity of the RNA isolated through this analysis, as a prior analysis of gene expression (Ghangal et al., 2009).

RNA extraction, optimization, and validation in this study were aimed at enabling optimal performance of RT-qPCR experiments in samples grown under abiotic stress conditions. A more suitable method for the extraction of RNA from *A. vera* cortex was implemented, which produced RNA with high integrity for this purpose. The difference in the value of C_T among the samples of each extraction protocol could be related to the differences in the purity of the total RNA (Deng et al., 2005).

Particularly, RT and PCR can be inhibited by many factors that have been shown to be present in the samples, in addition to exogenous contaminants. Some of these factors are derived from the tissue removed or may be the result of inefficient or haphazard laboratory handling. For instance, cell constituents, $Ca^{2+,}$ and the high concentration of genomic DNA are important factors (Fleige & Pfaffl, 2006; Wilson, 1997).

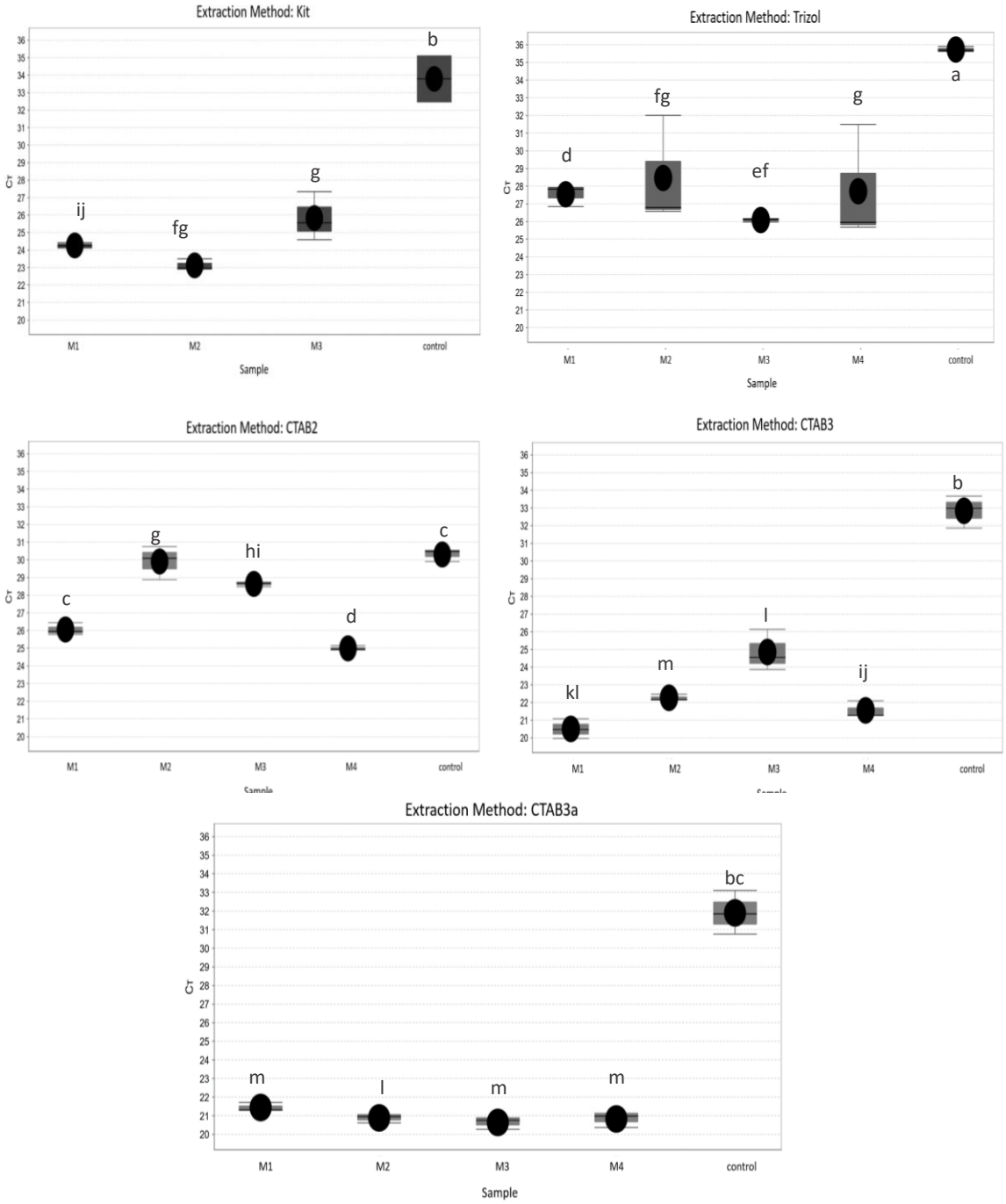

Figure *2*. Quantitative real-time PCR assay (RT-qPCR) using RNA extracted from *Aloe vera* grown under abiotic stress conditions, using five different isolation methods. *A. thaliana*, grown under normal conditions, was used as a control. The bars show the values of C_T were used as an indirect measure of efficiency (n = 4 biological replicates). Bars with the same literal are statistically equal according to the LSD test ($P \leq 0.05$). Samples: M1 (FC and 20 mM), M2 (FC and 80 mM), M3 (PWP and 20 mM) and M4 (PWP and 80 mM).

The specificities of the RT-qPCR amplification were confirmed by the melting curves. A single peak corresponds to a single amplification (Fig. 3) and indicates the purity and specificity of the amplified PCR fragment. The results showed that the specific amplification of the total RNA extracted from the *A. vera* plants was achieved in all the samples with the CTAB3a method. With the remaining four protocols (Kit, Trizol, CTAB2, CTAB3), it is possible to observe other peaks, in addition to the main one that represents the Tm; these extra peaks per curve represent non-specific products or dimers of primers (Li et al., 2021). Some various inhibitors or contaminants influence the presence of these non-specificities, such as pulp, polysaccharides, and polyphenols, among others (Li et al., 2021). No melting curves were detected in the negative control, so the extra peaks do not correspond to the inefficiency of the primers or poor handling of the samples. The negative control is the one that does not contain RNA and the volume is replaced with water (nuclease-free), it is the so-called «control without sample». This control makes it possible to detect contamination of the reagents.

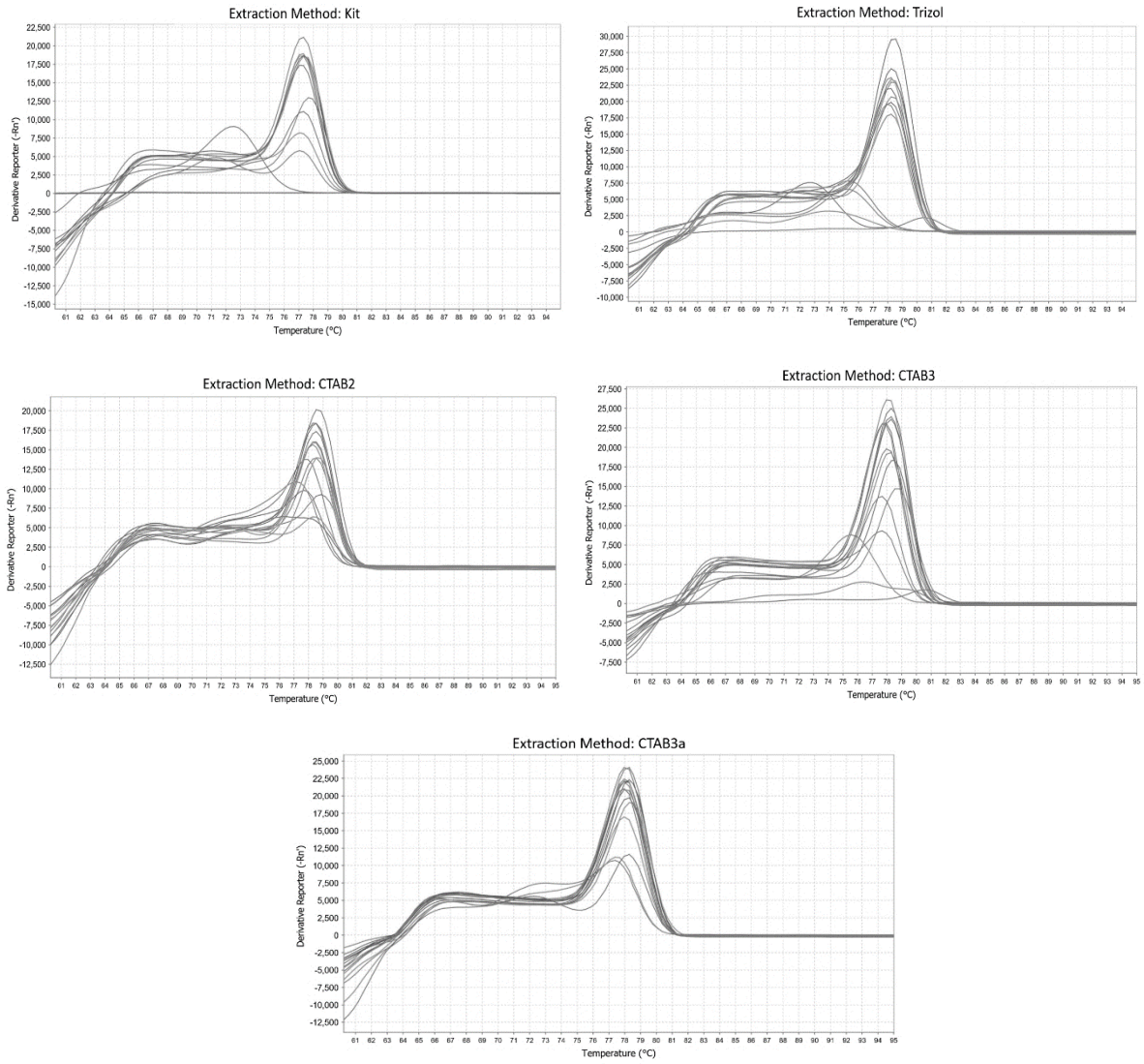

Figure 3. Melting curves of the RT-qPCR assay. A single peak corresponds to a single amplification. Specific amplification of total RNA extracted from *A. vera* using five extraction protocols: Kit, Trizol, CTAB2, CTAB3, and CTAB3a.

Conclusions

The RNA samples extracted using the CTAB3a method, ratios of purity $A_{260/280}$ close to two. Also, these RNA samples displayed sharp and strong bands in the electrophoresis agarose gel, showing a good integrity and high efficiency in the RT-qPCR assay. They show that the RNA extracted by this method has better quality compared to other RNA isolation methods evaluated, even for samples grown under higher stress conditions. The CTAB3a allowed an optimization in the amount of reagents, which is useful when it is required to process a large number of samples.

References

Afifah, A., Nounurai, P., Ferniah, R. S., Kusumaningrum, H. P., Wulandari, D., & Budiharjo, A. (2021). An Optimised TRIzol-based Protocol for the Improvement of RNA Extraction Yield of Tomato Stem. Pertanika Journal of Tropical Agricultural Science, 44(3).

Barlow, J., Mathias, A., Williamson, R., & Gammack, D. (1963). A simple method for the quantitative isolation of undegraded high molecular weight ribonucleic acid. Biochemical and Biophysical Research Communications, 13(1), 61-66. https://doi.org/10.1016/0006-291X(63)90163-3

Bilgin, D. D., DeLucia, E. H., & Clough, S. J. (2009). A robust plant RNA isolation method suitable for Affymetrix GeneChip analysis and quantitative real-time RT-PCR. Nature protocols, 4(3), 333-340. https://doi.org/10.1038/nprot.2008.249

Blanca, J., Cañizares, J., Roig, C., Ziarsolo, P., Nuez, F., & Picó, B. (2011). Transcriptome characterization and high throughput SSRs and SNPs discovery in Cucurbita pepo (Cucurbitaceae). BMC genomics, 12(1), 104. https://doi.org/10.1186/1471-2164-12-104

Clarke, J. D. (2009). Cetyltrimethyl ammonium bromide (CTAB) DNA miniprep for plant DNA isolation. Cold Spring Harbor Protocols, 2009(3), pdb. prot5177. https://doi.org/10.1101/pdb.prot5177

Deepa, K., Sheeja, T. E., Santhi, R., Sasikumar, B., Cyriac, A., Deepesh, P. V., & Prasath, D. (2014). A simple and efficient protocol for isolation of high quality functional RNA from different tissues of turmeric (Curcuma longa L.). Physiology and Molecular Biology of Plants, 20(2), 263-271. https://doi.org/10.1007/s12298-013-0218-y

Deng, M. Y., Wang, H., Ward, G. B., Beckham, T. R., & McKenna, T. S. (2005). Comparison of six RNA extraction methods for the detection of classical

swine fever virus by real-time and conventional reverse transcription–PCR. Journal of Veterinary Diagnostic Investigation, 17(6), 574-578. https://doi.org/10.1177/104063870501700609

Farrell Jr, R. E. (2009). RNA Methodologies: laboratory guide for isolation and characterization. Academic Press.

Favreau, B., Denis, M., Ployet, R., Mounet, F., Peireira da Silva, H., Franceschini, L., Carrer, H. (2019). Distinct leaf transcriptomic response of water deficient Eucalyptus grandis submitted to potassium and sodium fertilization. PLoS One, 14(6), e0218528. https://doi.org/10.1371/journal.pone.0218528

Fleige, S., & Pfaffl, M. W. (2006). RNA integrity and the effect on the real-time qRT-PCR performance. Molecular Aspects of Medicine, 27(2), 126-139. https://doi.org/10.1016/j.mam.2005.12.003

Gallagher, S. R. (2000). Quantitation of DNA and RNA with absorption and fluorescence spectroscopy. Current Protocols in Neuroscience, 10(1), A. 1K. 1-A. 1K. 8. https://doi.org/10.1002/0471142727.mba03ds93

Gehrig, H., Winter, K., Cushman, J., Borland, A., & Taybi, T. (2000). An improved RNA isolation method for succulent plant species rich in polyphenols and polysaccharides. Plant molecular biology reporter, 18(4), 369-376.

Ghangal, R., Raghuvanshi, S., & Sharma, P. C. (2009). Isolation of good quality RNA from a medicinal plant seabuckthorn, rich in secondary metabolites. Plant Physiology and Biochemistry, 47(11-12), 1113-1115. https://doi.org/10.1016/j.plaphy.2009.09.004

Gonzalez-Mendoza, D., Moreno, A. Q., & Zapata-Perez, O. (2008). An improved method for the isolation of total RNA from Avicennia germinans leaves. Zeitschrift für Naturforschung C, 63(1-2), 124-126. https://doi.org/10.1515/znc-2008-1-222

Imbeaud, S., Graudens, E., Boulanger, V., Barlet, X., Zaborski, P., Eveno, E. Auffray, C. (2005). Towards standardization of RNA quality assessment using user-independent classifiers of microcapillary electrophoresis traces. Nucleic acids research, 33(6), e56-e56. https://doi.org/10.1093/nar/gni054

Kasajima, I., Sasaki, K., Tanaka, Y., Terakawa, T., & Ohtsubo, N. (2013). Large-scale extraction of pure DNA from mature leaves of Cyclamen persicum Mill. and other recalcitrant plants with alkaline polyvinylpolypyrrolidone (PVPP). Scientia Horticulturae, 164, 65-72. https://doi.org/10.1016/j.scienta.2013.09.011

Li, J., Li, J., Liu, R., Wei, Y., & Wang, S. (2021). Identification of eleven meat species in foodstuff by a hexaplex real-time PCR with melting curve analysis. Food Control, 121, 107599. https://doi.org/10.1016/j.foodcont.2020.107599

Li, J., Zhang, Y., Liao, C., & Liu, X. (2014). Optimization of the procedure for extracting nucleic acids from aloe. Genetics and Molecular Research, 13(1), 276-282. https://doi.org/10.4238/2014.January.17.12

Li, S., Chen, L., Li, J., & Liu, J. (2021). Comparison of different protocols of RNA preparation from circulating blood for RNA sequencing. Biotechnology Letters, 43(9), 1685-1698.

Liu, L., Han, R., Yu, N., Zhang, W., Xing, L., Xie, D., & Peng, D. (2018). A method for extracting high-quality total RNA from plant rich in polysaccharides and polyphenols using Dendrobium huoshanense. PLoS One, 13(5), e0196592. https://doi.org/10.1371/journal.pone.0196592

Ludyga, N., Grünwald, B., Azimzadeh, O., Englert, S., Höfler, H., Tapio, S., & Aubele, M. (2012). Nucleic acids from long-term preserved FFPE tissues are suitable for downstream analyses. Virchows Archiv, 460(2), 131-140. https://doi.org/10.1007/s00428-011-1184-9

Ma, X., & Yang, J. (2011). An optimized preparation method to obtain high-quality RNA from dry sunflower seeds. Genet Mol Res, 10(1), 160-168. https://doi.org/10.4238/vol10-1gmr979

Manchester, K. L. (1996). Use of UV methods for measurement of protein and nucleic acid concentrations. Biotechniques, 20(6), 968-970. https://doi.org/https://doi.org/10.2144/96206bm05

Mota-Ituarte, M., Pedroza-Sandoval, A., Minjares-Fuentes, R., Trejo-Calzada, R., Zegbe, J. A., & Quezada-Rivera, J. J. (2023). Water deficit and salinity modify some morphometric, physiological, and productive attributes of Aloe vera (L.). Botanical Sciences, 101(2), 463-475. https://doi.org/10.17129/botsci.3175

Mushtaq, S., Tayyeb, A., & Firdaus e, B. (2022). A comparison of total RNA extraction methods for RT-PCR based differential expression of genes from Trichoderma atrobrunneum. Journal of Microbiological Methods, 200, 106535. https://doi.org/https://doi.org/10.1016/j.mimet.2022.106535

Nashimoto, M. (1992). Characterization of the spermidine-dependent, sequence-specific endoribonuclease that rquires transfer RNA for its activity. Nucleic acids research, 20(14), 3737-3742. https://doi.org/10.1093/nar/20.14.3737

Pavoković, D., Križnik, B., & Krsnik-Rasol, M. (2012). Evaluation of protein extraction methods for proteomic analysis of non-model recalcitrant plant tissues. Croatica Chemica Acta, 85(2), 177-183. https://doi.org/10.5562/cca1804

Prasad, S., Ajinath, L., & Mathew, D. (2022). Rapid and efficient protocol for genomic DNA extraction from leaf tissues of coconut (Cocos nucifera L.). Horticult Int J, 6(1), 17-21. https://doi.org/10.15406/hij.2022.06.00236

Ramachandran, R., Vishnu, B., Gangaprasad, A., & Siril, E. (2022). Effective and Reliable Protocol for DNA Isolation from Polyphenolic Leaves and

Fruits of Garcinia gummi-gutta (L.) Robs. Proceedings of the National Academy of Sciences, India Section B: Biological Sciences, 1-7.

Rezadoost, M. H., Kordrostami, M., & Kumleh, H. H. (2016). An efficient protocol for isolation of inhibitor-free nucleic acids even from recalcitrant plants. 3 Biotech, 6(1), 61. https://doi.org/10.1007/s13205-016-0375-0

Rodrigues, S. P., Ventura, J. A., Zingali, R., & Fernandes, P. (2009). Evaluation of sample preparation methods for the analysis of papaya leaf proteins through two-dimensional gel electrophoresis. Phytochemical Analysis: An International Journal of Plant Chemical and Biochemical Techniques, 20(6), 456-464. https://doi.org/10.1002/pca.1147

Roy, D., Tomo, S., Modi, A., Purohit, P., & Sharma, P. (2020). Optimising total RNA quality and quantity by phenol-chloroform extraction method from human visceral adipose tissue: A standardisation study. MethodsX, 7, 101113. https://doi.org/10.1016/j.mex.2020.101113

Rubio-Piña, J. A., & Zapata-Pérez, O. (2011). Isolation of total RNA from tissues rich in polyphenols and polysaccharides of mangrove plants. Electronic journal of Biotechnology, 14(5), 11-11. https://doi.org/10.2225/vol14-issue5-fulltext-10

Salinas, P., Salinas, C., Contreras, R. A., Zuniga, G. E., Dupree, P., & Cardemil, L. (2019). Water deficit and abscisic acid treatments increase the expression of a glucomannan mannosyltransferase gene (GMMT) in Aloe vera Burm. F. Phytochemistry, 159, 90-101. https://doi.org/10.1016/j.phytochem.2018.12.009

Šamec, D., Karalija, E., Šola, I., Vujčić Bok, V., & Salopek-Sondi, B. (2021). The Role of Polyphenols in Abiotic Stress Response: The Influence of Molecular Structure. Plants, 10(1), 118. https://www.mdpi.com/2223-7747/10/1/118

Sánchez-Rodríguez, A., Portal, O., Rojas, L. E., Ocana, B., Mendoza, M., Acosta, M., Höfte, M. (2008). An efficient method for the extraction of high-quality fungal total RNA to study the Mycosphaerella fijiensis–Musa spp. interaction. Molecular Biotechnology, 40(3), 299-305. https://doi.org/10.1007/s12033-008-9092-1

Schumann, U., Smith, N. A., & Wang, M.-B. (2013). A fast and efficient method for preparation of high-quality RNA from fungal mycelia. BMC Research Notes, 6(1), 71. https://doi.org/10.1186/1756-0500-6-71

Sundell, D., Street, N. R., Kumar, M., Mellerowicz, E. J., Kucukoglu, M., Johnsson, C., Hvidsten, T. R. (2017). AspWood: High-Spatial-Resolution Transcriptome Profiles Reveal Uncharacterized Modularity of Wood Formation in Populus tremula The Plant Cell, 29(7), 1585-1604. https://doi.org/10.1105/tpc.17.00153

Vasanthaiah, H. K. N., Katam, R., & Sheikh, M. B. (2008). Efficient protocol for isolation of functional RNA from different grape tissue rich in polyphenols

and polysaccharides for gene expression studies. Electronic journal of Biotechnology, 11, 42-51. http://www.scielo.cl/scielo.php?script=sci_arttext&pid=S0717-34582008000300005&nrm=iso

Vennapusa, A. R., Somayanda, I. M., Doherty, C. J., & Jagadish, S. (2020). A universal method for high-quality RNA extraction from plant tissues rich in starch, proteins and fiber. Scientific Reports, 10(1), 1-13. https://doi.org/10.1038/s41598-020-73958-5

Vining, K. J., Romanel, E., Jones, R. C., Klocko, A., Alves-Ferreira, M., Hefer, C. A., Strauss, S. H. (2015). The floral transcriptome of Eucalyptus grandis. New Phytol, 206(4), 1406-1422. https://doi.org/10.1111/nph.13077

Wilson, I. G. (1997). Inhibition and facilitation of nucleic acid amplification. Applied and environmental microbiology, 63(10), 3741-3751. https://doi.org/10.1128/aem.63.10.3741-3751.1997

CHAPTER V. Key genes regulating a glucomannan production of *Aloe vera* (L.) under salinity and salt-water stress combination

Abstract

Although the combined effects of different types of abiotic stress on plants are becoming more common due to climate change and its environmental consequences, studies that analyze them jointly are rare. This study aimed to identify the mannose-rich polysaccharide content and the expression of *glucomannan mannosyltransferase (GMMT)* gene, *xanthoxin dehydrogenase (ABA2)* gene, and *abscisic acid-glucosyltransferase (AOG*) in *Aloe vera* under salinity and salt-water stress combination. The experiment consisted of applying different concentrations of salt (0, 20, 40, 60, and 80 Mm NaCl) and two levels of soil moisture (field capacity (FC) and permanent wilting point (PWP), corresponding to 20.7 and 12.3 % of soil water content, respectively), to create ten treatments. Five of the treatments were with salinity (S) and irrigation at FC (S0, S20, S40, S60 y S80), while the other five included salinity and water deficit (WD) with irrigation at PWP (S0WD, S20WD, S40WD, S60WD y S80WD). By means of RT-qPCR, the relative expression of the *GMMT, ABA2,* and *AOG* genes was measured. The sugar composition was measured by means of gas chromatography. Both, salinity and saltwater stress combination, differences were found in the sugar content. In salinity, an upregulation of *GMMT* and *AOG,* and a downregulation of *ABA2* were found. In the salt-water stress combination, upregulation of *GMMT* and *ABA2*, while there was a downregulation of *AOG*. To conclude, in *A. vera*, the transcriptional regulation of ABA-related gene expression, as well as the content of mannose-rich polysaccharides, are factors that control tolerance to salt stress and a combination of salt-water stress.

Keywords: abiotic stress, combined stress, real-time PCR, acemannan

Resumen

Aunque los efectos combinados de diferentes tipos de estrés abiótico en las plantas son cada vez más comunes debido al cambio climático y sus consecuencias ambientales, los estudios que los analizan en conjunto son escasos. El objetivo de este estudio fue identificar el contenido de polisacáridos ricos en manosa y la expresión de los genes *glucomanano manosiltransferasa* (*GMMT), xantoxina deshidrogenasa* (*ABA2)* y *abscísico-glucosiltransferasa* (*AOG)* en *Aloe vera* en salinidad y una combinación de estrés hídrico-salino. El experimento consistió en aplicar diferentes concentraciones de sal (0, 20, 40, 60 y 80 mM) y dos niveles de humedad del suelo (capacidad de campo (FC) y punto de marchitez permanente (PWP), correspondientes a 20.7 & 12.3 %, respectivamente) para crear diez tratamientos. Cinco de los tratamientos fueron con salinidad y riego a FC (S0, S20, S40, S60 y S80), mientras que los otros cinco incluyeron salinidad y déficit hídrico con riego al PWP (S0WD, S20WD, S40WD, S60WD y S80WD). La composición de azúcares se midió por medio de cromatografía de gases. Por medio de RT-qPCR, se midió la expresión relativa de los genes *GMMT, ABA2* y *AOG*. Tanto para salinidad como combinación de estrés salino-hídrico se encontraron diferencias en el contenido de azúcares. En salinidad, fue encontrado un incremento la transcripción de *GMMT* y de *AOG,* y disminución en la transcripción de *ABA2*. En combinación de estrés salino-hídrico, incrementó la transcripción de *GMMT* y *ABA2*, mientras que hubo una disminución en la transcripción de *AOG*. Para concluir, en *A. vera*, la regulación transcripcional de la expresión génica relacionada con ABA, así como el contenido de polisacáridos ricos en manosa, son factores que controlan la tolerancia al estrés salino y a una combinación de estrés salino-hídrico.

Palabras clave: estrés abiótico, estrés combinado, PCR en tiempo real, acemanano

Introduction

Studies to analyze the effect of a combination of abiotic stresses on plants are not very common, even though, with climate change and its effects on the environment, the interaction of various types of stress is increasingly. Water and salt stress often coincide due to brackish water irrigation and the salt left after the water evaporates in irrigated agricultural areas (Xue et al., 2021). In the combination of salinity and drought stress, its main impact is through the osmotic effect, so the immediate downstream signaling process in response to these stresses is similar; however, it differs substantially at the end of a long period of stress. There is a large amount of evidence showing that the excessive accumulation of sodium (Na^+) and chloride (Cl^-) leads to irreversible damage. However, NaCl-mediated enhancement of plants is also reported to resist extreme drought and salt stress, enhance plant photosynthesis and metabolism (Chakraborty et al., 2022). Assessing the individual and combined effects of water deficit and salt stress on plant establishment, growing and develompment can help inform plant production management in terms of yield stabilization (Ors et al., 2021).

Abscisic acid (ABA) is a plant hormone involved in the control of a wide range of physiological processes, including adaptation to environmental stress (Audran et al., 1998). Expression of the *abscisic acid-glucosyltransferase AOG* or *ABA-GTase* gene regulates the conversion of ABA to its glucose ester, an inactive storage form of ABA (Jieyang et al., 2021). On the other hand, the *ABA2* gene of *xantoxin dehydrogenase* is involved in the biosynthesis of ABA (Chen et al., 2022). Moreover, Salinas et al. (2019) carried out a study where they found that the expression of the *glucomannan mannosyltransferase (GMMT)* gene, which codes for skeletal precursors of acemannan, the storage glucomannan of *A. vera*, increases with the water stress to which the plant is subjected.

These three genes, *GMMT, AOG,* and *ABA2*, were chosen in this study for their role in the pathways that are induced under abiotic stress conditions, and their reliability as stress indicators in *A. vera*. The objective of this study was to identify the mannose-rich polysaccharide content and the expression of *GMMT, AOG,* and *ABA2* in *Aloe vera* under salinity and salt-water stress combination.

Materials and methods

Sample

The experiment was carried out in the University Regional Unit for Arid Zones of the Autonomous University of Chapingo (Durango, Mexico), during spring to summer 2020. Salinity and water deficit treatments in *A. vera* plants, as well as morphometric, physiological and productive attributes, have been previously published in Mota-Ituarte et al. (2023). Five saline concentrations were applied: 0, 20, 40, 60, and 80 mM NaCl; and two levels of soil moisture were field capacity (FC) and permanent wilting point (PWP), corresponding to 20.7 and 12.3 % of soil water content, respectively. Resulting in ten treatments; five with salinity (S) and irrigation at FC (S0, S20, S40, S60, and S80) and five more with a combination of salinity and water deficit (WD), with irrigation at PWP (S0WD, S20WD, S40WD, S60WD, and S80WD). S0 was the control treatment.

At the end of the stress treatments, the plants were thoroughly washed by means of a sprinkler with distilled water. For RNA extraction and subsequent RT-qPCR, the third leaf from the inside out of the rosette of each plant was sectioned separately. Samples were immediately frozen in liquid nitrogen and stored at -70 °C until analysis.

After separating the leaves for RNA extraction, the samples were prepared to evaluate the polysaccharide content. The fillets containing the gel were separated from the leaves as described by Femenia et al. (1999). The leaves were cut, and the rind was separated from the fillet. Subsequently, the fillets were cut into small cubes and crushed, to later be homogenized. Approximately 500 g of homogenized fillets were frozen and freeze-dried in a LABCONCO FreeZone Triad Cascade Benchtop laboratory-scale freeze-dryer (LABCONCO, Kansas City, Missouri, USA) operated at 70×10^{-3} mBar with a condenser temperature of -40 ºC. The lyophilized *Aloe vera* gel was packaged and stored in anhydrous conditions until analysis.

Extraction of water-soluble polysaccharides

First, the Alcohol Insoluble Residues (AIR) of the *A. vera* samples were obtained as described by Femenia et al. (1999). Subsequently, the isolation of water-soluble polysaccharides was carried out as described by Minjares-Fuentes, Rodríguez-González, et al. (2017) with some modifications. The AIR suspension (500 mg AIR/L) was centrifuged at 13,000 g for 1 h at 20 °C and the supernatant, containing water-soluble polysaccharides, was lyophilized.

Sugar composition

For the samples of the soluble fraction, which mainly contains reserve glucomannan, acemannan, hydrolysis was carried out according to what was reported by Minjares-Fuentes, Rodríguez-González, et al. (2017). The soluble fractions were dispersed in H_2SO_4 1 M and hydrolyzed at 100 °C for 2.5 h. The neutral sugars released from the hydrolysis were derivatized as their corresponding alditol acetates and separated isothermally at 220 °C by gas chromatography (Hewlett-Packard 5890A, Waldbronn, Germany) with an FID detector, equipped with a 30 m DB column -225 (J&W Scientific, Folsom, CA, USA) with 0.25 mm ID and 0.15 µm film thickness.

RNA extraction

The bark was separated from the frozen leaves and stored at -70 °C, avoiding contamination with plant mucilage, due to the presence of polysaccharides that interfere with RNA extraction. The tissues were ground to an opaque green fine powder using a mortar and pestle and liquid nitrogen. RNA extraction was made as follows.

Extraction Buffer 700 µL (2% w/v CTAB, 2% w/v PVP, 100 mM Tris-HCl pH 8, 25 mM EDTA, 2 M NaCl, 0.05% spermidine) and 100 µL of β-mercaptoethanol were added to the sample. The mix was shaken for 30 s at maximum speed in a vortex. The next step was an incubation for 10 min at 65 ºC, inverting the tube four times. Chloroform 500 µL was added and vortexed again for 30 s at maximum speed. After, it was centrifuged for 10 min, at 4 ºC at 10,000 rpm. The supernatant was transferred to a new tube and 350 µL of phenol: chloroform: isoamyl alcohol (25:24:1) was added to it. It was vortexed for 30 s

at maximum speed and centrifuged for 10 min at 4 ºC at 10,000 rpm. The supernatant was transferred to a new tube and an equal volume of the recovered supernatant of chloroform: isoamyl alcohol (24:1) was added to it. It was shaken for 30 s in a vortex at maximum speed and centrifuged for 10 min, at 4 ºC and 10,000 rpm. Again, the supernatant was transferred to a new tube and 1/3 volume of 10 M LiCl was added. It was then allowed to precipitate overnight at 4 °C. Afterwards, it was centrifuged for 20 min at 4 ºC at 10,000 rpm, decanted, and the pellet was washed with 800 µL of 96% ethanol. It was shaken gently and centrifuged for 5 min, at 4 ºC at 10,000 rpm. Additionally, the pellet was washed with 800 µL of 70% ethanol and gently agitated. It was centrifuged for 5 min at 4 ºC at 10,000 rpm and decanted. Finally, the pellet was dried at room temperature and resuspended in 20 µL of DEPC water. Subsequently, a DNase treatment was carried out using DNase I RNase free (Ambion Life Technologies) with the supplier protocol.

Primers

An amount of 40 ng of RNA total per reaction was used to estimate the expression levels of the *GMMT, ABA2,* and *AOG* genes, with *actin* as the reference gene, using specific primers (Table 8). The primers were manufactured by Sigma-Aldrich.

Table 8. Primers used in the RT-qPCR assay.

Primer	Sequence	Tm (°C)	Expected product size (pb)	Reference
GMMT-F	5′-GTCCAGATCCCCATGTTCAACGAG-3′	60	107	Salinas et al. (2019)
GMMT-R	CCAACAGAATTGAGAAGGGTGAT-3′			
ABA2-F	5′-GGACAGTACAGAGGTCCAATTC-3′	55	80	Rodríguez-González et al., (2023)*
ABA2-R	5′- TCCTCAGCAACCTCCAAATC-3′			
AOG-F	5′- GGTGCCCACCCTCTTATTATC-3′	54	97	
AOG-R	5′- AAGGTGAAGGAGGAGGAGAA-3′			
Actin-F	5′- AGCCGTCGATGATTGGGATG -3′	60	116	Salinas et al. (2019)
Actin-R	5′- CCACTGAGCACAATGTTGCC -3′			

Tm: melting temperature, pb: base pairs. *Article in progress

Quantitative analysis RT-qPCR

RT-qPCR was carried out in triplicate, using Power SYBR® Green RNA-to-C_T™ 1-Step Kit in a real-time PCR system Step One™ of Applied Biosystems, with the manufacturer protocol, for a reaction volume of 10 µL. Negative control was used for RT-qPCR. The thermal cycle used was as follows: reverse transcription at 48 °C for 30 min, followed by activation of the taq polymerase at 95 °C for 10 min. Subsequently, an initial denaturation at 95 °C for 15 s, followed by alignment at 60 °C for 1 min. This process is for 40 cycles, followed by an analysis of the melting curve: 95 °C for 15 s, 60 °C for 15 s, and 95 °C for 15 s. Relative expression levels were determined using the Quantitation – Comparative C_T ($\Delta\Delta C_T$) method (Schmittgen & Livak, 2008):

$$Normalized\ level\ of\ expression\ of\ the\ target\ gene\ in\ the\ sample = 2\text{^}\ (-\Delta\Delta C_T)$$

After real-time PCR, melting curves were generated to verify the specificity of the amplification. The sizes of the PCR products were verified by agarose gel electrophoresis.

Statistical analysis

ANOVA and Fisher LSD post hoc test were performed to determine significant differences among the results obtained from each quantitative analysis. Statistical analyzes were performed using SAS 9.0 software (SAS Institute Inc., Cary, NC, USA).

Results and discussion

Sugar composition

The carbohydrate composition (g sugar/kg plant) showed significant differences among treatments ($P \leq 0.05$) for mannose and galactose. For glucose, no significant differences were found ($P \leq 0.05$). Table 9 shows the content of sugars present in the different soluble fractions obtained from the AIRs. All samples were characterized by the predominant presence of mannose, followed by minor amounts of glucose and galactose. These results are consistent with those obtained by Kumar and Kumar (2019) and Minjares-Fuentes, Rodríguez-González, et al. (2017).

Some trends can be observed, such as that the samples subjected to salinity with abundant irrigation (S20, S40, S60, and S80) were statistically equal to the control in the three sugars reported in this study. For manose, the samples with the highest levels of salt-water stress combination, with PWP irrigation (S60WD, and S80WD) were statistically equal to the control, whereas, treatments S20WD and S40WD had a decrease. This response of the plants may be because when the plant is under salinity conditions, it needs to store water to maintain constant hydration levels, since salt retains part of the irrigation water and, therefore, can cause dehydration. Hence, the glucomannan which has the reserve function, remains constant in concentration as the plant experiences salinity stress, since the amount of available water is abundant and constant, at FC in all plants. This could have been through ion exclusion strategies, as well as tissue tolerance, which plays

an important role since *A. vera* plants can use Na^+ as an osmoregulator (Chakraborty et al., 2022).

The polysaccharides content of *A. vera* is directly related to the culture conditions. Irrigation affects the amount of polysaccharides (Liu et al., 2019). Separately, salinity and water stress decrease the glucomannan content, according to what has been reported by some authors. In the case of irrigation with seawater at 42%, Jiang et al. (2014) reported that the concentration of polysaccharides decreases in the leaves at the base of the plant, but it is maintained in the upper and middle leaves. Minjares-Fuentes, Medina-Torres, et al. (2017) found that, with a water deficit of 60%, the mannose content decreases up to 41%. Quezada et al. (2017), reported that glucomannan increases in quantity under water stress.

In stress combination, plants may prioritize an acclimatization/adaptation strategy over the other, using a combination of the two responses, and/or using an entirely new strategy (Rivero et al., 2022). In this study, the effects of salinity on the sugar content were statistically equal to those obtained at extreme levels of combined salt-water stress (S60WD and S80WD). Therefore*, A. vera* plants could be using a synergistic response to salinity and water deficit. Production of polysaccharides under stress combination, a response driven by extreme drought, allowed the *A. vera* plants to enhance the overall response to the stress combination.

During stress combination, the main problem plants face is that the two types of stress can demand different and sometimes opposite metabolic and physiological responses (Rivero et al., 2022). Plants are generally stressed by salinity due to high osmotic pressure inhibiting water uptake, and crop symptoms are generally the same as those of water stress. In a salt-water stress combination, the response required of *A. vera* was to store as much water as possible since both, salinity and water deficit, cause water scarcity.

Table 9. Carbohydrate composition of the water-soluble polysaccharides isolated from *Aloe vera* under salinity and salt-water stress combination.

(g sugar/kg plant)

Treatment	Manose	Galactose	Glucose
S0*	2.079 ± 0.31 ab	0.064 ± 0.02 abc	0.189 ± 0.04 a
S20	2.599 ± 0.28 a	0.077 ± 0.01 a	0.194 ± 0.01 a
S40	1.801 ± 0.18 b	0.055 ± 0.00 abcd	0.170 ± 0.01 a
S60	1.964 ± 0.11 ab	0.077 ± 0.02 ab	0.195 ± 0.02 a
S80	1.748 ± 0.11 b	0.036 ± 0.00 cde	0.157 ± 0.02 a
S0WD	1.652 ± 0.54 b	0.048 ± 0.01 bcde	0.153 ± 0.03 a
S20WD	0.616 ± 0.48 c	0.024 ± 0.01 e	0.057 ± 0.03 a
S40WD	0.875 ± 0.35 c	0.040 ± 0.01 cde	0.090 ± 0.02 a
S60WD	1.659 ± 0.27 b	0.035 ± 0.01 de	0.149 ± 0.02 a
S80WD	1.727 ± 0.23 b	0.055 ± 0.00 abcd	0.205 ± 0.01 a

Values presented are the mean ± SD of three replicate technical samples. Values in the same column with the same literal are statistically equal according to the LSD test ($P \leq 0.05$). Treatments: S0 (salinity 0 mM NaCl & FC, *control treatment), S20 (salinity 20 mM NaCl & FC), S40 (salinity 40 mM NaCl & FC), S60 (salinity 60 mM NaCl & FC), S80 (salinity 80 mM NaCl & FC); S0WD (salinity 0 mM NaCl & PWP), S20WD (salinity 20 mM NaCl & PWP), S40WD (salinity 40 mM NaCl & PWP), S60WD (salinity 60 mM NaCl & PWP) and S80WD (salinity 80 mM NaCl & PWP).

GMMT expression in A. vera plants

The *GMMT* gene encodes the enzyme for the synthesis of acemannan (Salinas et al., 2019), the water reserve polysaccharide of *A. vera*. The relative expression *GMMT/Actin* showed significant differences among treatments ($P \leq 0.05$) (Figure 1). Expression was quantified using the RT-qPCR technique. The relative expression of *GMMT* was upregulated in *A. vera* plants under salinity, on average more compared to plants under salt-water stress combination. These results agree with those of other authors, who report that under salinity conditions, *A. vera* plants maintained adequate productivity in terms of gel production (Mota-Ituarte et al., 2023; Sifuentes-Rodríguez et al., 2020).

It is worth highlighting the S0WD treatment, with severe water restriction and no salinity, where the relative expression of GMMT is statistically equal to the control. In the research carried out by Salinas et al. (2019), where they identified the GMMT of *A. vera*, they found that its expression increased in the leaves of the plants with water deficit (50% FC), but not with the most severe water restriction treatment (25% FC), compared to control.

On the other hand, no significant difference was found in most of the salt-water stress combination levels, except in the highest level of stress where, as in the treatments with only salinity, the relative expression increased, up to four times more than the control. This is explainable since the water content in the tissues of plants classified as succulents plays a role more for survival than physiological availability for productivity (Sifuentes-Rodríguez et al. 2020). Under a combination of salt-water stress, *A. vera* prioritizes survival, thus reducing its photosynthetic activity and water storage.

Salinas et al. (2019) found, by means of an *in-silico* analysis, that *GMMT* belongs to the *cellulose synthase-like A type-9* (*CSLA9*) subfamily. *Cellulose synthase-like A (CSLA)* family genes have previously been reported to be involved in the plant response to abiotic stress and the biosynthesis of bioactive mannan polysaccharides (Davé & McCarthy, 1997; Goubet et al., 2009; He et al., 2015; Speicher et al., 2018). In addition, several investigations have shown the participation of members of the *CSLA* family of various plant species in the synthesis of 1,4-β-mannan and glucomannan skeletons. (Dhugga, 2012; Gille et al., 2011; Goubet et al., 2009; Liepman et al., 2007; Suzuki et al., 2006). The results of the expression of the *GMMT* gene in this study provide more information about the biosynthetic capacities of *CSLA* proteins in *A. vera* plants under salt-water stress combination.

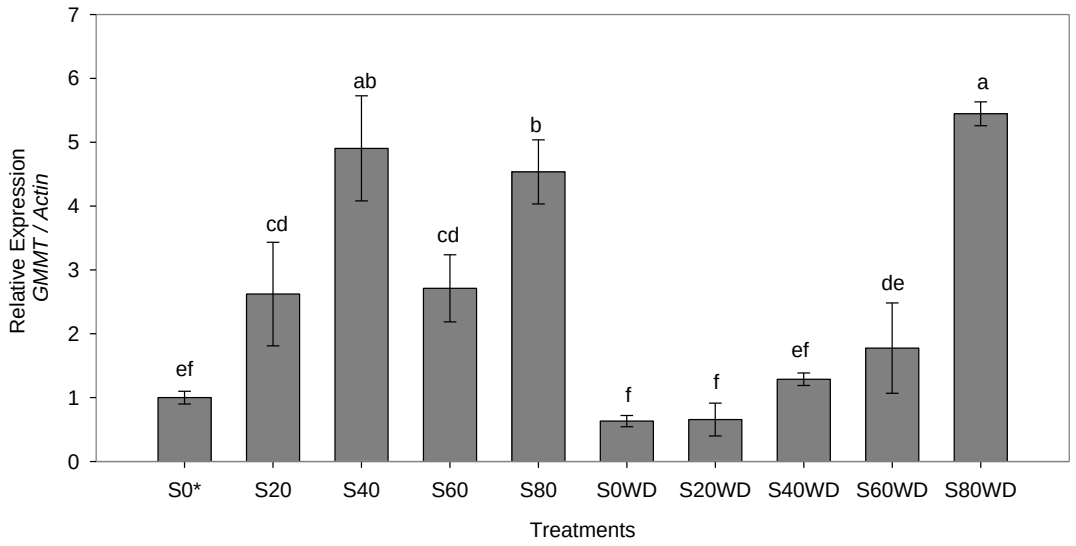

Figure 1. *GMMT* expression levels in *Aloe vera* plants subjected to salinity and salt-water stress combination. Treatments: S0 (salinity 0 mM NaCl & FC), S20 (salinity 20 mM NaCl & FC), S40 (salinity 40 mM NaCl & FC), S60 (salinity 60 mM NaCl & FC), S80 (salinity 80 mM NaCl & FC); S0WD (salinity 0 mM NaCl & PWP), S20WD (salinity 20 mM NaCl & PWP), S40WD (salinity 40 mM NaCl & PWP), S60WD (salinity 60 mM NaCl & PWP) and S80WD (salinity 80 mM NaCl & PWP). *S0 treatment was used as a calibrator and the expression levels of *ACTIN* as normalized. Mean values ± SD. Different letters represent significant differences among treatments (LSD test, $P \leq 0.05$). Four biological replicates and three technical replicates were used.

ABA2 expression in A. vera plants

The relative expression *ABA2/Actin* showed significant differences among treatments ($P \leq 0.05$) (Figure 2). Expression was quantified using the RT-qPCR technique. The relative expression of *ABA2* showed a higher presence in *A. vera* plants with salt-water stress combination treatments. The medium level (S40WD) of this stress combination is the one with the highest positive regulation, up to four times more than the control. The most severe stress combination treatments tend to decrease the expression of *ABA2*. As for the plants under salinity conditions, most showed lower relative expression values with respect to the control.

The *ABA2* biosynthetic enzyme *xanthoxin dehydrogenase* is a member of the *short-chain dehydrogenase/reductase* family, whose function is to catalyze the conversion of xanthoxin to the abscisic aldehyde in the ABA biosynthetic

pathway (Chen et al., 2022; Cheng et al., 2002; Jia et al., 2022). As is known, the importance of ABA lies in the fact that it is the main hormone that regulates the response of plants to abiotic stress (Brookbank et al., 2021; Jia et al., 2022; Li et al., 2022).

The action of *ABA2* is determinant for a basal level of ABA in plants (Léon-Kloosterziel et al., 1996). Lin et al. (2006) reported that *ABA2* expression in *A. thaliana* (*AtABA2*) slightly increased ABA levels and promoted salt stress tolerance. Endo et al. (2014) identified a *xanthoxin dehydrogenase* called *OsABA2* in rice and found that *OsABA2* mRNA expression did not change in response to dehydration treatment. In *A. thaliana*, *AtABA2* is expressed constitutively, at a relatively low level, and is not induced by dehydration stress (Cheng et al., 2002; González-Guzmán et al., 2002). Zhang et al. (2022) using genetic analysis showed that *OsABA2* can improve the sensitivity of rice to ABA and the tolerance of rice to drought and salt stress.

In this study, the expression of *ABA2* in *A. vera* plants under salinity conditions is lower than in plants in salt-water stress combination. Previously, it has been proposed that there are other minor ABA biosynthetic pathways or other genes, such as *SDRs (short-chain dehydrogenases/reductases*), which could replace the absence of *ABA2* with low efficiency (González-Guzmán et al., 2002; Seo & Koshiba, 2002).

In multifactorial stress combination of six different types of stress, Zandalinas et al. (2021) found that the function of *ABA2*, among other genes, was necessary for plant survival. That research was carried out on wild-type seedlings and different *Arabidopsis* mutants. The results of those investigations and the obtained in the present study reaffirm *ABA2* as one of the main actors in the response of plants to abiotic stress, and more importantly, its determining role when there is a combination of several stress factors.

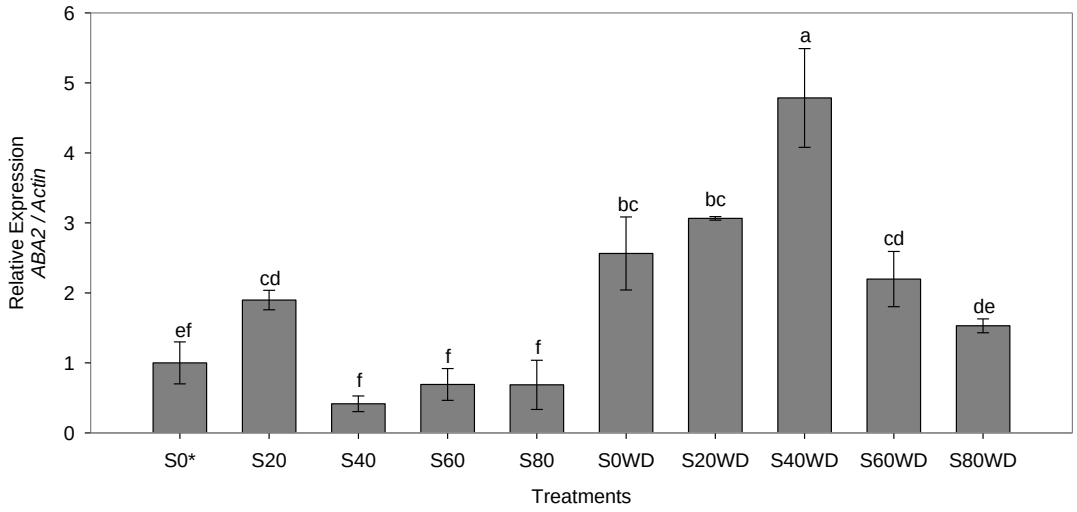

Figure 2. *ABA2* expression levels in *Aloe vera* plants subjected to salinity and combination salt-water stress. Treatments: S0 (salinity 0 mM NaCl & FC), S20 (salinity 20 mM NaCl & FC), S40 (salinity 40 mM NaCl & FC), S60 (salinity 60 mM NaCl & FC), S80 (salinity 80 mM NaCl & FC); S0WD (salinity 0 mM NaCl & PWP), S20WD (salinity 20 mM NaCl & PWP), S40WD (salinity 40 mM NaCl & PWP), S60WD (salinity 60 mM NaCl & PWP) and S80WD (salinity 80 mM NaCl & PWP). *S0 treatment was used as a calibrator and the expression levels of *ACTIN* as normalizer. Mean values ± SD. Different letters represent significant differences among treatments (LSD test, $P \leq 0.05$). Four biological replicates and three technical replicates were used.

AOG expression in A. vera plants

The relative expression *AOG/Actin* showed significant differences among treatments ($P \leq 0.05$) (Figure 3). Plants under salinity conditions show an important presence of *AOG* expression; increased expression can be observed in the first levels of stress, up to three times more than the control; subsequently, its decreases for the last two levels, still being double that of the control.

In plants under salt-water stress combination (S0WD to S80WD), it is observed a tendency to decrease the *AOG* expression. The salt-water stress combination represses the expression of the *AOG* gene, as the stress increases in severity.

The gene encoding *ABA glucosyltransferase* was initially identified in adzuki bean (*Vigna angularis),* and named *AOG*, it was also reported that its expression increased by water stress, so its expression is regulated by

environmental stress (Xu et al., 2002). When *ABA* is no longer required in an abiotic stress response, it can be degraded, catabolized, or stored in a bound *ABA-glucosyl-ester* (*ABA-GE*) form, catalyzed by *ABA-glucosyltransferase* (Sun et al., 2010; Zeevaart & RA, 1988). *ABA* in its glycosylated form has been identified in many plant species and is known to be a way to catabolize and reduce its levels. In this glycosylation process, *ABA-glucosyltransferase* (*ABA-GTase* or *AOG*) plays a key role (Sagar & Singh, 2019).

Felix et al. (2019) found that some genes involved in ABA activation/deactivation, including *AOG*, were subsequently elevated with osmotic stress applied to *Medicago sativa*. Pharmacological experiments have shown ABA catabolism may be beneficial for de novo ABA synthesis and critical for rapid early adaptation under abiotic stress (Yang et al., 2015).

In maize, the rate of ABA catabolism increases eleven-fold in response to water stress (Ren et al., 2006). Xu et al. (2002) analyzed the expression level of the *AOG* gene in adzuki beans and found that it was very low under normal conditions, while drought treatments increased its expression level.

Abiotic stress can not only cause an increase in ABA synthesis but can also promote the metabolism of the plants to lower its level. Consequently, both ABA biosynthesis and metabolic pathways and environmental stress have different degrees of effect on the ABA level in plants (Castellarin et al., 2007).

In the combination of salt-water in this study, a decrease in the expression of *AOG* is explainable since the glycosylated form of ABA is stored in the vacuoles to be used as energy in other functions of the plant. A characteristic of CAM plants, such as *A. vera*, is that under severe stress conditions, they prioritize survival over productivity, thus entering a state of dormancy where energy expenditure is minimal (March & Espinosa, 1988; Raven et al., 2005), where the presence of *AOG* is no longer required.

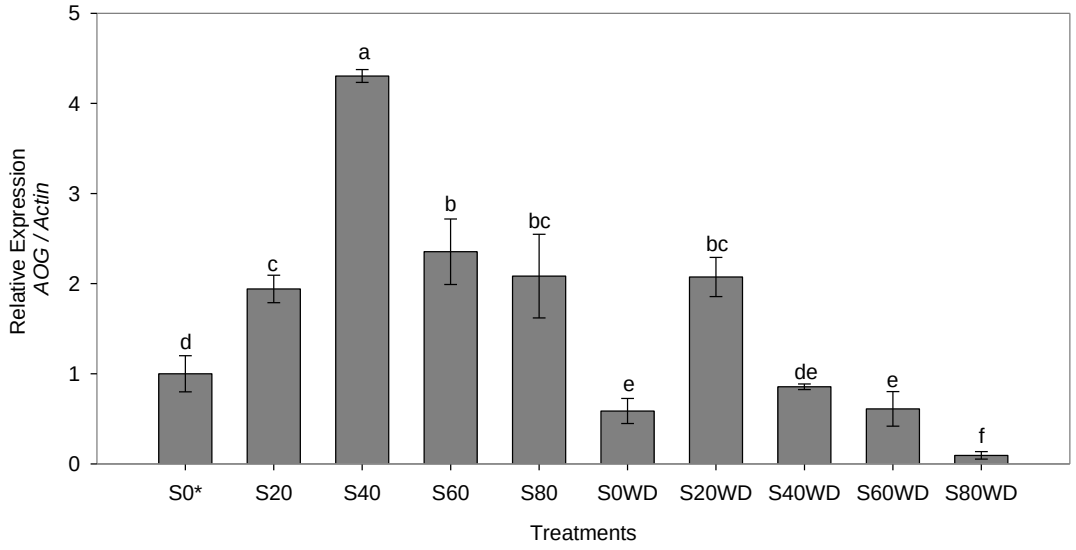

Figure3. *AOG* expression levels in *Aloe vera* plants subjected to salinity and combination salt-water stress. Treatments: S0 (salinity 0 mM NaCl & FC), S20 (salinity 20 mM NaCl & FC), S40 (salinity 40 mM NaCl & FC), S60 (salinity 60 mM NaCl & FC), S80 (salinity 80 mM NaCl & FC); S0WD (salinity 0 mM NaCl & PWP), S20WD (salinity 20 mM NaCl & PWP), S40WD (salinity 40 mM NaCl & PWP), S60WD (salinity 60 mM NaCl & PWP) and S80WD (salinity 80 mM NaCl & PWP). *S0 treatment was used as a calibrator and the expression levels of ACTIN as normalizer. Mean values ± SD. Different letters represent significant differences among treatments (LSD test, $P \leq 0.05$). Four biological replicates and three technical replicates were used.

Acemannan biosynthesis from A. vera

The main biological processes overrepresented in this study are associated with cellular carbohydrate metabolism and its relationship with ABA synthesis, including the metabolic process of acemannan, a mannose-rich polysaccharide, both under salt stress (Fig. 4A) and under salt-water stress combination (Fig. 4B). ABA seems to be involved in the control of *GMMT* expression in *A. vera* plants subjected to water deficit.

For the two soil moisture levels, no statistical differences were found in the acemannan content ($P \leq 0.05$). *GMMT*, *ABA2,* and *AOG* did show statistical differences for the two moisture levels (Fig. 4A). On the other hand, the acemannan content showed a substantial increase at the highest salinity levels

(Fig. 4B), showing statistical differences in both, acemannan content and in the relative expression of *GMMT*, *ABA2,* and *AOG* ($P \leq 0.05$).

Figure 4A shows that there is no statistical difference in the acemannan content in plants under water stress as compared to plants well irrigated. Therefore, we cannot conclude if the *AOG* and *ABA2* genes have any relationship with acemannan accumulation. However, it can be seen in Fig. 4B that at moderate salt stress levels (0, 20, and 40), *ABA2* expression increases. Indicating increased production of this hormone, since *ABA2* product participates in the early stages of ABA synthesis. Similarly, *AOG*, which participates in ABA catabolism, increases at these stress levels. The average level of stress (40) is the one with the highest presence for these two genes. This same level produced the lowest acemannan content. Measures of *ABA-GE* at a stress level of 60 may detect a significant presence of this compound stored in the vacuoles. This is possibly used as an energy source to produce other compounds, such as acemannan, which leads to a better response of *A. vera* to severe stress. Thus, at this level, a greater presence of *GMMT* is observed, as well as an increase in the acemannan content.

There are no previous reports on ABA regulation in *CSLA* family genes. The study carried out by Salinas et al. (2019) found that *GMMT* expression correlates with increased endogenous ABA levels in *A. vera* under water stress. Rai et al. (2016) found a positive regulation by ABA of a *xyloglucan galactosyltransferase* encoded by a *cellulose synthase-like C* (*CSLC*) in *Sorghum bicolor*.

High levels of ABA are critical to initiate signaling in the response to salt and water stress. Therefore, ABA levels should have some influence on acemannan content. Indeed, most genes involved in de novo ABA biosynthesis are upregulated by dehydration and salt stress (Jaiswal et al., 2021; Vafaei et al., 2023). Evidence to date indicates that various cell wall polysaccharides catalyzed by members of the *CSL* gene family are crucial for plant development and tolerance to biotic or abiotic stress. Several members of the Arabidopsis *CslA* family function in the synthesis of mannose-rich polysaccharides

(Dhugga, 2012; Goubet et al., 2009; Liepman & Cavalier, 2012; Liepman et al., 2007).

Further biochemical and molecular research on how *A. vera* components are synthesized and assembled under abiotic stress conditions will undoubtedly lead to a more complete understanding of the functions and properties of these bioactive components, such as acemannan. On the other hand, ABA metabolic genes are promising targets for molecular breeding work on tolerance to a combination of several simultaneous stress factors in crops.

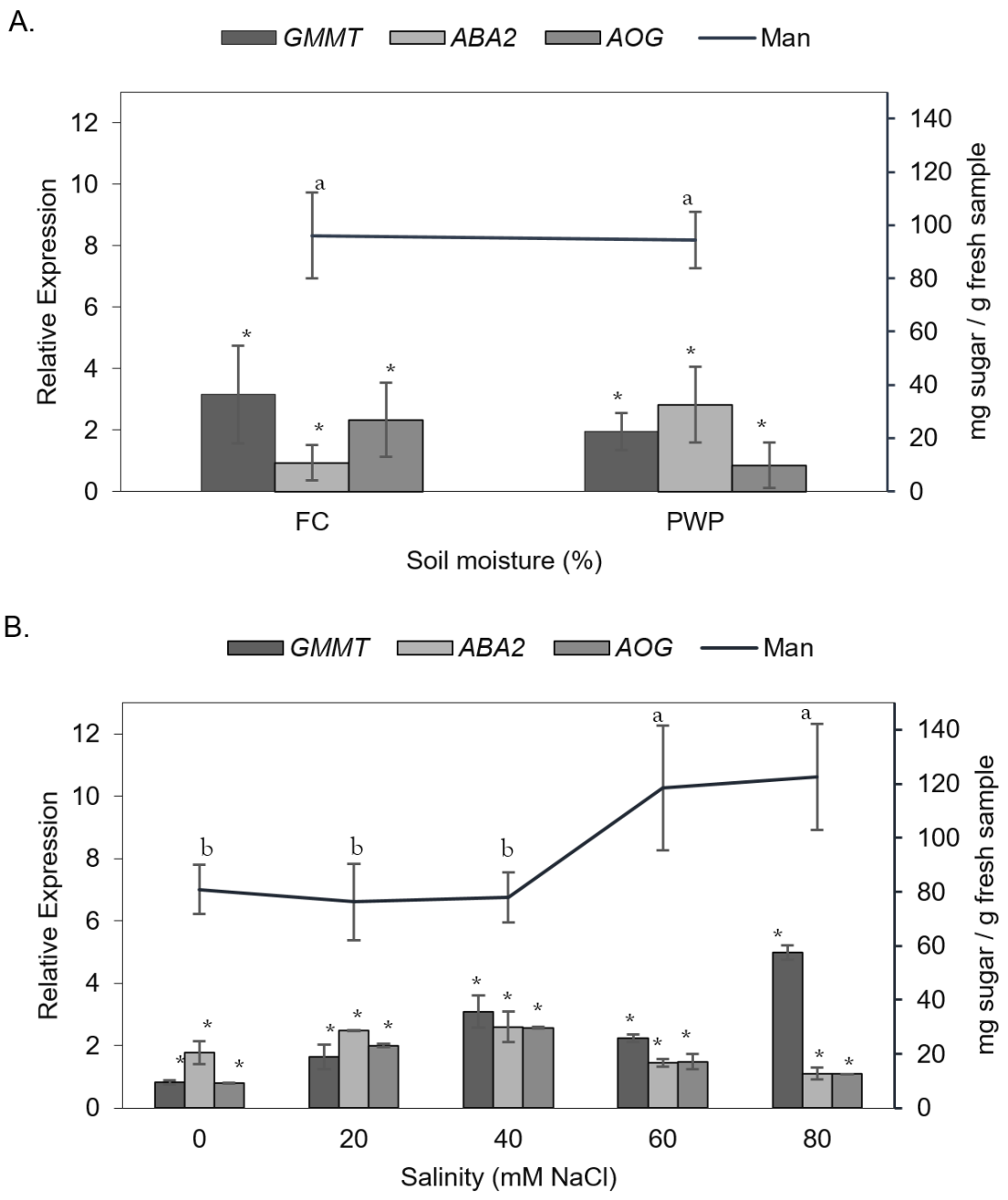

Figure 4. Expression of *GMMT, ABA2,* and *AOG* and mannose-rich polysaccharides content (mg sugar / g fresh sample) in *A. vera*. Values represent means ± SD. Asterisks indicate statistical significance (LSD, $P \leq 0.05$). The same test was applied to compare the mannose (Man) content in (A) two soil moisture levels (FC and PWP, corresponding to 20.7 & 12.3 % of soil moisture, respectively) and (B) five salinity levels. Salinity treatment 0 was used as a calibrator and *ACTIN* expression levels as normalizer. Four biological replicates and three technical replicates were used.

Conclusions

Upregulation of a *glucomannan mannosyltransferase* (*GMMT*) gene of *A. vera* was induced by salinity, as well as by salt-water stress combination.

For the *xantoxin dehydrogenase (ABA2)* gene a downregulation in salinity conditions, whereas that upregulation in salt-water stress combination was observed. Regarding *abscisic acid-glucosyltransferase* (AOG) gene, it was found upregulation in salinity conditions and a downregulation in salt-water stress combination. Therefore, the transcriptional regulation of ABA-related gene expression is a factor controlling tolerance to salt stress, as well as a combination of salt-water stress, in *A. vera*. The polysaccharide content shows a high tolerance of *Aloe vera* to abiotic stress, since no statistical difference was observed, even for the highest levels of salinity and combination of stress. The role of ABA-mediated polysaccharides and the mechanisms underlying their long-distance transport to the parenchyma require further clarification. These results could serve as a basis for further research on genes of interest that could be involved in the biosynthesis of *A. vera* acemannan. Studying the structure, function, and synthesis of acemannan will help to improve the quality of *A. vera* and increase the content of this polysaccharide.

References

Afifah, A., Nounurai, P., Ferniah, R. S., Kusumaningrum, H. P., Wulandari, D., & Budiharjo, A. (2021). An Optimised TRIzol-based Protocol for the Improvement of RNA Extraction Yield of Tomato Stem. *Pertanika Journal of Tropical Agricultural Science*, *44*(3). https://doi.org/10.47836/pjtas.44.3.10

Audran, C., Borel, C., Frey, A., Sotta, B., Meyer, C., Simonneau, T., & Marion-Poll, A. (1998). Expression Studies of the Zeaxanthin Epoxidase Gene inNicotiana plumbaginifolia 1. *Plant physiology*, *118*(3), 1021-1028. https://doi.org/10.1104/pp.118.3.1021

Barlow, J., Mathias, A., Williamson, R., & Gammack, D. (1963). A simple method for the quantitative isolation of undegraded high molecular weight ribonucleic acid. *Biochemical and Biophysical Research Communications*, *13*(1), 61-66. https://doi.org/10.1016/0006-291X(63)90163-3

Bilgin, D. D., DeLucia, E. H., & Clough, S. J. (2009). A robust plant RNA isolation method suitable for Affymetrix GeneChip analysis and quantitative real-time RT-PCR. *Nature protocols*, *4*(3), 333-340. https://doi.org/10.1038/nprot.2008.249

Brookbank, B. P., Patel, J., Gazzarrini, S., & Nambara, E. (2021). Role of Basal ABA in Plant Growth and Development. *Genes*, *12*(12), 1936. https://www.mdpi.com/2073-4425/12/12/1936

Castellarin, S. D., Pfeiffer, A., Sivilotti, P., Degan, M., Peterlunger, E., & Di Gaspero, G. (2007). Transcriptional regulation of anthocyanin biosynthesis in ripening fruits of grapevine under seasonal water deficit. *Plant, Cell & Environment*, *30*(11), 1381-1399. https://doi.org/10.1111/j.1365-3040.2007.01716.x

Chakraborty, K., Mondal, S., Bhaduri, D., Mohanty, A., & Paul, A. (2022). Chapter 11 - Interplay between sodium and chloride decides the plant's fate under salt and drought stress conditions. In V. Kumar, A. K. Srivastava, & P. Suprasanna (Eds.), *Plant Nutrition and Food Security in the Era of Climate Change* (pp. 271-314). Academic Press. https://doi.org/10.1016/B978-0-12-822916-3.00020-2

Chen, H., Yang, Q., Fu, H., Chen, K., Zhao, S., Zhang, C., . . . Zhuang, W. (2022). Identification of Key Gene Networks and Deciphering Transcriptional Regulators Associated With Peanut Embryo Abortion Mediated by Calcium Deficiency. *Frontiers in plant science*, *13*, 814015-814015. https://doi.org/10.3389/fpls.2022.814015

Cheng, W.-H., Endo, A., Zhou, L., Penney, J., Chen, H.-C., Arroyo, A., . . . Sheen, J. (2002). A Unique Short-Chain Dehydrogenase/Reductase in Arabidopsis Glucose Signaling and Abscisic Acid Biosynthesis and Functions. *The Plant Cell*, *14*(11), 2723-2743. https://doi.org/10.1105/tpc.006494

Clarke, J. D. (2009). Cetyltrimethyl ammonium bromide (CTAB) DNA miniprep for plant DNA isolation. *Cold Spring Harbor Protocols*, *2009*(3), pdb. prot5177. https://doi.org/10.1101/pdb.prot5177

Davé, V., & McCarthy, S. P. (1997). Review of konjac glucomannan. *Journal of Environmental Polymer Degradation*, *5*(4), 237-241.

Deepa, K., Sheeja, T. E., Santhi, R., Sasikumar, B., Cyriac, A., Deepesh, P. V., & Prasath, D. (2014). A simple and efficient protocol for isolation of high quality functional RNA from different tissues of turmeric (Curcuma longa L.). *Physiology and Molecular Biology of Plants*, *20*(2), 263-271. https://doi.org/10.1007/s12298-013-0218-y

Deng, M. Y., Wang, H., Ward, G. B., Beckham, T. R., & McKenna, T. S. (2005). Comparison of six RNA extraction methods for the detection of classical swine fever virus by real-time and conventional reverse transcription–

PCR. *Journal of Veterinary Diagnostic Investigation*, *17*(6), 574-578. https://doi.org/10.1177/104063870501700609

Dhugga, K. S. (2012). Biosynthesis of non-cellulosic polysaccharides of plant cell walls. *Phytochemistry*, *74*, 8-19. https://doi.org/https://doi.org/10.1016/j.phytochem.2011.10.003

Endo, A., Nelson, K. M., Thoms, K., Abrams, S. R., Nambara, E., & Sato, Y. (2014). Functional characterization of xanthoxin dehydrogenase in rice. *Journal of Plant Physiology*, *171*(14), 1231-1240. https://doi.org/https://doi.org/10.1016/j.jplph.2014.05.003

Farrell Jr, R. E. (2009). *RNA Methodologies: laboratory guide for isolation and characterization*. Academic Press.

Favreau, B., Denis, M., Ployet, R., Mounet, F., Peireira da Silva, H., Franceschini, L., . . . Carrer, H. (2019). Distinct leaf transcriptomic response of water deficient Eucalyptus grandis submitted to potassium and sodium fertilization. *PLoS One*, *14*(6), e0218528. https://doi.org/10.1371/journal.pone.0218528

Felix, K., Su, J., Lu, R., Zhao, G., Cui, W., Wang, R., . . . Shen, W. (2019). Hydrogen-induced tolerance against osmotic stress in alfalfa seedlings involves ABA signaling. *Plant and Soil*, *445*(1), 409-423. https://doi.org/10.1007/s11104-019-04328-y

Femenia, A., Sánchez, E. S., Simal, S., & Rosselló, C. (1999). Compositional features of polysaccharides from Aloe vera (Aloe barbadensis Miller) plant tissues. *Carbohydrate Polymers*, *39*(2), 109-117.

Fleige, S., & Pfaffl, M. W. (2006). RNA integrity and the effect on the real-time qRT-PCR performance. *Molecular Aspects of Medicine*, *27*(2), 126-139. https://doi.org/10.1016/j.mam.2005.12.003

Gallagher, S. R. (2000). Quantitation of DNA and RNA with absorption and fluorescence spectroscopy. *Current Protocols in Neuroscience*, *10*(1), A. 1K. 1-A. 1K. 8. https://doi.org/10.1002/0471142727.mba03ds93

Ghangal, R., Raghuvanshi, S., & Sharma, P. C. (2009). Isolation of good quality RNA from a medicinal plant seabuckthorn, rich in secondary metabolites. *Plant Physiology and Biochemistry*, *47*(11-12), 1113-1115. https://doi.org/10.1016/j.plaphy.2009.09.004

Gille, S., Cheng, K., Skinner, M. E., Liepman, A. H., Wilkerson, C. G., & Pauly, M. (2011). Deep sequencing of voodoo lily (Amorphophallus konjac): an approach to identify relevant genes involved in the synthesis of the hemicellulose glucomannan. *Planta*, *234*(3), 515-526.

González-Guzmán, M., Apostolova, N., Bellés, J. M., Barrero, J. M., Piqueras, P., Ponce, M. a. R., . . . Rodríguez, P. L. (2002). The Short-Chain Alcohol Dehydrogenase ABA2 Catalyzes the Conversion of Xanthoxin to Abscisic Aldehyde[W]. *The Plant Cell*, *14*(8), 1833-1846. https://doi.org/10.1105/tpc.002477

Gonzalez-Mendoza, D., Moreno, A. Q., & Zapata-Perez, O. (2008). An improved method for the isolation of total RNA from Avicennia germinans leaves. *Zeitschrift für Naturforschung C*, *63*(1-2), 124-126. https://doi.org/10.1515/znc-2008-1-222

Goubet, F., Barton, C. J., Mortimer, J. C., Yu, X., Zhang, Z., Miles, G. P., . . . Dupree, P. (2009). Cell wall glucomannan in Arabidopsis is synthesised by CSLA glycosyltransferases, and influences the progression of embryogenesis. *The Plant Journal*, *60*(3), 527-538. https://doi.org/https://doi.org/10.1111/j.1365-313X.2009.03977.x

He, C., Zhang, J., Liu, X., Zeng, S., Wu, K., Yu, Z., . . . Duan, J. (2015). Identification of genes involved in biosynthesis of mannan polysaccharides in Dendrobium officinale by RNA-seq analysis. *Plant Molecular Biology*, *88*(3), 219-231.

Imbeaud, S., Graudens, E., Boulanger, V., Barlet, X., Zaborski, P., Eveno, E., . . . Auffray, C. (2005). Towards standardization of RNA quality assessment using user-independent classifiers of microcapillary electrophoresis traces. *Nucleic acids research*, *33*(6), e56-e56. https://doi.org/10.1093/nar/gni054

Jaiswal, S. K., Mahajan, S., Chakraborty, A., Kumar, S., & Sharma, V. K. (2021). The genome sequence of Aloe vera reveals adaptive evolution of drought tolerance mechanisms. *iScience*, *24*(2), 102079. https://doi.org/https://doi.org/10.1016/j.isci.2021.102079

Jia, K.-P., Mi, J., Ali, S., Ohyanagi, H., Moreno, J. C., Ablazov, A., . . . Al-Babili, S. (2022). An alternative, zeaxanthin epoxidase-independent abscisic acid biosynthetic pathway in plants. *Molecular Plant*, *15*(1), 151-166. https://doi.org/https://doi.org/10.1016/j.molp.2021.09.008

Jiang, C.-Q., Quan, L.-T., Shi, F., Yang, N., Wang, C.-H., Yin, X.-M., & Zheng, Q.-S. (2014). Distribution of Mineral Nutrients and Active Ingredients in Aloe vera Irrigated with Diluted Seawater. *Pedosphere*, *24*(6), 722-730. https://doi.org/https://doi.org/10.1016/S1002-0160(14)60059-X

Jieyang, J., Mingyue, Z., Gao, T., Tingting, J., Zhang, N., Wang, J., . . . Chuankui, S. (2021). Amplification of early drought responses caused by volatile cues emitted from neighboring plants. *Horticulture Research*, *8*(1). https://doi.org/10.1038/s41438-021-00704-x

Kasajima, I., Sasaki, K., Tanaka, Y., Terakawa, T., & Ohtsubo, N. (2013). Large-scale extraction of pure DNA from mature leaves of Cyclamen persicum Mill. and other recalcitrant plants with alkaline polyvinylpolypyrrolidone (PVPP). *Scientia Horticulturae*, *164*, 65-72. https://doi.org/10.1016/j.scienta.2013.09.011

Kumar, S., & Kumar, R. (2019). Role of acemannan O-acetyl group in murine radioprotection. *Carbohydrate Polymers*, *207*, 460-470. https://doi.org/https://doi.org/10.1016/j.carbpol.2018.12.003

Léon-Kloosterziel, K. M., Gil, M. A., Ruijs, G. J., Jacobsen, S. E., Olszewski, N. E., Schwartz, S. H., . . . Koornneef, M. (1996). Isolation and characterization of abscisic acid-deficient Arabidopsis mutants at two new loci. *The Plant Journal*, *10*(4), 655-661. https://doi.org/10.1046/j.1365-313X.1996.10040655.x

Li, J., Li, J., Liu, R., Wei, Y., & Wang, S. (2021). Identification of eleven meat species in foodstuff by a hexaplex real-time PCR with melting curve analysis. *Food Control*, *121*, 107599. https://doi.org/10.1016/j.foodcont.2020.107599

Li, Y., Wang, M., Guo, T., Li, S., Teng, K., Dong, D., . . . Han, L. (2022). Overexpression of abscisic acid-insensitive gene ABI4 from Medicago truncatula , which could interact with ABA2, improved plant cold tolerance mediated by ABA signaling. *Frontiers in plant science*, *13*, 982715. Retrieved 2022, from

Liepman, A. H., & Cavalier, D. M. (2012). The cellulose synthase-like A and cellulose synthase-like C families: recent advances and future perspectives. *Frontiers in plant science*, *3*, 109. https://doi.org/10.3389/fpls.2012.00109

Liepman, A. H., Nairn, C. J., Willats, W. G. T., Sørensen, I., Roberts, A. W., & Keegstra, K. (2007). Functional Genomic Analysis Supports Conservation of Function Among Cellulose Synthase-Like A Gene Family Members and Suggests Diverse Roles of Mannans in Plants. *Plant Physiology*, *143*(4), 1881-1893. https://doi.org/10.1104/pp.106.093989

Lin, P.-C., Hwang, S.-G., Endo, A., Okamoto, M., Koshiba, T., & Cheng, W.-H. (2006). Ectopic Expression of ABSCISIC ACID 2/GLUCOSE INSENSITIVE 1 in Arabidopsis Promotes Seed Dormancy and Stress Tolerance. *Plant Physiology*, *143*(2), 745-758. https://doi.org/10.1104/pp.106.084103

Liu, C., Cui, Y., Pi, F., Cheng, Y., Guo, Y., & Qian, H. (2019). Extraction, Purification, Structural Characteristics, Biological Activities and Pharmacological Applications of Acemannan, a Polysaccharide from Aloe vera: A Review. *Molecules*, *24*(8), 1554. https://www.mdpi.com/1420-3049/24/8/1554

Ludyga, N., Grünwald, B., Azimzadeh, O., Englert, S., Höfler, H., Tapio, S., & Aubele, M. (2012). Nucleic acids from long-term preserved FFPE tissues are suitable for downstream analyses. *Virchows Archiv*, *460*(2), 131-140. https://doi.org/10.1007/s00428-011-1184-9

Ma, X., & Yang, J. (2011). An optimized preparation method to obtain high-quality RNA from dry sunflower seeds. *Genet Mol Res*, *10*(1), 160-168. https://doi.org/10.4238/vol10-1gmr979

Manchester, K. L. (1996). Use of UV methods for measurement of protein and nucleic acid concentrations. *Biotechniques*, *20*(6), 968-970. https://doi.org/https://doi.org/10.2144/96206bm05

March, K., & Espinosa, S. (1988). *Cactus y suculentas*. Blume.

Minjares-Fuentes, R., Medina-Torres, L., González-Laredo, R. F., Rodríguez-González, V. M., Eim, V., & Femenia, A. (2017). Influence of water deficit on the main polysaccharides and the rheological properties of Aloe vera (Aloe barbadensis Miller) mucilage. *Industrial crops and products*, *109*, 644-653. https://doi.org/https://doi.org/10.1016/j.indcrop.2017.09.016

Minjares-Fuentes, R., Rodríguez-González, V. M., González-Laredo, R. F., Eim, V., González-Centeno, M. R., & Femenia, A. (2017). Effect of different drying procedures on the bioactive polysaccharide acemannan from Aloe vera (Aloe barbadensis Miller). *Carbohydrate Polymers*, *168*, 327-336. https://doi.org/https://doi.org/10.1016/j.carbpol.2017.03.087

Mota-Ituarte, M., Pedroza-Sandoval, A., Minjares-Fuentes, R., Trejo-Calzada, R., Zegbe, J. A., & Quezada-Rivera, J. J. (2023). Water deficit and salinity modify some morphometric, physiological, and productive attributes of Aloe vera (L.). *Botanical Sciences*, *101*(2). https://doi.org/10.17129/botsci.3175

Nashimoto, M. (1992). Characterization of the spermidine-dependent, sequence-specific endoribonuclease that rquires transfer RNA for its activity. *Nucleic acids research*, *20*(14), 3737-3742. https://doi.org/10.1093/nar/20.14.3737

Ors, S., Ekinci, M., Yildirim, E., Sahin, U., Turan, M., & Dursun, A. (2021). Interactive effects of salinity and drought stress on photosynthetic characteristics and physiology of tomato (Lycopersicon esculentum L.) seedlings. *South African Journal of Botany*, *137*, 335-339. https://doi.org/10.1016/j.sajb.2020.10.031

Pavoković, D., Križnik, B., & Krsnik-Rasol, M. (2012). Evaluation of protein extraction methods for proteomic analysis of non-model recalcitrant plant tissues. *Croatica Chemica Acta*, *85*(2), 177-183. https://doi.org/10.5562/cca1804

Quezada, M. P., Salinas, C., Gotteland, M., & Cardemil, L. (2017). Acemannan and fructans from Aloe vera (Aloe barbadensis Miller) plants as novel prebiotics. *Journal of agricultural and food chemistry*, *65*(46), 10029-10039.

Rai, K. M., Thu, S. W., Balasubramanian, V. K., Cobos, C. J., Disasa, T., & Mendu, V. (2016). Identification, Characterization, and Expression Analysis of Cell Wall Related Genes in Sorghum bicolor (L.) Moench, a Food, Fodder, and Biofuel Crop [Original Research]. *Frontiers in plant science*, *7*. https://doi.org/10.3389/fpls.2016.01287

Raven, P. H., Evert, R. F., & Eichhorn, S. E. (2005). *Biology of plants*. Macmillan.

Ren, H., Gao, Z., Chen, L., Wei, K., Liu, J., Fan, Y., . . . Zhang, J. (2006). Dynamic analysis of ABA accumulation in relation to the rate of ABA catabolism in maize tissues under water deficit. *Journal of Experimental Botany*, *58*(2), 211-219. https://doi.org/10.1093/jxb/erl117

Rivero, R. M., Mittler, R., Blumwald, E., & Zandalinas, S. I. (2022). Developing climate-resilient crops: improving plant tolerance to stress combination. *The Plant Journal*, *109*(2), 373-389. https://doi.org/10.1111/tpj.15483

Rodrigues, S. P., Ventura, J. A., Zingali, R., & Fernandes, P. (2009). Evaluation of sample preparation methods for the analysis of papaya leaf proteins through two-dimensional gel electrophoresis. *Phytochemical Analysis: An International Journal of Plant Chemical and Biochemical Techniques*, *20*(6), 456-464. https://doi.org/10.1002/pca.1147

Roy, D., Tomo, S., Modi, A., Purohit, P., & Sharma, P. (2020). Optimising total RNA quality and quantity by phenol-chloroform extraction method from human visceral adipose tissue: A standardisation study. *MethodsX*, *7*, 101113. https://doi.org/10.1016/j.mex.2020.101113

Sagar, S., & Singh, A. (2019). Chapter 22 - Abscisic Acid, a Principal Regulator of Plant Abiotic Stress Responses. In M. I. R. Khan, P. S. Reddy, A. Ferrante, & N. A. Khan (Eds.), *Plant Signaling Molecules* (pp. 341-353). Woodhead Publishing. https://doi.org/https://doi.org/10.1016/B978-0-12-816451-8.00021-6

Salinas, P., Salinas, C., Contreras, R. A., Zuniga, G. E., Dupree, P., & Cardemil, L. (2019). Water deficit and abscisic acid treatments increase the expression of a glucomannan mannosyltransferase gene (GMMT) in Aloe vera Burm. F. *Phytochemistry*, *159*, 90-101. https://doi.org/10.1016/j.phytochem.2018.12.009

Šamec, D., Karalija, E., Šola, I., Vujčić Bok, V., & Salopek-Sondi, B. (2021). The Role of Polyphenols in Abiotic Stress Response: The Influence of Molecular Structure. *Plants*, *10*(1), 118. https://www.mdpi.com/2223-7747/10/1/118

Sánchez-Rodríguez, A., Portal, O., Rojas, L. E., Ocana, B., Mendoza, M., Acosta, M., . . . Höfte, M. (2008). An efficient method for the extraction of high-quality fungal total RNA to study the Mycosphaerella fijiensis–Musa spp. interaction. *Molecular Biotechnology*, *40*(3), 299-305. https://doi.org/10.1007/s12033-008-9092-1

Schmittgen, T. D., & Livak, K. J. (2008). Analyzing real-time PCR data by the comparative CT method. *Nature protocols*, *3*(6), 1101-1108. https://doi.org/10.1038/nprot.2008.73

Schumann, U., Smith, N. A., & Wang, M.-B. (2013). A fast and efficient method for preparation of high-quality RNA from fungal mycelia. *BMC Research Notes*, *6*(1), 71. https://doi.org/10.1186/1756-0500-6-71

Seo, M., & Koshiba, T. (2002). Complex regulation of ABA biosynthesis in plants. *Trends in Plant Science*, *7*(1), 41-48. https://doi.org/https://doi.org/10.1016/S1360-1385(01)02187-2

Sifuentes-Rodríguez, N., Pedroza-Sandoval, A., Zegbe-Dominguez, J., & Trejo-Calzada, R. (2020). Indicadores de productividad y calidad de gel de sábila en condiciones de estrés salino. *Revista Fitotecnia Mexicana*, *43*(2), 181-181.

Speicher, T. L., Li, P. Z., & Wallace, I. S. (2018). Phosphoregulation of the plant cellulose synthase complex and cellulose synthase-like proteins. *Plants*, *7*(3), 52. https://doi.org/10.3390/plants7030052

Sun, L., Zhang, M., Ren, J., Qi, J., Zhang, G., & Leng, P. (2010). Reciprocity between abscisic acid and ethylene at the onset of berry ripening and after harvest. *BMC Plant Biology*, *10*(1), 257. https://doi.org/10.1186/1471-2229-10-257

Suzuki, S., Li, L., Sun, Y.-H., & Chiang, V. L. (2006). The Cellulose Synthase Gene Superfamily and Biochemical Functions of Xylem-Specific Cellulose Synthase-Like Genes in Populus trichocarpa *Plant Physiology*, *142*(3), 1233-1245. https://doi.org/10.1104/pp.106.086678

Vafaei, A. H., Gohari Kia, M. R., Gougerdchi, V., Dehghanian, Z., Asgari Lajayer, B., Aftab, T., & Astatkie, T. (2023). Chapter seventeen - Insight on abiotic stress management in plants by improving plant nutritional status. In T. Aftab & K. R. Hakeem (Eds.), *Sustainable Plant Nutrition* (pp. 381-402). Academic Press. https://doi.org/https://doi.org/10.1016/B978-0-443-18675-2.00007-9

Vasanthaiah, H. K. N., Katam, R., & Sheikh, M. B. (2008). Efficient protocol for isolation of functional RNA from different grape tissue rich in polyphenols and polysaccharides for gene expression studies. *Electronic journal of Biotechnology*, *11*, 42-51. http://www.scielo.cl/scielo.php?script=sci_arttext&pid=S0717-34582008000300005&nrm=iso

Vennapusa, A. R., Somayanda, I. M., Doherty, C. J., & Jagadish, S. (2020). A universal method for high-quality RNA extraction from plant tissues rich in starch, proteins and fiber. *Scientific Reports*, *10*(1), 1-13. https://doi.org/10.1038/s41598-020-73958-5

Wilson, I. G. (1997). Inhibition and facilitation of nucleic acid amplification. *Applied and environmental microbiology*, *63*(10), 3741-3751. https://doi.org/10.1128/aem.63.10.3741-3751.1997

Xu, Z. J., Nakajima, M., Suzuki, Y., & Yamaguchi, I. (2002). Cloning and characterization of the abscisic acid-specific glucosyltransferase gene

from adzuki bean seedlings. *Plant Physiol*, *129*(3), 1285-1295. https://doi.org/10.1104/pp.001784

Xue, F., Liu, W., Cao, H., Song, L., Ji, S., Tong, L., & Ding, R. (2021). Stomatal conductance of tomato leaves is regulated by both abscisic acid and leaf water potential under combined water and salt stress. *Physiologia Plantarum*, *172*(4), 2070-2078. https://doi.org/doi.org/10.1111/ppl.13441

Yang, Y.-W., Chen, H.-C., Jen, W.-F., Liu, L.-Y., & Chang, M.-C. (2015). Comparative transcriptome analysis of shoots and roots of TNG67 and TCN1 rice seedlings under cold stress and following subsequent recovery: insights into metabolic pathways, phytohormones, and transcription factors. *PloS one*, *10*(7), e0131391.

Zandalinas, S. I., Sengupta, S., Fritschi, F. B., Azad, R. K., Nechushtai, R., & Mittler, R. (2021). The impact of multifactorial stress combination on plant growth and survival. *New Phytologist*, *230*(3), 1034-1048.

Zeevaart, J., & RA, C. (1988). Metabolism and Physiology of Abscisic Acid. *Annual Review of Plant Physiology and Plant Molecular Biology*, *39*(1), 439-473. https://doi.org/10.1146/annurev.pp.39.060188.002255

Zhang, G., Shen, T., Ren, N., & Jiang, M. (2022). Phosphorylation of OsABA2 at Ser197 by OsMPK1 regulates abscisic acid biosynthesis in rice. *Biochemical and Biophysical Research Communications*, *586*, 68-73. https://doi.org/https://doi.org/10.1016/j.bbrc.2021.11.067

VI. GENERAL CONCLUSIONS

The productivity, as well as morphological and molecular characteristics of *Aloe vera* grown under water deficit and salinity was studied. Thus, the statistical evidence confirmed the hypothesis that water deficit in interaction with salinity negatively affects the productivity of *Aloe vera*, in terms of crop yield and growth as well as some molecular properties.

As a consequence of salinity, fresh biomass, gel content, and harvest index were moderately reduced, affecting the width and thickness of the *A. vera* leaves. However, the gel quality increased therefore the increase of total solids. Total solids are valued by the international market since polysaccharides and other bioactive compounds, such antioxidant compounds, which have been considered as the responsible for the beneficial properties attributed to *A. vera* plant, mainly compose these. *Aloe vera* showed high tolerance to water deficit and moderate tolerance to salinity in leaf width and thickness, fresh biomass, and gel production.

On the other hand, the total RNA extracted by the optimized method in this study showed acceptable purity values, good integrity level, and high efficiency in the RT-qPCR assay. Thus, the total RNA extracted from plants grown under salinity and water deficit conditions, by the optimized method in this study, had better quality compared to the isolate with the other methods evaluated. Also, this method minimized the use of reagents during the RNA extraction procedure, which could be considered as an important advantage from an environmental point of view.

Moreover, *Aloe vera* showed high tolerance to salinity in polysaccharide content, as no statistical difference was observed, even at the highest stress levels. Further, *glucomannan mannosyltransferase* (*GMMT*) gene showed positive regulation as consequence of salinity and the combination with water stress/deficit on *Aloe vera* while the *xanthoxin dehydrogenase* (*ABA2*) and *abscisic-acid glucosyltransferase* (*AOG*) genes showed opposite relative expression. In fact, the *ABA2* gene showed downregulation under salinity conditions and upregulation under the combination of salt-water stress,

whereas *AOG* gene revealed positive regulation under salinity conditions and downregulation under the combination of salt-water stress. Therefore, the transcriptional regulation of gene expression related to ABA could be a key factor involved in stress tolerance by salt, as well as a combination of salt-water stress in *A. vera*.

Further studies are required in order to evaluate the role of ABA-mediated stress tolerance mechanisms and their relation to polysaccharides and their long-distance transport to the parenchyma. These results could serve as a basis for future research on genes of interest that could be involved in the biosynthesis of *Aloe vera* acemannan. Finally, the results of this study suggest that moderate abiotic stress, given by salinity or a combination of salinity and water deficit, may be a strategy to obtain *A. vera* gel with good quality and polysaccharide content for different agro-industrial purposes in arid and semi-arid areas. The relative expression of the genes observed in the present study has great potential to be used in the study of *A. vera* response to different types and combinations of stress factors.

These findings may support the development of similar studies in arid areas and may be useful for the proper management of future experiments with the combination of two or more types of stress. As in other studies, it is proposed that moderate stress can be an interesting and useful tool to implement in the agriculture of plant species with bioactive components and stress tolerance.