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Standardization of the GUS Reporter System and Different Methodologies for the Quantification of Sulphur-related Compounds to Evaluate the Effect of Nutritional Stress in *Arabidopsis thaliana*

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RESUMEN

La nutrición vegetal ha sido estudiada por décadas, y su principal enfoque yace en entender las funciones de los elementos químicos que son necesarios para el crecimiento y desarrollo vegetal. El azufre es un macroelemento esencial para la supervivencia de las plantas, ya que desempeña una variedad de roles estructurales y fisiológicos dentro de las mismas. En los años 80, se logró establecer una conexión definitiva entre el azufre y la defensa vegetal, y dado que la deficiencia nutricional de azufre parecía aumentar la incidencia de enfermedad en los cultivos, se acuñó el término Resistencia Inducida por Azufre (SIR, por sus siglas en inglés). La Resistencia Sistémica Adquirida (SAR, por sus siglas en inglés) es una respuesta inmune innata de las plantas mediada por la hormona vegetal ácido salicílico (AS), y se conoce que el metabolismo del azufre interactúa con las rutas de defensa relacionada al AS mediante compuestos como la cisteína y el glutatión. Existen contradicciones en cuanto al efecto real que tiene el estrés nutricional de azufre en la activación de genes de defensa relacionados al AS tales como el gen *PR1*, pues se han reportado casos en los que la deficiencia de azufre promueve la expresión de estos genes mientras que el exceso promueve su supresión, y viceversa. Tampoco existe suficiente información acerca de los cambios en los reservorios de compuestos sulfurados dentro de la célula cuando existe estrés nutricional de azufre y como estos impactan la expresión de genes de defensa mediados por el AS. En el presente estudio se trabajó en la estandarización del sistema reportero GUS con *Arabidopsis thaliana* PR1::GUS para determinar el efecto que el estrés nutricional de azufre tiene sobre la expresión del gen *PR1*. Paralelamente, se trabajó en la estandarización de métodos de detección de sulfato, cisteína, y especies reactivas de oxígeno en tejido de *Arabidopsis thaliana*. Se demostró que la deficiencia nutricional de azufre promueve la expresión del gen *PR1*, mientras que el exceso suprime su expresión. Si bien los resultados obtenidos no replican por completo lo reportado por otros autores, se espera que el análisis planteado acerca del rol del glutatión en la activación o supresión del gen *PR1* aporte al entendimiento del efecto del estrés nutricional de azufre sobre la defensa vegetal. Por otro lado, en este estudio se logró la estandarización de los métodos para la determinación de sulfato y especies reactivas de oxigeno, mientras que no se logró realizar la determinación de cisteína en extractos de proteína vegetal. Se anticipa que la utilización de las metodologías estandarizadas proveerá información novedosa acerca de los reservorios de compuestos sulfurados dentro de la célula, ampliando así el entendimiento del rol del azufre en la defensa vegetal.

Palabras clave: azufre, SAR, SIR, ácido salicílico, sulfato, cisteína, ROS

ABSTRACT

Plant nutrition has been studied for decades, and its focus lies on understanding the role of the chemical elements that are necessary for plant growth and development. Sulphur is an essential macronutrient for plant survival, because it has structural and functional roles inside plants. In the 80's, a connection was established between sulphur and plant defense, and given that sulphur deficiency seemed to increase disease incidence in crops, the term Sulphur Induced Resistance (SIR) was coined. Systemic Acquired Resistance (SAR) is an innate immune response found in plants, which is mediated by the plant hormone salicylic acid (SA), and it is known that sulphur metabolism interacts with the defense routes related to SA via compounds like cysteine or glutathione. Contradictions exist regarding the real effect that sulphur nutritional stress has over the expression of defense genes related to SA, such as *PR1*. Some studies have reported that sulphur deficiency promotes the expression of *PR1* and sulphur excess suppresses its expression, while other authors report the exact opposite. Information is also scarce in relation to the changes in the pool of sulphur containing compounds inside the cell whilst the plant is undergoing sulphur nutritional stress, and how this can affect the expression of defense genes related to SA. In the present study, it was aimed to standardize the GUS reporter system using *Arabidopsis thaliana* PR1::GUS in order to determine the effect that sulphur stress has over the expression of the *PR1* gene. Parallel to this, the standardization of methods for the detection of sulphate, cysteine, and reactive oxygen species in *Arabidopsis thaliana* tissue was carried out. The results show that sulphur deficiency promotes the expression of the *PR1* gene, while sulphur excess prompts its suppression. While it was evident that it was not possible to fully replicate the results reported in other publications, it is expected that the proposed analysis regarding the role of glutathione in the activation or suppression of the *PR1* gene contributed to the understanding of the effect that sulphur stress has on plant defense. On the other hand, the standardization of protocols for the detection of sulphate and reactive oxygen species in *A. thaliana tissue* proved to be successful, while it was not possible to determine cysteine on plant protein extracts. It is anticipated that the use of the standardized protocols will yield novel information about the pool of sulphur-containing compounds, thus increasing the knowledge of sulphur's role in plant defense.

Key words: sulphur, SAR, SIR, salicylic acid, sulphate, cysteine, ROS

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1 INTRODUCTION

1.1 Sulphur and nutrition

Plant nutrition has been studied for decades, and its focus lies on understanding the function of chemical elements and compounds that are necessary for plant growth and metabolism. Essential elements are those which plants cannot complete their life cycles without, and/or constitute some essential metabolite for plant functioning (Marschner, 2012). Nutrients have been classified in two main categories: macronutrients and micronutrients. Macronutrients are needed in relatively big amounts for plant development, and include nitrogen, carbon, oxygen, hydrogen, phosphorus, potassium, calcium, magnesium, and sulphur (Allen & Pilbeam, 2007). On the other hand, micronutrients are needed in minimum amounts to ensure plant development, and include nickel, iron, chloride, boride, manganese, zinc, copper, and molybdenum (Allen & Pilbeam, 2007).

Sulphur is a macronutrient that has several functions in plants, in both structural and functional aspects, and it is of great importance to many intracellular processes that take place in plant metabolism, despite being the least abundant macronutrient. Sulphur is essential for the construction of the amino acid cysteine and some vitamins, and it is involved in the correct functioning of the electron transport chain and chloroplasts (Maruyama-Nakashita, Inoue, Watanabe-Takahashi, Yamaya, & Takahashi, 2003). In the latter, sulphur forms conjugates with iron that make up part of the electron transport that takes place during photosynthesis, therefore, sulphur is directly linked to glucose production in plants (Saito, 2004). Sulphur aids in the process of nitrogen fixation in legumes, and it is also involved in the process of synthetizing amino acids from nitrate (Saito, 2004).

1.2 Sulphur and defense

Plants' sessile nature has pressured them to evolve several defense mechanisms and strategies to defend themselves from disease, some of which are quite complex. The two main mechanisms for plant defense consist in the formation of physical barriers or the synthesis of natural defense molecules (Datnoff, Elmer, & Huber, 2007). Physical barriers essentially consist in the formation of thick cell walls that do not allow pathogens to enter the plant; nitrogen and calcium have proven to be the most important nutrients for this mechanism. In contrast, the production of defense molecules is based on the generation of compounds that attack pathogens once they have already entered the plant, therefore stopping the pathogen from proliferating internally. Sulphur and nitrogen are the two elements of most importance to produce these antimicrobial compounds. For instance, phytoalexins and isothiocyanates are two kinds of sulphur-containing molecules that play an important role in plant defense, for it has been proven that they have antimicrobial activity, mainly against fungal pathogens (Saito, 2004).

During the 1970's, sulphur was considered a contaminant, since it was the main contributor for the formation of acid rains. Sulphur mainly came from industrial sources and it represented a great concern for many environmentalist parties. There was no discussion regarding sulphur's role as a contaminant, but it also happened to indirectly fertilize soils, since part of the sulphur that emanated from industrial plants made its way to the soil, and then it could be used by crops as a nutrient (Bloem, Haneklaus, & Schung, 2015). During the 1980's, environmental reforms to reduce pollution came into action and accomplished their goal to reduce sulphur emissions coming from factories. Short time later, an increased disease incidence was reported on several crops, mainly on plants from the Brassicaceae family (cabbage, beet, canola, etc.) which have a higher nutritional demand of sulphur in comparison with other plants. Studies were conducted and, for the

first time, a connection was established between sulphur levels and disease incidence. It was in that moment that the term SIR (sulphur induced resistance) was coined (Bloem, Haneklaus, & Schung, 2015). From that point on, considerable progress has been made in order to elucidate the role that sulphur plays in the defense mechanisms of plants.

For instance, studies have been made in several crops in order to further understand the SIR phenomenon; these crops include canola, maize, tomato, cotton, potato, and some others. The vast majority of results suggest that at higher levels of sulphur, resistance to disease increases, with both necrotrophic and biotrophic pathogens (Bloem, Haneklaus, & Schung, 2015). These findings seem to corroborate the initial hypothesis surrounding SIR, in which the indirect fertilization of the soil with higher levels of sulphur coming from industrial sources seemed to increase plant resistance to disease. Interestingly, opposite results have been reported by Criollo and Gonzales in 2013 and 2015, respectively. Both studies conducted trials using *Arabidopsis thaliana*, in which nutritional treatments with complete sulphur deficiency caused the activation of pathogenesis-related genes (PR-genes). Furthermore, Criollo (2013) showed that *A. thaliana* resisted the infection of the hemibiotrophic pathogen *Pseudomonas syringae* when treated with a nutritional diet with complete absence of sulphur. These contradictions reveal that the phenomenon of SIR is not as straight forward as initially believed, and that further studies are necessary to fully understand the role of sulphur in plant defense.

1.3 Systemic Acquired Resistance (SAR)

A cornerstone of plant defense is the systemic acquired resistance (SAR), which is an innate immune response against pathogens found in plants. This type of response takes place when a plant is exposed to a pathogen that it has been exposed before (Durrant & Dong, 2004). Despite that most infections in plants tend to be localized, SAR is an

organism-wide response and it is of vital importance for the plant's capacity to defend itself, as well as recover from a past infection. SAR is considered a broad-spectrum immune response, since it is efficient for a vast array of pathogens, mainly (but not restricted to) necrotrophic organisms (Durrant & Dong, 2004). The cellular mechanisms that control SAR have been studied in recent years, and some of the cellular and genetic components involved have been understood (Maleck, et al., 2000). Plants use pattern-recognizing receptors (PRRs) to identify microbial structures with conserved characteristics that trigger an immune response (Maleck, et al., 2000). SAR is also related to the expression of pathogenesis-related genes, which produce pathogenesis-related (PR) proteins. These proteins are active components of plant defense, because some act as antimicrobial molecules, and others act as signaling molecules that regulate immune response in the plant (Durrant & Dong, 2004).

The plant hormone salicylic acid (SA) is a phenolic hormone that is involved in several metabolic processes in plants. It has been found that SA plays a role in plant growth, photosynthesis, nutrient uptake from soil, transpiration, and plant defense (Vlot, Dempsey, & Klessig, 2009). Salicylic acid is directly related to SAR, since it promotes the expression of PR genes, and therefore, the production of PR proteins (Tripathi, Raikhy, & Kumar, 2019). The accumulation of salicylic acid is required in order for SAR to take place, which is corroborated by previous studies that have determined that SA increases in the site of infection, as well as systemically, when a plant is undergoing infection (van Loon, 2016). A derivative of SA of volatile nature, methyl salicylate, acts as a plant pheromone, which warns nearby plants of pathogens, prompting them to activate their immune response (Shulaev, Silverman, & Raskin, 1997).

1.4 The Link between Sulphur Metabolism and SAR

Sulphur enters the plant in the form of sulphate (SO_4^{2-}), which is then converted into adenosinephosphosulphate (APS) with the help of ATP. APS then converts into sulphite (SO_3^{2-}), and then into sulphur (S^{2-}). Sulphur then reacts with O-acetylserine (OAS) to form the amino acid cysteine (Bloem, Haneklaus, & Schung, 2015). Cysteine is at the heart of sulphur metabolism in plants, because it is a building block of proteins and the main precursor for all sulphur-containing compounds inside the plant. These compounds include isothiocyanates, glucosinolates, phytoalexins, hydrogen sulphide (H_2S), and glutathione (Bloem, Haneklaus, & Schung, 2015). It has been proven that cysteine homeostasis is essential for plant immunity, and an adequate concentration of this amino acid in the cell is vital to initiate and regulate defense mechanisms inside the plant (Alvarez, Bermudez, Romero, Gotor, & Garcia, 2012).

Sulphur metabolism inside plants is directly linked with salicylic acid, and therefore with SAR. It is known that PR-proteins are rich in cysteine content, and therefore cysteine is of vital importance for the deployment of SAR. As previously mentioned, SA promotes de expression of PR-genes, so an accumulation of SA would require a sufficient concentration of cysteine in order to produce the PR-proteins involved in SAR. If a cysteine deficiency were to take place, SAR would be directly affected by the lack of building blocks for PR-proteins (Alvarez, Bermudez, Romero, Gotor, & Garcia, 2012). Glutathione is also a key component in plant defense, because it acts as a redox buffer inside the cell. Glutathione can be found in two ways inside the cell, as GSH (reduced) or GSSG (oxidized), and it oscillates between these two states depending on the oxidative stress inside the cell (Leustek, Martin, Bick, & Davies, 2000). Here, SA comes into play once again, since it has the ability to increase the amount of reactive oxygen species (ROS) inside the cell by binding to peroxidases and inhibiting their activity, which then

causes a rise in the oxidative stress inside the cell (Dzhavakhiya, Ozeretskovskaya, & Zinovyeva, 2007). It is clear that sulphur and plant defense are connected, but there is still not enough information regarding the cellular mechanics that control this interaction. As previously addressed, there are contradictory findings regarding the role of sulphur in plant defense, so it is necessary to conduct further studies that analyze how the pool of sulphur-containing molecules inside the cell behaves at different dietary levels of sulphur, and then establish a clear connection with SA and SAR that will explain the SIR phenomenon.

1.5 Methods for Sulphur Metabolism Analysis

Throughout the process of studying plant defense and sulphur metabolism, several techniques have been developed to study the metabolic intermediates of those cellular mechanisms, including sulphate, cysteine, ROS, and PR-proteins. Sulphate is usually determined utilizing analytical chemistry methods, including ICP-AES, ion chromatography, and turbidimetric analysis. The first two are methods of high precision, often used in specialized laboratories of analysis when detecting compounds even in trace amounts and, because of their precision and sensibility, tend to be more expensive than other detection methods (Coskun, 2016). On the other hand, turbidimetric determination of sulphate is based on the utilization of barium chloride ($BaCl_2$), which forms insoluble crystals upon reacting with sulphate, and then the turbidity of the solution can be analyzed (Verma, 1977). In regard to cysteine determination, chromatography and colorimetric analysis are the norm (Friedman, Krull, & Cavins, 1970). The latter is based on a simple but very specific reaction of ninhydrin with cysteine at a low pH, which yields a bright pink color, and can then be analyzed in a spectrophotometer (Gaitonde, 1967). Reactive oxygen species (ROS) can be easily determined in a fluorometric assay, utilizing 2',7' – dichlorofluorescin diacetate (DCFDA), which forms a fluorescent compound after being

oxidized by ROS (Da, et al., 2019). Lastly, PR-gene expression and PR-proteins can also be analyzed in several ways. RT-PCR and qPCR are methods commonly utilized to detect gene expression qualitatively and quantitatively, respectively, and therefore, they provide information regarding the activation of defense mechanisms in the plant under specific conditions. The GUS reporter system is another way to detect gene expression, which is characterized by its simplicity and rapidness; this method will be explained in greater detail in following sections of this text. It is common to carry out this type of analyses in model organisms, which are well characterized and provide a solid foundation for the generation of new knowledge that can be extrapolated into particular organisms later on, such as the case of *Arabidopsis thaliana*.

1.6 *Arabidopsis thaliana*

Arabidopsis thaliana is a small plant native to Eurasia that belongs to the Brassicaceae family, which also includes plants such as mustard and cabbage (Hoffmann, 2002). *A. thaliana*'s leaves form a characteristic rosette on the base of the plant, and its flowers grow from the center of the rosette up to 25cm high. The leaves of this plant are green, occasionally with a purple tinge around the edges, and they are 1.5 to 5 cm in length. Each individual plant has the ability to produce thousands of seeds, which are contained inside pods and are very small and light, so they can be easily dispersed through the environment. *A. thaliana* is a plant of rapid growth, its life cycle is completed in a total of six weeks from germination up to the production of seeds, and it has the ability to grow in rocky, sandy, and calcareous soils, often as a pioneer plant (Mitchell-Olds, 2001). Due to its ease of dispersion and speed of growth, *A. thaliana* is regarded as a weed plant, but since it is not a strong competitor for nutrients, it is not a plant of concern for agricultural practices (Mitchell-Olds, 2001).

All the characteristics mentioned above make *Arabidopsis thaliana* a perfect candidate for laboratory work, and that is why it has become one of the most important model organism for the fundamental investigation of molecular biology and genetics in plants. *A. thaliana* grows easily under controlled conditions, including Petri dishes, pots, hydroponic culture, and greenhouses; and it requires little space for its growth. *A. thaliana*'s genetic construction is relatively simple, consisting of 135 megabases arranged in five separate chromosomes of diploid nature (Coelho, et al., 2007). In addition to this, *A. thaliana*'s metabolic pathways are also well described and studied, which has led to many important discoveries regarding topics like plant immunity, growth, and development (Durvasula, et al., 2017). The extensive use that has been given to *A. thaliana* has yielded a wide variety of well-characterized mutants, which have been useful to study the function and regulation of several genes inside the plant.

1.7 GUS Reporter System

The GUS reporter system is a histochemical technique that allows for the evaluation of gene expression in a qualitative or quantitative fashion. This technique utilizes the *E. coli* gene *uidA* as a reporter gene; this gene codes for enzyme *β*-glucoronidase, which is a hydrolase that can catalyze the cleavage of *β*-glucoronides, and in the process forms an easily detectable compound (Jefferson, Burgess, & Hirsh, 1986). The most common compound used in this type of reporter gene assays is 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc), which reacts with *β*-glucoronidase and yields a blue precipitate that indicates that enzyme activity is taking place in the analyzed tissue. The reaction that takes place breaks down X-Gluc into glucoronic acid and an indoxyl derivative (chloro-bromoindigo), that then undergoes an oxidative dimerization to produce the insoluble and blue colored compound (dichloro-dibromoindigo) (Karcher, 2002).

With the help of recombinant DNA techniques, a vast array of transgenic lines of *Arabidopsis thaliana* have been obtained. In relation with the GUS reporter system, the common practice relies on replacing the original promoter of the *uidA* gene with the promoter of a particular gene of interest, yielding a chimeric gene that is then inserted inside the plant (Karcher, 2002). This will enable the identification of transcriptional activity of the promoter of the gene that is being studied, because if the enzyme is being produced, the plants will turn blue after the application of the chromogenic substrate. Examples of transgenic lines of *A. thaliana* are PG15 (*CaMV35S::GUS*) and PR1::GUS. The PG15 line has the CaMV 35S promoter inserted before the *uidA* gene. The CaMV 35S is a strong promoter isolated from the Cauliflower Mosaic Virus (CaMV) which is known to be of constitutive expression throughout the plant. This means that *A. thaliana* PG15 expresses *β*-glucoronidase in all of its tissues regardless of the growth conditions, and therefore, it can be used as the positive control for the GUS reporter assay (Jefferson, Kavanagh, & Bevan, 1987). On the other hand, the PR1::GUS line contains the *PR1* promoter before the *uidA* gene, which will be expressed only under certain conditions. Given that the *PR1* gene is a marker for salicylic acid and also a component of plant defense, its detection via the GUS system provides information about the state of the SA metabolic pathways and defense mechanisms inside the plant (Shapiro & Zhang, 2001). It is important to mention that the function of *PR1* has just recently been known to be a sterol binding protein in plant cells, and it shows the action of an antimicrobial protein (Gamir, et al., 2017)

In order to further elucidate the mechanisms that govern SIR and the true relationship between sulphur nutritional stress and the activation of the plant's defense mechanisms it is necessary to study the metabolites involved in those processes, and determine how each of them changes when the plant is subjected to specific conditions. Considering that the

analysis of particular compounds and metabolic intermediaries inside plants is a very specific and delicate task, it is mandatory to establish robust and standardized protocols that allow for a proper determination of each molecule of interest (Montgomery, 2017). Simultaneously, it is crucial to determine the conditions in which PR-genes are expressed when the plant is exposed to a diet with sulphur deficiency or excess in order to make sense of the apparent contradictions that are reported in the literature. That is why the main objectives of this work are to standardize protocols for the determination of sulphate, cysteine, and ROS in plant tissue, and using the GUS reporter system to determine the conditions in which the *PR1* gene (marker for SA) is expressed when exposing the *Arabidopsis thaliana* mutant PR1::GUS to different nutritional stresses of sulphur.

2 JUSTIFICATION

For hundreds of years, agriculture has relied upon soil fertilization practices to promote and maximize crop production around the world (Roberts, 2009). This has led to the current state of agricultural production worldwide, but in present day, it has become of great importance to optimize soil fertility and production in order to catch up with the rapidly increasing global population. Each macro and micronutrient plays an essential part in plant growth and development, because they all serve specific functions in the plant, whether it is a structural and/or functional role (Marschner, 2012). It is necessary to have all nutrients present in the right proportions in the soil in order to avoid deficiencies or toxicity in crops (Marschner, 2012). The study and understanding of the SIR phenomenon and the intracellular process that govern the interactions between plant defense and sulphur metabolism could lead to the development of specialized fertilization techniques that optimize the nutrient ratio in the soil, promoting growth and strengthening plant defense (Bloem, Haneklaus, & Schung, 2015). This could also signify the reduction of commonly overused agrochemicals, which can have detrimental effects on the environment and human health (Roberts, 2009). In order to properly study the cellular processes and interactions between plant defense and sulphur metabolism, standardized protocols should be designed in order to gather useful information regarding the state of the intermediary compounds of sulphur metabolism that are related to plant defense.

3 OBJECTIVES

3.1 General Objective

- Standardize the use of the GUS reporter system and different methodologies for the quantification of sulphur containing compounds in order to evaluate the effect of sulphur nutritional stress on the defense pathways related to salicylic acid in *Arabidopsis thaliana*

3.2 Specific objectives

- Cultivate *Arabidopsis thaliana* lines PR1::GUS, Col-0 and PG15 *in vitro* and in sand.
- Determine the effect of salicylic acid over the expression of the *PR1* gene in relation to treatments with sulphur excess and deficiency via the GUS reporter system.
- Standardize quantification protocols for products of sulphur metabolism and salicylic acid pathway such as cysteine, sulphate, and ROS present in *A. thaliana* leaf tissue.

4 AREA OF STUDY

In the present study, *Arabidopsis thaliana* PR1::GUS and Col-0 seeds were utilized. The PR1::GUS line is a mutant of *A. thaliana* modified with the *PR1* promoter inserted before the *uidA* gene of *E. coli*. *A. thaliana* Col-0 is the wild ecotype of this organism. The Agrobiotechnology and Food Biotechnology Laboratory of USFQ and Utrecht University provided all seeds. This investigation was realized in the Agrobiotechnology and Food Biotechnology Laboratory of USFQ and in Utrecht University. The turbidimetric determination of sulphate and the fluorescent measurement of ROS were done in the LIA-USFQ, and the CENBIO-UTE facilities, respectively.

5 MATERIALS

5.1 *Arabidopsis thaliana* in vitro culture

- Nylon stockings
- 3cm diameter plasticized PVC tubes
- Murashige-Skoog (MS) Medium
- *Arabidopsis thaliana* Col-0 seeds
- Plastic wrap
- Plastic zip ties
- Rey Biofood Square food container 0.28L
- Autoclave
- Micropipettes
- Sodium hypochlorite (NaOCl) 5% (Clorox)
- Distilled water
- Heathrow Vortexer
- Laminar flow cabinet
- Centrifuge

5.2 *Arabidopsis thaliana* sand culture

- Six-well plates for cellular culture
- Washed and autoclaved river sand
- MS Medium
- *Arabidopsis thaliana* Col-0 seeds
- *Arabidopsis thaliana* PR1::GUS seeds

5.3 Sulphur nutritional stress treatments

- Sulphur deficient MS medium

- Sulphur excess MS medium
- Sigma Salicylic Acid
- Distilled water
- Tween 80
- Air brush kit aerograph model EW-110
- Gast DOA-P704-AA Vacuum pump

5.4 GUS Reporter System

- Disodium phosphate (Na_2HPO_4)
- Monosodium phosphate (NaH_2PO_4)
- EDTA
- Sigma Triton X-100
- Sigma N,N-Dimethylformamide
- Duchefa Biochemie X-GlcA

5.5 Sulphate Determination

- Loba Chemie nitric acid 69%
- Sigma D-Sorbitol
- Distilled water
- Barium chloride ($BaCl_2$)
- 50 ml Falcon tubes
- 10 ml glass pipette
- Sodium sulphate
- Thermofisher spectrophotometer
- Concentrated hydrochloric acid

5.6 Protein extraction

- EDTA
- Tris-HCl
- Sigma Sodium lauryl sulphate (SDS)
- Sigma B-mercaptoethanol
- Distilled water
- Thermofisher Stdrd DryBath
- Ethanol 96%
- 1.5 ml Eppendorf tubes
- Explants of *A. thaliana* Col-0
- Liquid nitrogen
- MP 4mm Glass Beads

5.7 Protein quantification

- Bradford reagent
- 96-well reading plate
- Sirio S ELISA plate reader

5.8 Cysteine determination

- Glacial acetic acid
- Loba Chemie orthophosporic acid
- Loba Chemie ninhydrin
- Loba Chemie L-Cysteine hydrochloride (monohydrate) 98.5%
- Distilled water
- Thermal cycler
- PCR 96 well plates

- Jenway 7305 Spectrophotometer
- Multichannel micropipette
- Protein extracts of *A. thaliana* Col-0

5.9 ROS determination

- DCFDA
- Biotek Cytation 5
- Hydrogen peroxide
- Distilled water
- MS medium
- Sulphur deficient MS medium
- Sulphur excess MS medium
- 96-well black plate for fluorescence analysis
- *Arabidopsis thaliana* Col-0 grown *in vitro*

6 METHODS

6.1 *Arabidopsis thaliana* in vitro culture

For the culture of *A. thaliana in vitro*, MS medium was prepared, and 30ml of the medium were poured into Rey Biofood Square food containers. In order to prepare a matrix in which the seeds of *A. thaliana* could grow, plasticized PVC tubes of 3cm in diameter were cut into rings of 0.5cm in height. Squares of nylon stockings were cut out and stretched over the PVC rings in such a way that the seeds would not fall through, yet leaving enough space for the seeds to germinate and the roots to pass through. The nylon cloths were secured around the PVC tube utilizing plastic zip ties. One PVC ring with nylon cloth was put inside every food container with the nylon cloth facing outward. Each container was then covered with aluminum foil, and autoclaved for 20 minutes at 121°C.

Arabidopsis thaliana Col-0 seed were sterilized using a liquid disinfection protocol. A sodium hypochlorite solution 2.5% with 1-2 drops of tween 80 was prepared, and 1ml of the solution was added to a 1.5ml Eppendorf tube along with *A. thaliana* seeds. The tubes were vortexed for 10 minutes and then spinned in a centrifuge. Then, inside the laminar flow cabinet, the sodium hypochlorite solution was removed, and 1ml of sterile distilled water was added. The seeds were rinsed in water five times. After the last rinse, the seeds were left in water and allowed to rest for 4-5 days at 4°C to break dormancy (Park, Kwak, Oh, Kim, & Kang, 2009). The sterile seeds were distributed in the plastic containers on top of the nylon cloth utilizing a 20-200µl micropipette. The containers were covered with plastic wrap and secured with a rubber band. The seeds were grown at 20°C, under white led lights with a 12-hour photoperiod.

6.2 *Arabidopsis thaliana* sand culture

For the culture of *A. thaliana* on sand, river sand was thoroughly washed with water and then autoclaved for 20 minutes at 121°C. The sand was then spread out on a tray and allowed to dry. Then, the sand was distributed in 6-well plates for cellular culture, making sure that the sand filled half of the capacity of each individual well. Liquid MS medium was prepared, and it was distributed in all wells, using enough medium to wet all the sand in each well. In separate plates, *A. thaliana* Col-0 and PR1::GUS were sprinkled over the sand in each well. Each plate was covered with its lid, and then left to rest for 4-5 days at 4°C. The seeds were grown at 20°C, under white led lights with a 12-hour photoperiod. The liquid in each well was replenished as needed in order to avoid the plants from drying out.

6.3 Sulphur nutritional stress treatments

For the different sulphur nutritional stress treatments, MS medium and modified versions of the MS medium were prepared. One of the modified mediums was completely deficient with sulphur, while the other had a 4-fold excess in sulphur in comparison with the standard MS medium. A salicylic acid 10 mM solution was prepared by dissolving 0.207 g of salicylic acid in 5 ml of ethanol and then completed to 150 ml with distilled water. Afterwards, 1 ml of the 10 mM was diluted to 20 ml to achieve a final concentration of 0.5 mM, and 2-3 drops of tween 80 were added. A mock solution was prepared to maintain the same alcohol:water ratio without the addition of salicylic acid. Two batches of treatments were prepared, both containing treatments with sulphur deficiency, MS, and sulphur excess, but one was treated with salicylic acid while the other was treated with the mock solution.

The plants grown in sand were used for these treatments. The first step was to rinse the sand in each well to remove all the nutrients. This was done by utilizing distilled water

and a Pasteur pipette, rinsing the sand several times until its conductivity dropped below 200µS. Then, an airbrush was used to spray the plants with the salicylic acid or mock solution accordingly. The excess liquid remaining from the spraying was removed with a Pasteur pipette. Up next, each type of medium was distributed in two of the six wells of each plate, making sure that there was enough liquid to barely cover the sand in each well. The plants were left uncovered, at room temperature on the bench top overnight.

6.4 GUS Reporter System

Solutions of disodium phosphate (Na_2HPO_4) 1M and monosodium phosphate (NaH_2PO_4) 1M were prepared. Then, 34.2 ml and 15.8 ml of each solution respectively were mixed to yield 50ml of NaPi 1M solution. A solution of EDTA 0.25M pH: 8 was also prepared. In order to prepare the X-Gluc solution, 50 ml of the NaPi solution, 20 ml of the EDTA solution, and 5 ml of Triton X-100 were combined, and then diluted to 500 ml with distilled water. Finally, 250 mg of X-GlcA were dissolved in 12 ml of N,N-Dimethylformamide, which were then added to the final solution. The X-Gluc solution was stored at -4°C.

After the sulphur nutritional treatments, PR1::GUS plants were placed in a 24-well plate, with 4-5 plants per well. Next, 1 ml of the X-Gluc solution was added to each well. The plates were put inside a glass vacuum desiccator, which was attached to a vacuum pump, and they were left at -15 Bar for 1 hour. Afterwards, the plates were covered with their lids, sealed with parafilm, and left in an incubator at 37°C for 2-3 days. After incubation, the X-Gluc solution was removed and the plants were discolored with 96% ethanol.

6.5 Sulphate Determination by Turbidimetry

To determine sulphate in plant tissue, a turbidimetric method was used. Solutions of HCl 6M and 70% sorbitol were both prepared to carry out the procedure. A calibration curve was obtained by preparing sulphate standards of sodium sulphate ranging from 0 ppm to 50 ppm of sulphate concentration. 10 ml of each standard were placed in a 50 ml Falcon tube, followed by 1 ml of HCl 6M, and then 5 ml of the sorbitol solution. Approximately 1 g of barium chloride was added to each tube, and then vigorously shaken. Each tube was left to rest for at least 5 minutes, and then the samples were analyzed in a turbidimeter at 470nm (Verma, 1977). All of the standards were analyzed by duplicate. The mean of the measurements of each standard was used to construct the calibration curve.

For the analysis of sulphate from plant tissue, the first step was weighing the samples, followed by an acid digestion of the explants. This was done by submerging the samples either in 2 ml of 69% nitric acid or 2 ml of 69% nitric acid + 1 ml of hydrogen peroxide and letting them rest for 24 hours (Huang, 2004). Each extract was then carefully diluted to 25 ml with distilled water. In order to determine the recovery index of this method with the reagents used in the acid digestions, samples with a concentration of 30 ppm of sulphate were prepared with the same proportions of nitric acid and peroxide used in the analysis of plant tissue. Then, 10 ml aliquots of all samples were used to determine sulphate with the same procedure as the standards. Finally, calculations were made to determine the amount of sulphate in the initial mass of plant tissue. The following equation was developed to determine the concentration of sulphate in plant tissue:

$$SO_4\ in\ tissue\ \left[\frac{nmol\ of\ SO_4}{mg\ FW}\right] =$$

$$\frac{sample\ absorbance - intercept}{slope} \times$$

$$\frac{100}{recovery\ index\ of\ digestion\ method\ [\%]} \times$$

$$\frac{dilution\ volume\ of\ sample\ [L]}{sample\ weight\ [mg]} \times \frac{10^6}{SO_4\ MW}$$

Equation 1. Calculation of sulphate concentration in fresh weight of A. thaliana

6.6 Protein extraction

In order to determine cysteine, a prior protein extraction is required (Krueger, et al., 2009). Five different methods of extraction were conducted. The first method utilized an extraction buffer with the following reagents and concentrations: 0.1M EDTA pH:8, 0.12M Tris-HCl, pH:6.8, 4% w/v SDS, and 10% v/v β-ME. Plant samples in the range of 0.1-0.2 g were put inside a 1.5 ml Eppendorf tube alongside four 4mm glass beads, and then submerged in liquid nitrogen until frozen. The tubes were then vortexed until a paste was formed inside the tube. The samples were freezed in liquid nitrogen and vortexed again. Lastly, 1 ml of the extraction buffer was added to the tubes. The samples were then boiled for 10 minutes (Tsugama, Liu, & Takano, 2011). The second and third methods used only distilled water or ethanol for the extraction (Carrillo, 2011). Explants in the range of 0.1-0.2 g were subjected to the same freezing and vortexing procedure described for the previous method. Then, 1ml of distilled water or 96% ethanol was added accordingly to each tube. The samples were then boiled for 30 minutes. The fourth method of extraction consisted of a three-step process with a mixture of water/ethanol. The samples were weighed, frozen and vortexed as described for the previous methods. Then, 1ml of 80% ethanol was added to the tube, and it was boiled for 30 minutes; this was done twice, collecting the extracts in a separate tube. The third time, 1 ml of 50% ethanol was added, and the sample boiled for another 30 minutes. The extract was

removed once again and added to the previous extracts to yield a final volume of 3 ml in a proportion of 70:30 ethanol:water (Cross, et al., 2006). The last method of extraction started with the same freezing and vortexing than the previous methods. Then, 1 ml of 40% ethanol was added to the tube, and then it was left overnight at 4°C (Carillo, et al., 2008). All tubes were centrifuged and the supernatant was used for protein and cysteine determination.

All five methods of extraction were also conducted utilizing fortified solutions with a cysteine concentration of 0.6 mM. This was done with the intention of determining if cysteine could be detected on the protein extracts, and discard any possible interference in the measurement from the extraction media or plant contents.

6.7 Protein quantification

For protein quantification, the Bradford protein assay was employed (Bradford, 1976). 25µL of each extract was put inside an individual well in a 96-well plate, followed by 100µL of Bradford reagent. The plate was incubated in the dark at room temperature for 25 minutes and then read in the Sirio S ELISA plate reader at 620 nm (Bradford, 1976). Each sample was measured twice. Protein concentration was determined utilizing a standard curve provided by José Alvarez, professor of Chemical Engineering in at USFQ, which was realized in the Agrobiotechnology and Food Biotechnology Laboratory of USFQ (Appendix 1).

6.8 Cysteine determination

Cysteine was determined utilizing a modified version of the colorimetric method proposed by Gaitonde in 1967. A ninhydrin solution was prepared by adding 250 mg of ninhydrin to 6 ml of acetic acid and 4 ml of 0.6M phosphoric acid. A calibration curve was obtained by preparing standards with known cysteine mass utilizing cysteine

hydrochloride, ranging from 0.01 to 0.08 µmol of cysteine. The method was adapted to be realized in a thermal cycler instead of boiling the samples in a water or dry bath. Using a multichannel micropipette, 50 µl of each cysteine standard were added to a well of a 96-well PCR plate, followed by 50 µl of acetic acid, and 50 µl of the ninhydrin solution. Each standard was replicated 8 times. The plate was then introduced in the thermal cycler, under a program designed to boil the standards at 95°C for 10 minutes. Each sample was then diluted in 2 ml of 96% ethanol, and its absorbance at 560 nm was measured. The mean of the eight replicates of all standards was used to construct the calibration curve. The same procedure was followed to determine the cysteine concentration from the different protein extracts.

6.9 ROS determination

For the fluorescent determination of ROS, a working solution with the following reagents and concentrations was prepared: 20µM DCFDA, 1/10 MS medium, and 0.1% Tween 80. *Arabidopsis thaliana* Col-0 grown *in vitro* were used for this analysis. 2-3 plants were put into separate wells of a 24-well plate. Different groups of plants were treated for 5 minutes with MS medium, sulphur deficient medium, sulphur excess medium, or salicylic acid 0.5 M accordingly. After their respective treatments, plants were transferred to clean 24-well plates, and 3 ml of the working solution was added to each well. Another group of plants was mechanically damaged using tweezers and immediately submerged in 3 ml of working solution. The plates were incubated in the dark for 30 minutes at room temperature. After incubation, 200 µl of each sample was loaded four times into individual wells of a 96-well black plate for fluorescence analysis. Fluorescence was measured in a Biotek Cytation 5 fluorometer, with an excitation at 488 nm and emission at 525 nm. The remaining leaves were separated into 2 ml tubes, dried in an oven, and weighed.

6.10 Statistical analysis

The calibration curves modeled for the methods of turbidimetric sulphate determination and colorimetric cysteine determination were obtained in Microsoft Office Excel. The analysis for the results of protein extraction consisted of a one-way ANOVA and Tukey pairwise comparisons, both of which were done in Minitab 17. Lastly, the analysis of the results of fluorescent ROS determination consisted of a Welch's test and Games-Howell pairwise comparisons, which were also conducted in Minitab 17.

7 RESULTS

7.1 *In vitro* culture of *Arabidopsis thaliana*

The nature of the different nutritional experiments conducted in this study demanded a modified version of *in vitro* culture that allowed an easy way to transfer plants from one medium to another. The conventional method of *in vitro* culture proved to be inefficient in this regard, since every plant had to be removed from the growth medium and transferred to the nutritional assay individually. The proposed method of culture with PVC rings and nylon stockings as a matrix was successful, because it simplified the transfer procedure from one medium to the other.

Figure 1. Arabidopsis thaliana Col-0 grown in vitro over a nylon cloth matrix

Figure 1 shows the growth of *A. thaliana* Col-0 in vitro with the designed system of plasticized PVC rings and nylon cloth as a support matrix. The PVC tube kept its integrity after autoclaving, and the nylon cloth allowed the seeds to germinate.

7.2 *Arabidopsis thaliana* sand culture

The culture of *A. thaliana* in sand utilizing plastic plates normally used for cellular culture also proved to be successful. This method of culture made it easier to treat the plants with their respective nutritional diet without the need of transferring them from one vessel to another. The main appeal of this method is that healthy plants can be cultured without the use of a surface sterilization protocol, thus reducing the number of plants lost to infection, while also reducing the time needed to set up a batch of *A. thaliana*.

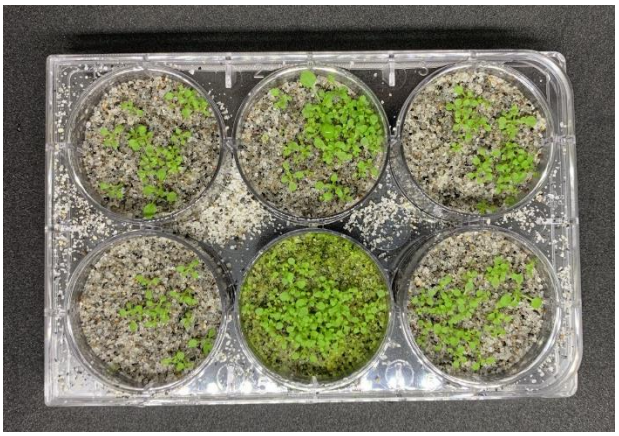

Figure 2. Arabidopsis thaliana PR1::GUS grown on river sand

Figure 2 shows the growth of *A. thaliana* PR1::GUS in a 6-well plate for cellular culture, with river sand and liquid MS medium as substrate.

7.3 Sulphur nutritional stress treatments and GUS Reporter System

In this analysis, the GUS reporter system allowed for easy detection of *PR1* expression inside the PR1::GUS plants. If the analyzed plant tissue stain blue after the addition of the chromogenic substrate X-Gluc, it can be inferred that *PR1* expression is being promoted by the particular treatment that they were subjected to.

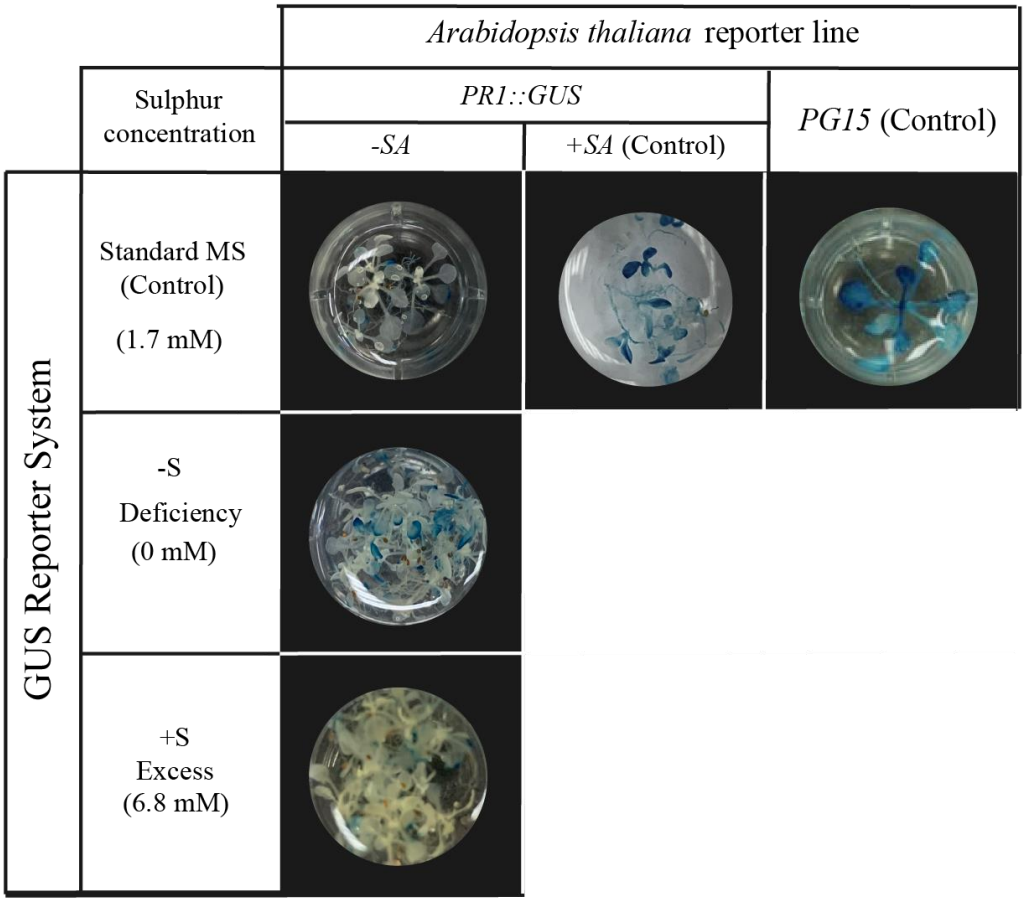

Figure 3. GUS stain after the application of sulphur treatments and salicylic acid (SA) in A. thaliana reporter lines PR1::GUS and PG15

Figure 3 shows the results of the sulphur treatments in *A. thaliana*. In the PR1::GUS reporter line, blue colored leaves indicates the expression of the *PR1* gene. Plants treated with the standard MS concentration of sulphur without SA did not show expression of the *PR1* gene. Plants treated with the standard MS concentration of sulphate plus SA showed expression of the *PR1* gene as expected (Shapiro & Zhang, 2001). Sulphur deficiency weakly promoted the activation of *PR1*, while sulphur excess did not promote the expression of *PR1*. Since *A. thaliana* PG15 is under constitutive expression of the *uidA* gene it should always turn blue when X-Gluc is added. For this reason, PG15 plants were used as a control to determine if the GUS staining assays were working correctly (Jefferson, Kavanagh, & Bevan, 1987).

7.4 Sulphate determination by turbidimetry

Sulphate is the main form of sulphur that plants assimilate from the soil into their cells, which is the first step in sulphur metabolism that then leads to the formation of all the other sulphur-containing compounds found in the cell (Bloem, Haneklaus, & Schung, 2015). If the shifts in concentration of specific products inside sulphur metabolism want to be analyzed, it is crucial to be able to determine how amount of sulphate in the plant is affected by the different nutritional stresses. Sulphate is commonly determined by a turbidimetric method that utilizes barium chloride ($BaCl_2$), which, upon reacting with sulphate, forms insoluble barium sulphate ($BaSO_4$) crystals, thus increasing the turbidity of the solution (Verma, 1977). To utilize this method, obtaining a calibration curve is necessary, because it will allow for the determination of sulphate in unknown samples based on the readings for standards with known concentrations of sulphate (Verma, 1977).

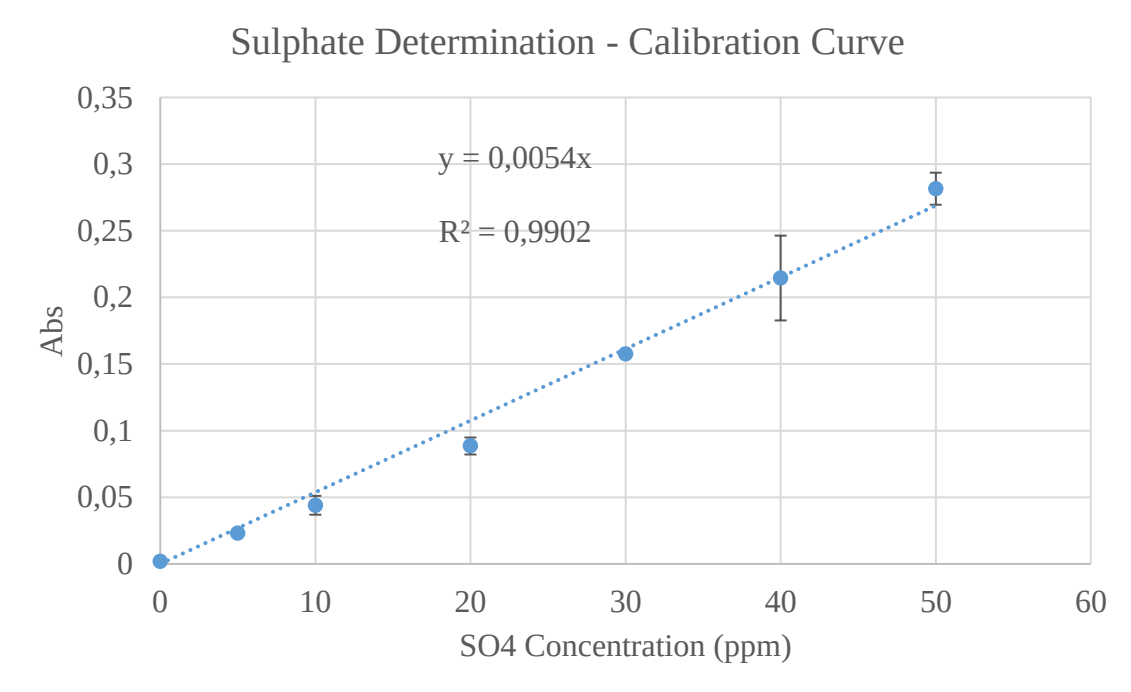

Figure 4. Calibration curve for the turbidimetric determination of sulphate

Figure 4 shows the calibration curve obtained for the turbidimetric determination of sulphate in plant tissue. The equation obtained was y = 0,0054x, with an R^2 = 0.9902, which indicates that the data have an appropriate fit and explain the variability of the model.

It is not common for a quantification procedure to be able to detect all of the target compound in a sample. The percentage of compound detected in relation to the real concentration in the sample is known as the recovery index. The recovery index of the determination of sulphate for both digestion protocols was calculated.

Table 1. Calculation of the recovery index for sulphate determination with two types of digested samples

Method	**SO_4 in sample (ppm)**	**Mean of SO_4 determination (ppm)**	**Recovery (%)**
Nitric acid	30	27.91	93.03
Nitric acid + peroxide	30	15.49	51.62

Table 1 shows the calculation of the recovery index for sulphate determination. Samples with a known concentration of sulphate were prepared and analyzed. The means of the measurements were compared against the real concentration and the recovery index was calculated. Samples digested only with nitric acid yielded a recovery index of 93%, which falls inside of the expected recovery for this method (Verma, 1977). The recovery index of samples with nitric acid + peroxide yielded a much lower recovery index of 51%.

After determining the recovery index, sulphate was determined in *A. thaliana* plants grown *in vitro*. Samples of different mass were digested and then subjected to the determination protocol.

Table 2. Sulphate determination in A. thaliana samples grown in vitro

Sample	Digestion method	Fresh weight (mg)	Mean of SO_4 detected in sample (ppm)	nmol SO4/mgFW
1	Nitric acid	27.50	0.019	34.77
2	Nitric acid	57.50	0.0335	30.41
3	Nitric acid + peroxide	35.60	0.0125	33.20
4	Nitric acid + peroxide	40.20	0.031	37.19
			Mean	33.89
			Standard Deviation	2.84
			Coefficient of variation	8.38%

Table 2 shows the results of sulphate determination in tissue of *A. thaliana*. Equation 1 was used to determine the concentration of sulphate in tissue. Adjusting the obtained measurements to the weight of each sample and recovery index of each method yielded similar final concentrations of sulphate for all samples. All mathematical adjustments needed for this calculation are already included in Equation 1.

7.5 Cysteine determination, protein extraction and quantification

Cysteine is at the heart of sulphur metabolism, since the synthesis of majority of the sulphur containing compounds uses cysteine as a precursor (Bloem, Haneklaus, & Schung, 2015). In order to properly analyze cysteine in plant tissue, it is necessary to carry out a protein extraction procedure, and the extracts can be used for cysteine quantification. There are numerous protocols for protein extraction, ranging from complex extraction buffers to minimalist ones. For this reason, five different methods of protein extraction were tested: protein extraction buffer, alcohol-only, water-only, alcohol + water, and cold extraction.

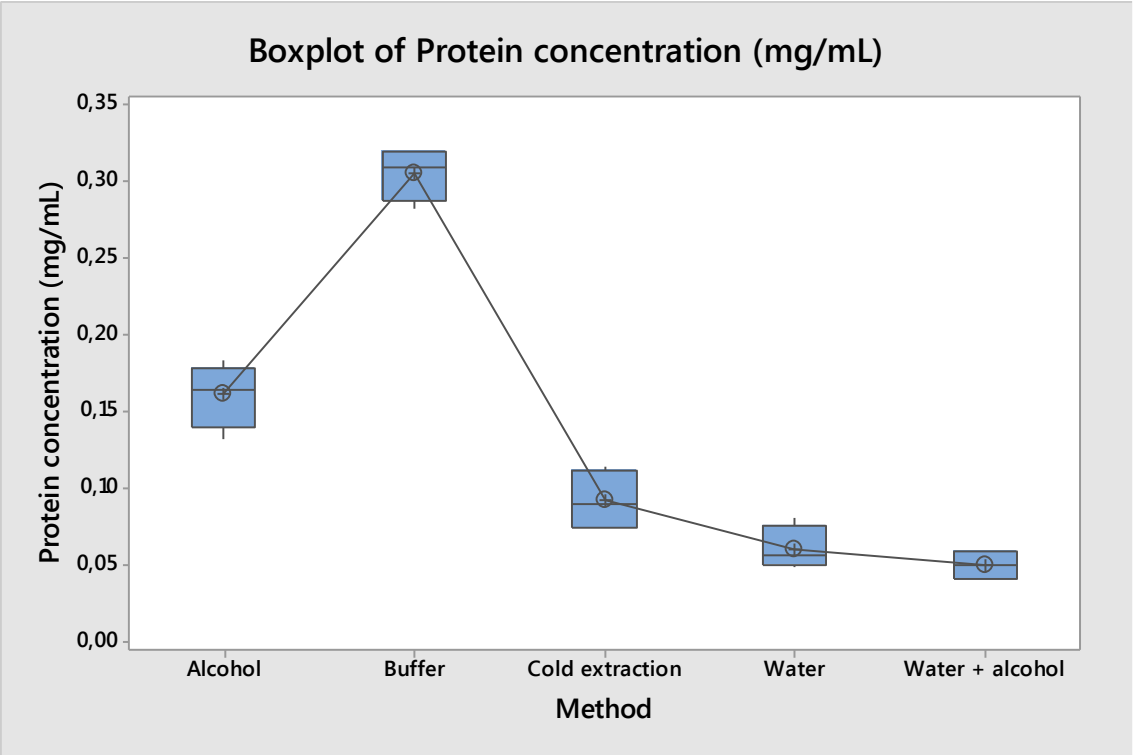

Figure 5. Boxplot of the concentration of protein after four methods of extraction

Figure 5 shows a boxplot of the protein concentrations for each extraction method. The graph shows that the extraction with the protein buffer has a higher extraction capacity than the other four methods. The means of protein concentration were: extraction buffer 0.305 mg/ml, alcohol only extraction 0.161 mg/ml, cold extraction 0.09160 mg/ml, water only extraction 0.05057 mg/ml, and water + alcohol extraction 0.04960 mg/ml. This indicates that all methods allowed for the extraction of protein to some degree.

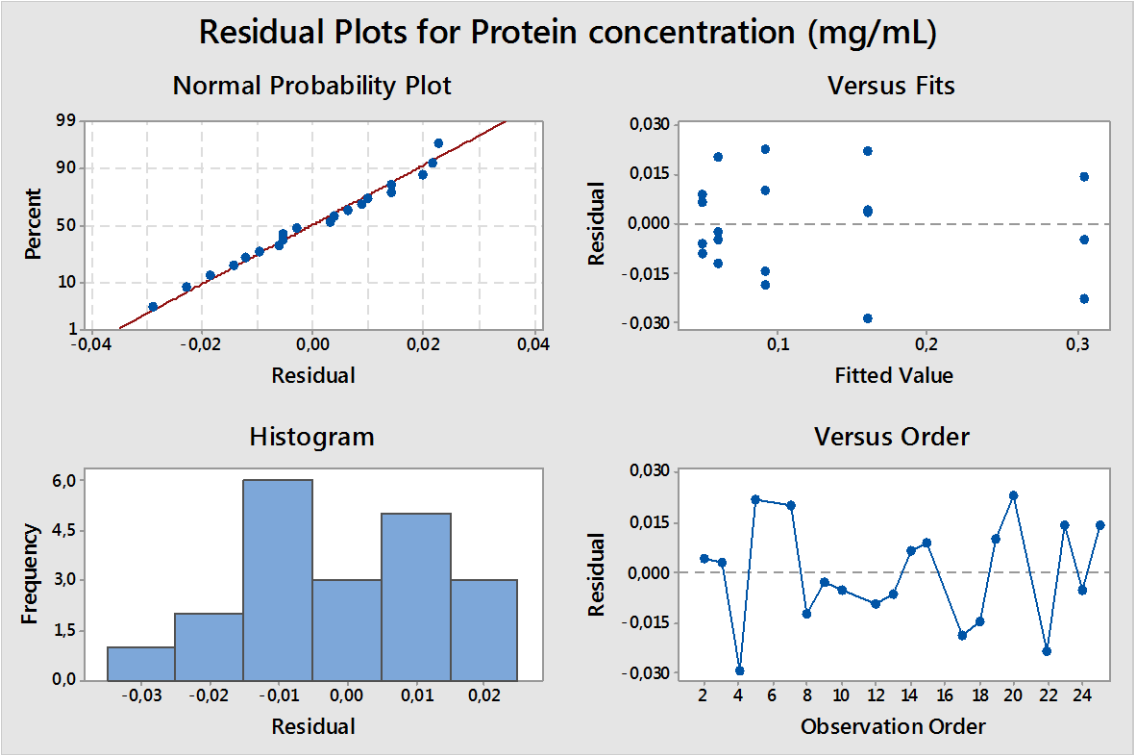

Figure 6. Four in one chart for the ANOVA of protein extraction

Figure 6 shows the four in one chart displayed in Minitab 17 along with the analysis of variance for protein extraction. All of the ANOVA assumptions were met (normality, independence, and equal variances) (Devore, 2008).

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Method	4	0,177689	0,044422	154,96	0,000
Error	15	0,004300	0,000287		
Total	19	0,181989			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0,0169311	97,64%	97,01%	95,80%

Figure 7. Minitab output for the ANOVA of protein extraction

Figure 7 shows the ANOVA table displayed in Minitab 17 for the analysis of protein extraction. The ANOVA table shows a p value <0.05 which indicates that at least one of the means of protein concentration was significantly different. The model summary presents an R^2 of 97.64%, which suggests that the variability observed in the response data is correctly explained by the model obtained (Devore, 2008).

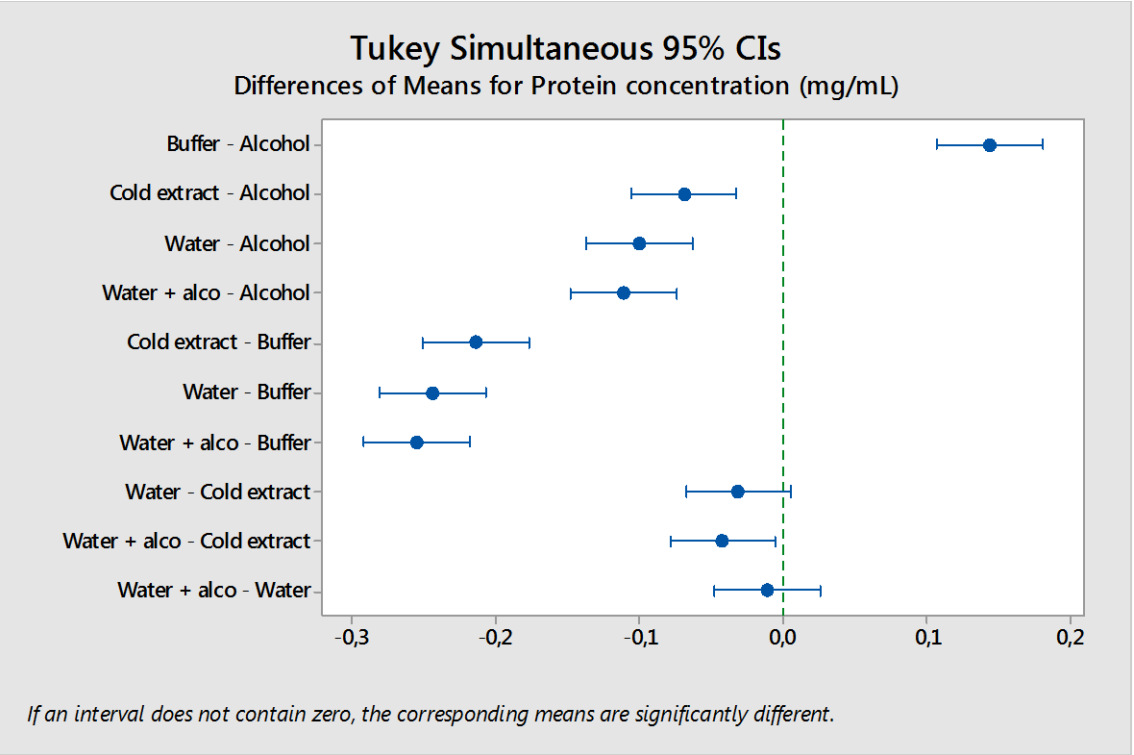

Figure 8. Tukey simultaneous comparisons for the means of protein concentration of the five methods of extraction

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Method	N	Mean	Grouping
Buffer	4	0,30540	A
Alcohol	4	0,1610	B
Cold extraction	4	0,09160	C
Water	4	0,06057	C D
Water + alcohol	4	0,04960	D

Means that do not share a letter are significantly different.

Figure 9. Minitab output for the groupings of Tukey pairwise comparisons for the four methods of extraction

Figure 8 and Figure 9 show the outputs of Minitab 17 for the Tukey pairwise comparisons of the means for each method of extraction. These results show that the mean of protein concentration for the buffer extraction is significantly different than the other four methods. The mean of protein concentration for the alcohol only extraction is significantly higher than the three remaining methods. The cold and water-only

extractions are statistically the same. Lastly, the water + ethanol extraction is statistically equal to the water-only extraction.

After protein extraction, a colorimetric method utilizing ninhydrin was used to determine cysteine content in the extracts. This method is based on the specific reaction that takes place between ninhydrin and cysteine at low pH, which yields a pink product that can be detected by colorimetry (Gaitonde, 1967). In order to utilize this method, a calibration curve had to be obtained, which will enable the determination of cysteine in an unknown sample based on the readings of standards with known masses of cysteine (Gaitonde, 1967).

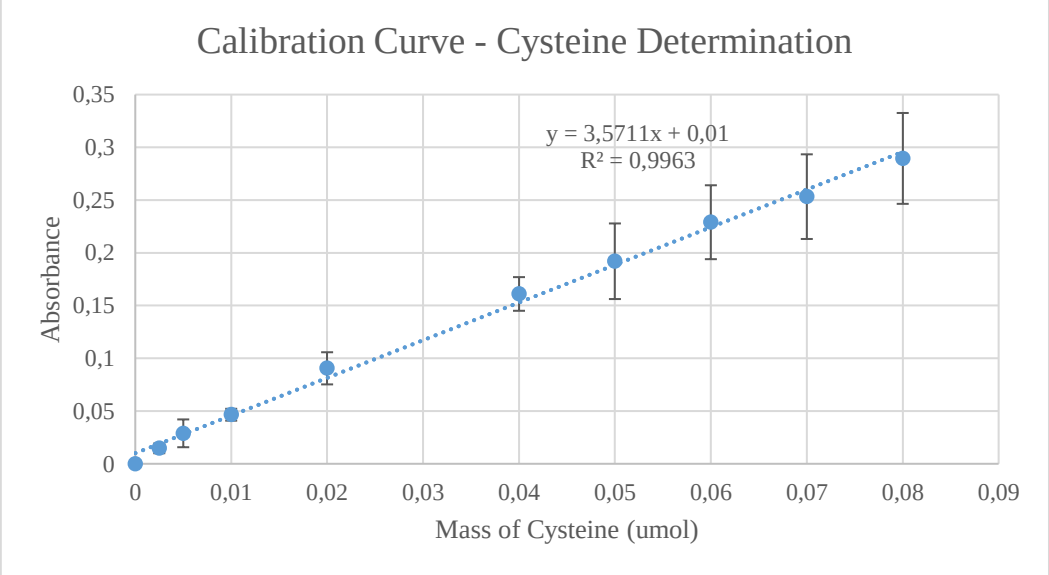

Figure 10. Calibration curve for the colorimetric determination of cysteine

Figure 10 shows the calibration curve obtained for the colorimetric determination of cysteine. The equation obtained was an $y = 3{,}5711x + 0{,}01$, with $R^2 = 0{,}9963$, which indicates that the data have an appropriate fit and explain the variability of the model.

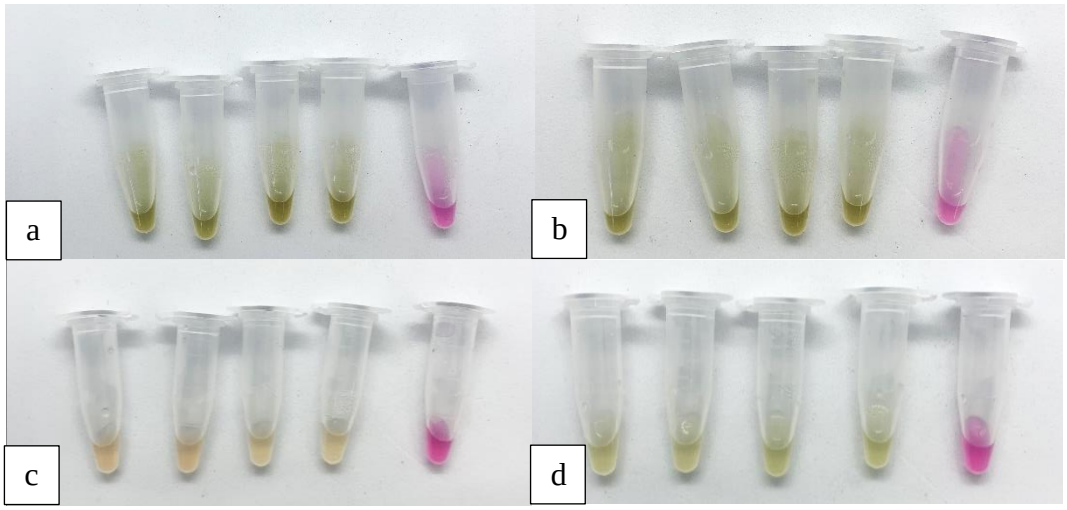

Figure 11. Protein extracts of the four methods of extraction with their respective cysteine control

Figure 11 shows the protein extracts used for the determination of cysteine in plant tissue: a) alcohol, b) water, c) alcohol + water, d) cold extraction. The tube on the right of all samples is a cysteine control ran parallel with the samples. It was not possible to determine the amount of cysteine in the extracts, since none of the samples turned pink similar to the control, which suggests that the reaction did not take place. Furthermore, detection of cysteine with the ninhydrin protocol yielded a reading of 0 µmol of cysteine in all samples.

7.6 ROS determination

The determination of ROS in relation to different nutritional sulphur stresses is of particular importance, since ROS are the link between the SA pathway and sulphur metabolism (Bloem, Haneklaus, & Schung, 2015). The most common protocol to determine ROS is utilizing the method of DCFDA, which is a molecule that becomes fluorescent when oxidized by ROS (Da, et al., 2019). Different assays were conducted in this study in order to determine the effect that sulphur nutritional stress has on the production of ROS. Plants treated with standard MS medium were used as a negative control, since optimal conditions should not show signs of increased ROS in the plants.

On the other hand, mechanically damaged plants were used as a positive control, for it is known that physical damage causes the production and accumulation of ROS inside the cell (Mittler, Vanderauwera, Gollery, & Van Breusegem, 2004).

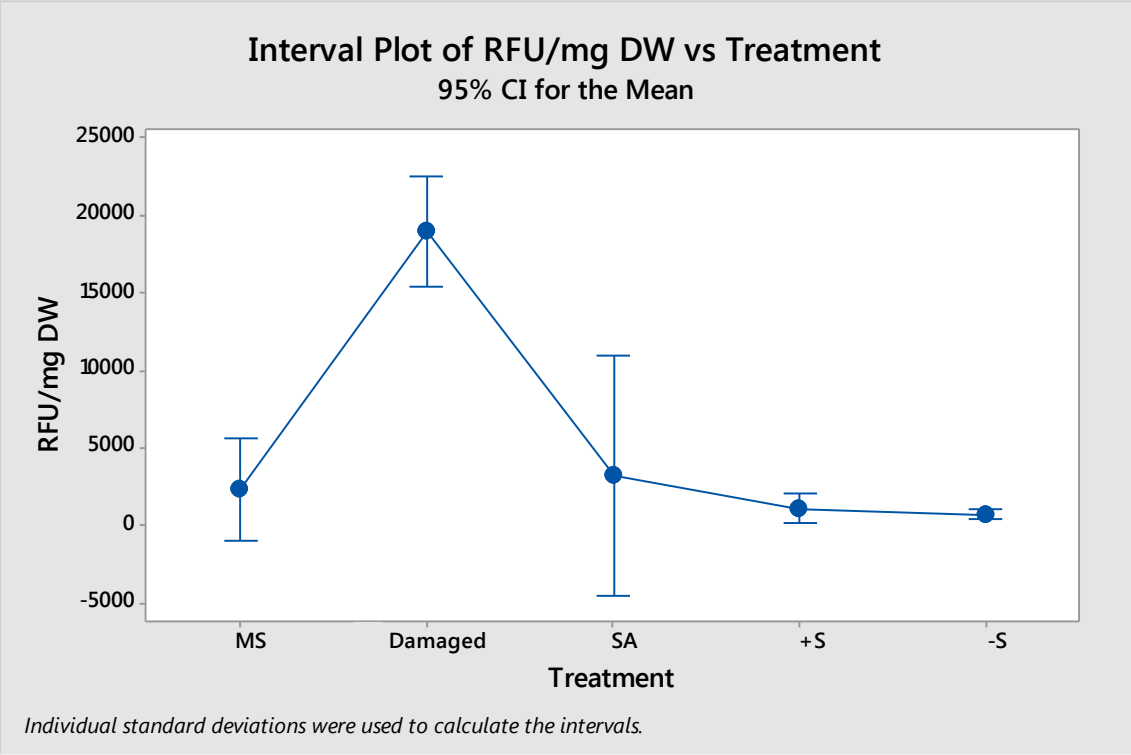

Figure 12. Interval plot of ROS determination for the five different treatments

Figure 12 shows an interval plot of the ROS determination for the five different treatments. The graph shows that the mechanically damaged plants produced a higher amount of ROS than the other four groups of plants. All the plants subjected to nutritional treatments (MS, SA, +S, and –S) where analyzed for ROS after 5 minutes in treatment. Damaged plants were analyzed for ROS immediately after inflicting mechanical damage. The means of each treatment were: Damaged 18947 RFU/mgDW, SA 3190 RFU/mgDW, MS 2314 RFU/mgDW, +S 1081 RFU/mgDW, and -S 695 RFU/mgDW.

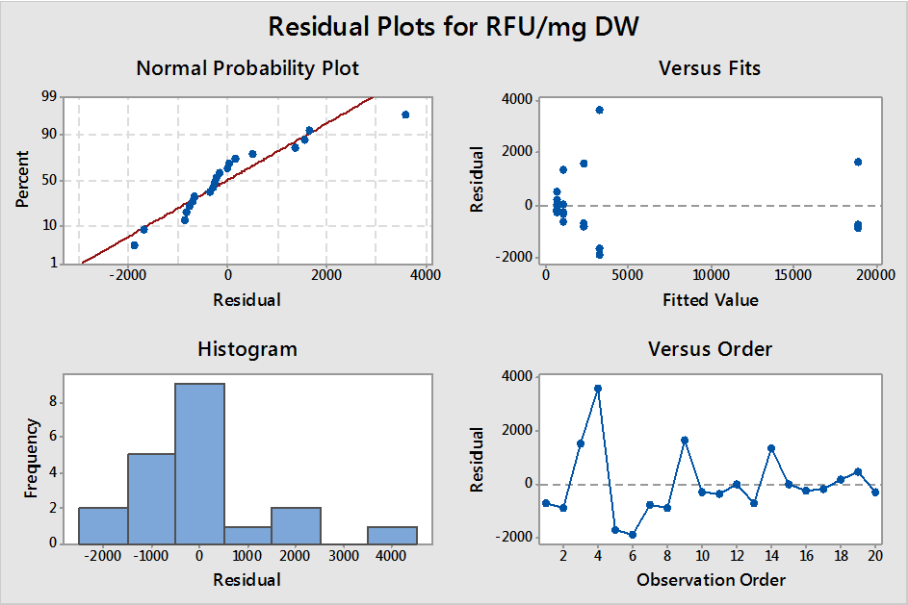

Figure 13. Four in one chart for the ANOVA of ROS determination

Figure 13 shows the four in one chart displayed in Minitab 17 along with the analysis of variance for ROS determination. The ANOVA assumptions were not met in this case. The data follow a normal distribution and are independent, but they do not show equal variances (Devore, 2008).

Welch's Test

Source	DF Num	DF Den	F-Value	P-Value
Treatment	4	4,80180	86,01	0,000

Model Summary

R-sq	R-sq(adj)	R-sq(pred)
96,33%	95,35%	91,99%

Figure 14. Minitab output for Welch's test of ROS determination

Figure 14 shows the Welch's test table displayed in Minitab 17 for the analysis of ROS determination. The table indicates that at least one of the means of ROS detection was significantly different ($p<0.05$) (Devore, 2008). The model summary presents an R^2 of 96.33%, which suggests that the variability observed in the response data was correctly explained by the model obtained (Devore, 2008).

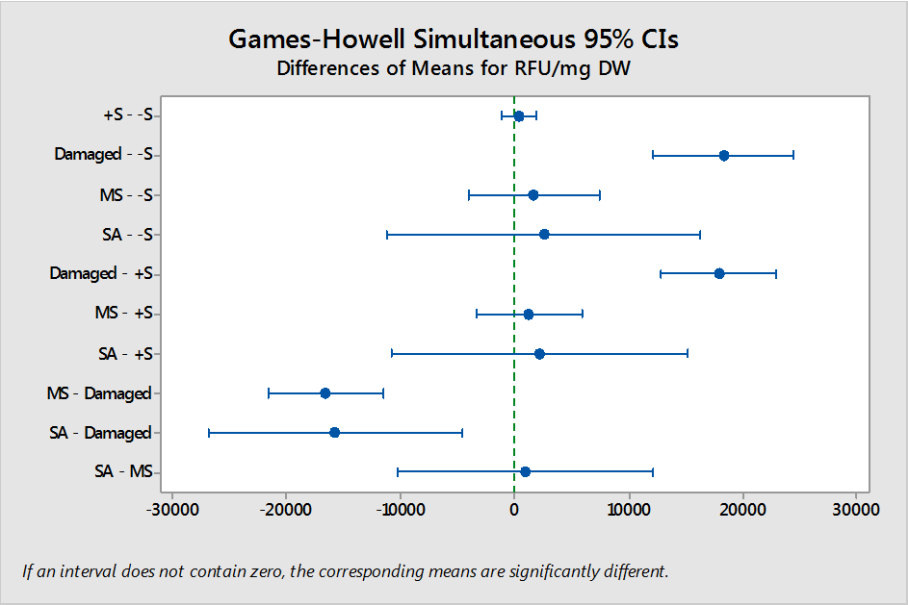

Figure 15. Games-Howell comparisons for the means of ROS determination of the five treatments

Games-Howell Pairwise Comparisons

Grouping Information Using the Games-Howell Method and 95% Confidence

Treatment	N	Mean	Grouping
Damaged	3	18947	A
SA	3	3190	B
MS	3	2314	B
+S	5	1081	B
-S	6	695	B

Means that do not share a letter are significantly different.

Figure 16. Minitab output for the groupings of Games-Howell pairwise comparisons for the five treatments

Figure 15 and Figure 16 show the outputs of Minitab 17 for the Games-Howell pairwise comparisons of the means for each group of plants. These results show that the damaged plants produced a significantly higher amount of ROS compared to the other four groups. All the other groups were statistically equal.

8 DISCUSSION

8.1 *In vitro* culture of *Arabidopsis thaliana*

The culture of *A. thaliana* utilizing PVC rings and nylon stockings proves to be easy and affordable. Similar protocols have been developed for other types of *in vitro* experiments, in which commercial nylon meshes are used as a matrix over the culture medium (Crombez, et al., 2016; Johnson, et al., 2011). In contrast, the method proposed in this study utilized nylon stockings, which are readily available everywhere and are quite inexpensive, and yet perform perfectly in order to let the seeds germinate (Figure 1). The plasticized PVC also performed well in setting up this culture system, since it resisted the autoclaving process. Other plastics were tested to make the rings, including polystyrene (from plastic Petri dishes) and polypropylene (from Falcon tubes), but they were not suited for this purpose since they deformed during autoclaving. This method of *in vitro* culture will prove to be of great use in the future when making experiments with different nutritional diets and plant hormones, since the ring can be easily removed from the medium while causing very little damage to the roots of the plants.

8.2 *Arabidopsis thaliana* sand culture

The proposed method for culture of *A. thaliana* on sand also proved an easy and practical way to work in batches for the different nutritional assays that were conducted (Figure 2). This method has some advantages and disadvantages in comparison with *in vitro* culture. Firstly, sand culture does not require the use of agar substrates that tend to be expensive, which are necessary when working *in vitro* (Davis, Hall, Millar, Darrah, & Davis, 2009). Also, the seeds do not have to go through a process of surface sterilization (Davis, Hall, Millar, Darrah, & Davis, 2009), so sand culture also reduces the time required to set up a batch of *A. thaliana* plants. It is also convenient to be able to subject the plants to their respective nutritional treatments in the same well, without having to do

any sort of transfer; the sand can be easily rinsed and medium can be replaced, all in the same container. Lastly, the application of plant hormone (in this case the salicylic acid solution) can be easily done with an airbrush, which ensures an even coating over the plants. The downside with this method of sand culture is that it is difficult to maintain even and steady conditions of humidity in each well, which is not the case with *in vitro* culture. It is necessary to check the plants regularly, since the medium evaporates quite rapidly, and it must be replenished constantly so the plants do not dry out. Removing the excess condensation on the lids of the plates is also important, since the risk of fungal contamination was reduced. Overall, this seems to be an appropriate and useful method to carry out nutritional studies in plants like *A. thaliana.*

8.3 Sulphur nutritional stress treatments and GUS Reporter System

The GUS reporter system was utilized to assess the expression of the *PR1* gene as promoted by different conditions of sulphur dietary stress and salicylic acid application. The *A. thaliana* plants cultivated on sand were subjected to different conditions of sulphur nutritional stress, and then were analyzed for the expression of the *PR1* gene. It is appropriate to mention that the GUS reporter system assesses the activity of the *PR1* promoter, which is reflected in the production of β-glucoronidase, and not the expression of *PR1* per se. Nevertheless, the presence of β-glucoronidase and the blue staining of the plant tissue is generally interpreted as expression of the gene of interest (Karcher, 2002). There were three control groups of plants which were used to validate the results observed in this assay. Plants subjected to the standard sulphur concentration of the MS medium should not show an expression of the *PR1* gene, while plants subjected to the standard sulphur concentration but treated with SA should turn blue indicating the expression of the *PR1* gene (Shapiro & Zhang, 2001). The third control group were *A. thaliana* PG15 plants, which have the *uidA* gene inserted after the CaMV 35S promoter, which is a

promoter of constitutive expression in plants. This means that the PG15 line should always turn blue, regardless of the conditions (Jefferson, Kavanagh, & Bevan, 1987); this is used as a control to determine that the staining process is taking place correctly. The three control groups showed the correct pattern of coloring in the assays realized (Figure 3).

Regrettably, the results obtained in relation to the sulphur treatments were not as evident as those reported by Gonzales and Criollo in 2015 and 2013, respectively. It has not been possible to fully replicate the results obtained in those studies, so it is clear that there are some conditions that are not being established exactly like the other authors, which difficults witnessing a more uniform expression of *PR1* when subjecting the PR1::GUS plants to sulphur deficiency. Nevertheless, it is clear to see that the expression of the *PR1* gene is more pronounced in the plants treated with sulphur deficiency (with or without SA) than in the plants treated with sulphur excess (with or without SA) (Figure 3). An explanation for this phenomenon requires the integration and analysis of several cellular mechanisms that regulate plant defense. It has been established that nutritional stress promotes the production and accumulation of ROS inside the cell (Cakmak, 2005), and it is also known that ROS promote the biosynthesis of salicylic acid as well as the expression of the *PR1* gene (Dzhavakhiya, Ozeretskovskaya, & Zinovyeva, 2007). SA and ROS work in a positive feedback loop, in which both promote the accumulation of each other inside the cell, which causes a shift in the redox potential due to the increased amount of ROS (Mittler, Vanderauwera, Gollery, & Van Breusegem, 2004). This causes a change in the protein NPR1, which shifts from its oligomeric form to its active monomeric form. The monomeric *PR1* then translocates to the nucleus, where it acts as a co-activator of the *PR1* gene by enhancing the binding of TGA transcriptional factors to

SA-responsive promoter elements in the DNA (Pieterse, Leon-Reyes, Van der Ent, & Van Wees, 2009).

Negative feedback for salicylic acid signaling comes from two primary sources, which are NPR1 and glutathione (GSH) (Shah, 2003; Herrera, Salinas, & Holuigue, 2015). If a plant accumulates high amounts of SA, levels of glutathione increase in order to mitigate the oxidative stress and suppress SA signaling (Herrera, Salinas, & Holuigue, 2015). In the case of a sulphur deficiency, the plant's cysteine pool will greatly reduce, and thus, it would not be able to synthesize new GSH (Wirtz, Droux, & Hell, 2004; Hasanuzzaman, Nahar, Islam, & Fujita, 2017). This means that the plant does not have the means to control the positive feedback loop that exists between SA and ROS, hence the expression of the *PR1* gene when treated with a sulphur deficiency (with or without SA). On the other hand, when the plants are subjected to an excess of sulphur in their diet, they are able to produce enough GSH to control the positive feedback loop of SA and ROS. It has been demonstrated that at higher sulphur concentrations, the cysteine pool inside the cell increases as long as there is enough O-acetylserine in the cell to carry out the reaction (Wirtz, Droux, & Hell, 2004). This implies that the excess of sulphur (with or without SA) will enable the plant to produce enough GSH to regulate the redox state of the cell to a degree in which the expression of *PR1* is greatly reduced. Still, it is necessary to find the proper conditions in which sulphur deficiency decisively promotes the expression of the *PR1* gene, and then the determination of the intermediary products of sulphur metabolism will provide valuable information as to what processes are taking place inside the cells of the plant.

8.4 Sulphate determination by turbidimetry

It is well known that the two main ways to determine sulphate concentration are ion chromatography and turbidimetric methods with barium chloride. Clearly, the

turbidimetric method has the advantage of being less expensive, and it does not need overly specialized equipment. Nevertheless, several roadblocks were met when standardizing the method for the determination of sulphate. Initially, a turbidimetric method optimized for the determination of sulphate in water was utilized (Rossum & Villarruz, 1961). In addition, different methods of extraction of sulphate were tested. Lyophilized and fresh tissue were frozen, ground, and mixed with distilled water, and then analyzed with the aforementioned method. The results obtained with the water analysis protocol were not satisfactory, since the measurements were extremely variable, and did not seem to be coherent. This may have been due to different factors, including that the working solution of that method was not designed to be used with plant extracts. Also, the barium sulphate crystals that form during the analysis are highly insoluble and precipitate quickly. Since the method for water analysis did not have any sort of stabilizer to keep the crystals evenly dispersed throughout the solution, the samples had to be processed in small batches, because if a sample was left to rest for more than five minutes, precipitation of the crystals affected the reading. It was also possible that the extraction methods were not good enough to draw out most of the sulphate from the tissue, which resulted in the uneven measurements observed with this method.

In order to make sure to extract all the sulphate form the plant tissue analyzed, an acid digestion protocol was adopted, which is common when realizing elemental analysis of the sort. A turbidimetric method specialized in the detection of sulphate from acid digestion extracts was adopted as well (Verma, 1977). This method has the particularity of using a 70% sorbitol solution as an added stabilizer, which promotes an even distribution of the barium sulphate crystals throughout the solution. The calibration curve obtained for this method clearly shows that the procedure is fit to determine sulphate concentration, since it shows high precision in its readings (Figure 4). The R^2 obtained

for the calibration curve is 0.9902, which indicates that the data is well fitted to the regression line. This also indicates that the variability observed in the response data (absorbance) is explained by the modeled curve (Montgomery, 2017). A high R^2 is a good indicator that precise predictions will be made when new samples are analyzed with this method (Montgomery, 2017).

In relation to the recovery index obtained for the turbidimetric determination of sulphate, the different methods of acid digestion yielded vastly different results. The samples prepared with 2 ml of nitric acid and diluted to 25 ml with 30 ppm of sulphate showed a recovery of 93% (Table 1). This recovery index falls in the acceptable range of recovery determined for most analytical chemistry procedures, and it reaches the levels of recovery described for this particular technique (Ravisankar, Navya, Pravallika, & Sri, 2015; Verma, 1977). The opposite is true for the samples prepared with nitric acid and hydrogen peroxide, which have a low recovery index (51%) (Table 1). Therefore, acid digestion with nitric acid only seems to be better suited for sulphate determination with the proposed method. Regarding the determination of sulphate in plant tissue, the results obtained demonstrated that the method is able to quantify sulphur in plants in a consistent manner. A single batch of plants was used for sulphate quantification since it was desired to assess if the readings were consistent within a homogeneous group of plants. Different increments of plant mass were used to make the determinations, and it was expected to obtain a similar concentration of sulphur in tissue after adjusting the readings of each sample with its initial mass. The results show that the method employed did in fact detect similar concentrations of sulphur in the tissue of the plants analyzed. The inter-assay coefficient of variation for this method is of 8.38%, which is acceptable, since values <10% for this parameter tend to be the standard (Reed, Lynn, & Meade, 2002). This assures that the quantifications made with this method are reliable and replicable,

therefore validating its use in future experiments. Nevertheless, it would be appropriate to further perfect this protocol in order to reduce the coefficient of variation to a value lesser that 5%, since this will guarantee that small differences can be detected in the tissues analyzed.

8.5 Cysteine determination, protein extraction and quantification

The modified protocol for determination of cysteine proposed in this study, based on Gaitonde's method (1967), seems to be a practical and efficient way to determine cysteine from a large group of samples simultaneously, while also reducing the amount of reagents needed to do the measurements. The proposed methodology reduces the reaction volume from 1.5 ml to 150 µl, while maintaining the same proportions of the reagents to ensure that the proper reaction takes place. In addition, the use of 96-well PCR plates, along with the multichannel micropipette speeds up the procedure, while reducing the variability that would arise from preparing a large number of samples by hand. Another advantage is that the thermal cycler allows an easy and safe way to boil the samples. This proved to be an issue in preliminary tests when the reactions were heated in a dry bath, because the plastic tubes popped open and the contents of the reaction were spilled. The calibration curve obtained with this method (Figure 10) also yielded a high R^2 (0.9963), which implies that the method is fit to make precise determinations of the mass of cysteine in a sample, similar to the interpretation for the sulphate calibration curve.

Sadly, it was not possible to make cysteine determination from plant extracts. None of the extract samples turned pink as they should have in order to make the determination. Several hypotheses were analyzed as to why it was not possible to make the determination on the samples. In the first extraction method utilizing a more complex extraction buffer, it was believed that at least one of the components in the buffer was interfering with the

reaction of ninhydrin with cysteine, so the simpler extraction methods were tried out. Still, it was not possible to make cysteine determinations in any of the other four methods of extraction (alcohol, water, water + alcohol, and cold extraction). To make sure that the extraction protocols used were in fact extracting protein from the samples, the Bradford assay was used to determine protein concentration on the extracts (Figure 5) (Bradford, 1976). An ANOVA was used to determine if there was any difference on the extraction yield with each method (Figure 6; Figure 7). It was finally determined that all of the methods of extraction worked to some degree, but the method of extraction using only alcohol yielded a significantly higher protein extraction than all the other methods (Figure 8). This proved that the protein extraction methods were in fact extracting protein, so the determination of cysteine could not be performed due to some other factor.

Then, the possibility that the amount of cysteine in the samples was so low that the reaction could not take place was also contemplated. In order to analyze this, fortified solutions with cysteine were prepared, and those were used to extract protein from plant tissue. A control was run parallel to the samples to determine that cysteine was not affected by any of the steps in the extraction procedures. Figure 11 shows the results after the extraction with fortified solutions, in which the reaction took place in all of the controls, but not in any of the extracts. The extracts should have turned at least as pink as the controls, which suggests that some component of the plant extract is interfering with the reaction. Other studies that perform cysteine determinations for plant protein extracts perform a desalinization step prior to the determination of cysteine (Krueger, et al., 2009). This step is most likely to remove anions from the extract, but nevertheless, the purpose of this step is unclear as to which specific element or compound is being removed in order for the reaction of ninhydrin and cysteine to take place.

8.6 ROS determination

The fluorescent determination of ROS yielded interesting results in regard to the effects of the different treatments that were applied to the plants. It is well known that perception of stresses (biotic and abiotic) causes the production of ROS inside the cell of the plant (Mittler, Vanderauwera, Gollery, & Van Breusegem, 2004). Figure 12 clearly shows that subjecting the plants to mechanical damage greatly increases the production of ROS. Even though the mean of ROS accumulation in the plants treated with salicylic acid is similar to the MS, -S, and +S nutritional treatments, the dispersion of the data is much greater, since some of the measurements within this group showed high concentrations of ROS in the tissue. This is to be expected, since hormonal perception inside the plant causes the accumulation of ROS, like is the case with salicylic acid (Mittler, Vanderauwera, Gollery, & Van Breusegem, 2004; Vlot, Dempsey, & Klessig, 2009). SA has the ability to bind specifically to catalase enzymes, which are part of the ROS scavenging mechanisms inside the cell. In turn, this causes the accumulation of ROS, like hydrogen peroxide, inside the cell (Dzhavakhiya, Ozeretskovskaya, & Zinovyeva, 2007). Concerning the sulphur nutritional treatments, the three treatments did not seem to cause an increase in ROS concentration inside the cell.

At this stage, it is not possible to draw conclusions as to the effect that sulphur nutritional stress has on the accumulation of ROS. ROS regulation inside plants is characterized by sudden spikes in concentration followed by drastic reductions controlled by the ROS scavenging mechanisms inside the cell (Mittler, Vanderauwera, Gollery, & Van Breusegem, 2004). It is possible that the five-minute period that the plants were left submerged in their respective treatments was too long, and the spike of ROS accumulation was missed for the determination, especially when considering that nutritional stress is known to promote ROS accumulation (Cakmak, 2005). This may be

argued in comparison to the high accumulation of ROS evidenced in the mechanically damaged plants, which were treated and immediately submerged in the working solution. It would be appropriate to realize a series of determinations within a specific time limit to assess if there is indeed an accumulation of ROS inside the cells caused by the sulphur stress treatments.

It is noteworthy to mention that a conventional ANOVA could not be performed to analyze the measurement of ROS, since the ANOVA assumptions were not met (Figure 13). Equal variances were not obtained for the sample groups in this analysis, so Welch's test was used to determine if the means of ROS concentration of the treatments were significantly different (Figure 14). Welch's tests is a variation of Student's t-test, with the difference that it can be used to compare samples with unequal variances or sample sizes (Devore, 2008), so it was an appropriate test to analyze the ROS determination data. After determining that at least one of the means was significantly different, Games-Howell comparisons were used to determine that the mean of ROS concentration in the mechanically damaged plants was indeed significantly higher than all the other groups (Figure 15; Figure 16).

9 CONCLUSIONS

1) In relation to the culture of *A. thaliana*, results were obtained for the two proposed methods of culture. Both *in vitro* and sand culture proved to be easy and effective ways to grow *A. thaliana* that facilitate the realization of the sulphur stress treatments, so the objectives of cultivating different lines of *A. thaliana* in sand and/or *in vitro* were accomplished.
2) It was possible to partially observe the effect that SA and sulphur stress have over the expression of *PR1*. Sulphur deficiency seems to activate the expression of *PR1*, while sulphur excess does not activate *PR1*.
3) Regarding determination of sulphate, physical methods of lysis and extraction of sulphate (freezing and milling), as well as the turbidimetric protocol optimized for water samples proved to be inadequate for the analysis of sulphate in plant tissue. Acid digestion of the plant samples coupled with the turbidimetric protocol proposed by Verma in 1977 appear to be a precise and convenient way to measure sulphate in plant tissue.
4) Cysteine determination in plant protein extracts was not possible, despite demonstrating that the protein extraction protocols analyzed were working correctly. It seems to be the case that some component inside the plant is released during protein extraction, and is interfering with the reaction of ninhydrin with cysteine.
5) The fluorescent measurement of ROS with DCFDA showed to be an appropriate procedure to determine the accumulation of ROS in plant tissue, as demonstrated by the positive (damaged plants) and negative (MS treatment) controls. Nevertheless, it is not yet possible to determine if sulphur stress has a significant impact on the accumulation of ROS. Since ROS accumulation is transient in nature (Mittler, Vanderauwera, Gollery, & Van Breusegem, 2004), the waiting period utilized during

the application of nutritional treatments could have masked the real readings, generating misleading results.

10 RECOMMENDATIONS

1) The use of the proposed methods of *A. thaliana* are recommended for future experiments regarding nutritional stress, since they provide a way to subject several plants to the same treatment uniformly.
2) In order to get the best out of the proposed culture systems, it is advisable to use fresh seeds obtained from healthy plants (e.g. free of fungal infection), since this will reduce the risk of persistent contamination, especially when working *in vitro*. Using fresh seeds is also recommended, since they germinated more quickly and evenly in both culture systems in comparison to older seeds (2 years old).
3) It is recommended to carry out the nutritional stress treatments under different conditions of light, temperature, and humidity to accurately determine the specific set of conditions in which *PR1* is expressed by sulphur deficiency. In order to speed up this process, it would be wise to have a constant supply of seeds at disposal; a small group of seeds from every batch should be destined exclusively for seed production.
4) For sulphate determination, it is recommended to use an acid digestion protocol that uses 2 ml 69% nitric acid only, and then dilute it to 25 ml. This will ensure that the barium chloride dissolves during the assay, so the reaction with sulphate ions takes place.
5) For cysteine determination, it is recommended to carry out the desalinization step of the protein extracts described in similar studies where Gaitonde's method to determine cysteine was used. (Krueger, et al., 2009). This step could not be realized due to the unavailability of desalinization columns, since it was impossible to get them on time for the completion of this work.
6) In a practical sense, it would be ideal to utilize chromatographic analysis to determine both sulphate and cysteine in plant tissue, because of the technique's sensibility and

precision (Coskun, 2016). Still, the economic cost of this type of analysis may outweigh its convenience.

7) For ROS determination, it is necessary to conduct further assays that monitor the accumulation of ROS within a limited time window when subjecting plants to sulphur nutritional stress. This will help answer the remaining question if in fact sulphur nutritional stress activates ROS, and if that is the case, how rapidly and intensely.

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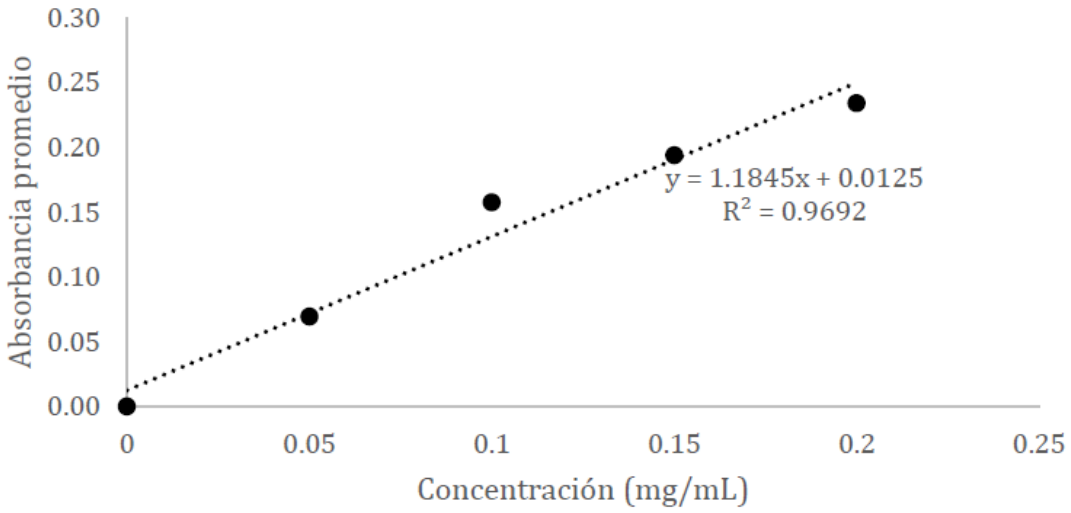
12 APPENDIX



Appendix 1. BSA calibration curve for protein determination in plant extracts using the Bradford Assay