UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
INSTITUTO DE BIOCIÊNCIAS – RIO CLARO

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS
(BIOLOGIA CELULAR, MOLECULAR E MICROBIOLOGIA)

CHITOSAN GEL PROPERTIES BY DRY TEMPERATURE AND INFLUENCE IN THE SWEET ORANGE PHYSIOLOGY AS FOLIAR SPRAY

RENATO HENRIQUE CALORE

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Dissertação apresentada ao Instituto de Biociências do Câmpus de Rio Claro, Universidade Estadual Paulista, como parte dos requisitos para obtenção do título de Mestre em Biologia Celular, Molecular e Microbiologia.

Orientador: Dr. Michel Brienzo.
Coorientador: Dr. Jaiber Humberto Rodriguez Llanos

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RESUMO

A produção dos citrus é uma atividade econômica de importância significativa em mais de 140 países ao redor do mundo, abrangendo uma vasta gama de produtos. Contudo essa prática é ameaçada por doenças tais como o cancro, o "Greening" e as pintas pretas, que reduzem a produção e causam perdas econômicas significativas. O tratamento usual para essas doenças é baseado na aplicação de cobre metálico, inseticidas sintéticos e fungicidas. A aplicação contínua desses materiais pode ser danosa para o meio ambiente. Alternativas naturais vêm sendo desenvolvidas para substituir a aplicação desses compostos. Apesar de serem efetivas, sua aplicação pode ser desafiadora em função de sua volatilidade e biodegradabilidade. O encapsulamento desses compostos com polímeros tais como gelatinas, gomas naturais e quitosana tem sido estudado como um meio de controlar a liberação e prevenir a degradação prematura, e a quitosana é particularmente notável nesse aspecto em função de sua versatilidade. Apesar de ser extensamente estudada, parâmetros tais como a temperatura de secagem e a interação do gel de quitosana com plantas não foram explorados. Esse estudo descreve o impacto causado pela temperatura nos parâmetros físico-químicos dos filmes de quitosana e a interação do gel com os parâmetros fisiológicos das folhas de citrus. Os filmes de quitosana foram preparados de 6 °C a 50 °C (simulando temperaturas ambientais) e tiveram seus parâmetros comparados. Já a aplicação de gel ocorreu em 3 diferentes dosagens simulando condições encontradas na aplicação em campo, como parâmetros de comparação foram analisados a troca gasosa e as respostas bioquímicas. Observou-se que a temperatura de secagem influenciou significativamente a tensão e elongação na ruptura, o incremento na temperatura de secagem ocasionou uma redução em ambos os parâmetros, nos demais parâmetros (solubilidade, umidade e espessura) a temperatura de secagem não ocasionou variações significativas. O gel aplicado nas plantas de citrus não teve impacto na assimilação de CO_2 e nos demais parâmetros de trocas gasosas em todas as 3 aplicações, os parâmetros da aplicação e do controle não apresentaram variações estatísticas significativas, indicando que o gel não apresentou nenhuma influencia negativa para a troca gasosa. Foi observado também que a aplicação do gel foi responsável por lesões nas folhas, em menores concentrações (C1 e C2), a média de folhas sem danos foi de aproximadamente 97%. Já para a aplicação com excesso de gel C3, ocorreram danos mais severos, apenas 73% das folhas não apresentaram danos enquanto 25% das folhas tiveram danos de até 25% da área foliar, danos superiores a 25% da área também foram observados. Apesar desses danos, a aplicação moderada do gel mostrou impactos mínimos no desenvolvimento da folha. Já os resultados de estresse (SOD, CAT, APX) sugerem que a aplicação do gel causou certo estresse nas folhas, evidenciado pela elevação dos níveis de catalase, o mesmo resultado foi percebido com as plantas que receberam o solvente. Após 21 dias da aplicação, os níveis de catalase retornaram aos valores ambientais. Os demais parâmetros permaneceram inalterados. Ainda foi observado que o gel de quitosana tem efeito contra a bactéria causadora do cancro *in vitro*, sugerindo que a utilização da quitosana como carreador de compostos bactericidas pode ter uma importante função de coadjuvante no controle do cancro.

Palavras-chave: quitosana, biodegradabilidade, cancro cítrico, citricultura, biogel, biofilme, polissacarídeos, biopolímeros.

ABSTRACT

Citriculture is an important economic activity in more than 140 countries worldwide, with a wide variety of products. However, it is threatened by various diseases such as canker, greening, and black spot, which reduce production and cause significant economic losses. The usual treatment for those diseases is based on the application of metallic copper, synthetic insecticides, and fungicides. However, continued application can be harmful to the environment. Natural alternatives have been developed to replace those chemicals. Although they can be effective, their application is challenging due to their volatility and biodegradability. Encapsulation with biopolymers such as gelatin, natural gums, chitosan, and neat has been studied as a means to control release and prevent premature degradation and chitosan is particularly noteworthy due to its versatility. Despite being extensively studied, parameters such as drying temperature and interaction of the chitosan gel with plants have not been explored. This study describes the impact of drying temperatures on the physical-chemical parameters of chitosan films and the interaction between chitosan gel and the physiological parameters of citrus leaves. The chitosan films were prepared from 6 °C to 50 °C (simulating the environmental temperatures) and had their parameters compared. The gel application was made in 3 dosages simulating possible situations encountered in the fields, as evaluation parameters were measured gas exchange and biochemical responses. The drying temperature was significant for tensile strength and elongation at rupture, and the increase in temperature caused a reduction in tensile strength and in the elongation, for the other parameters like solubility, humidity, and thickness, temperature caused no significant variation. The chitosan gel applied to the citrus plants had no significant impact on the CO_2 assimilation rate, in all three applications and its controls had no significant statistical differences, indicating that the chitosan had no negative influence on gas exchange. The application of chitosan gel was responsible for causing lesions (leaf burns) on orange leaves, at lower gel concentrations (C1 and C2) the average number of leaves without any considerable damage was 97%. An excess of gel 3.2% (C3) showed more severity, only 73% of the leaves had no considerable damage, 21% showed less than or equal damage to 25% foliar area, in this application, damage greater than half of the leaf occurred. Even with those damages, the average application showed minimal impact on the leaf development. The results found in the stress parameters (SOD, CAT, APX) suggest that the application of chitosan gel causes stress on the leaves, signaled by a punctual increase in catalase, and the same result was observed in plants treated with solvent only. After 21 days of application, catalase activity in the leaves returned to normal levels. The other stress parameters (SOD and APX) remained unchanged compared to the usual levels typically found in plants without application. The bacterial activity of the gel was evaluated against *X. citri*, and at a concentration of 0.8%, the chitosan gel proved effective against the canker bacteria *in vitro*, suggesting that, in addition to serving as a carrier for bactericidal compounds, the gel could also function as an important adjuvant in the control of citrus canker.

Keywords: chitosan, biodegradability, citrus canker, citriculture, biogel, biofilm, polysaccharides, biopolymers.

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List of abbreviations

Xac	*Xanthomonas citri* subsp. *citri*
NYG	Nitrogen, Yeast and Glycerol medium
CFU	Colony forming unit
C1	Gel 0.8% application as spray
C2	Gel 3.2% application by spreading; partial leaf covering
C3	Gel 3.2% application by spreading; total leaf covering
S1	Solvent application proportional to C1
S2	Solvent application proportional to C2
S3	Solvent application proportional to C3
IRGA	Infrared Gas Analyzer
ROS	Reactive oxygen species
APX	Ascorbate peroxidase
SOD	Superoxide dismutase
CAT	Catalase
FW	Fresh weight
DO	Leaves with no apparent damage
D1	Leaves with damage smaller than 25% of foliar area
D2	Leaves with damage smaller than 50% of foliar area
D3	Leaves with damage higher than 50% of foliar area

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1.Introduction

Citriculture is an important activity practiced widely in more than 140 countries around the world in different environments such as tropical, subtropical, and Mediterranean climates, with an emphasis on countries like China, the United States, Mexico, and Brazil. The range of citrus products is vast, including sweet oranges, lemons, limes, grapefruits, and tangerines. In Brazil, production is mainly focused on oranges, accounting for 34% of the global market share. Thus, that production is largely processed into orange juice for export—approximately 74% of the orange juice consumed worldwide comes from Brazil. China leads in the production of grapefruit and tangerines, holding around 75% and 71% of the respective market shares. In the case of limes and lemons, Mexico, Argentina, Turkey, and the United States are the leading producers (UNITED STATES DEPARTMENT OF AGRICULTURE FOREIGN AGRICULTURAL SERVICE, 2024).

Citrus production is susceptible to several diseases, such as canker, greening, and black spot (Barbieri et al., 2023). Citrus canker is caused by the gram-negative bacteria *Xanthomonas citri* subsp. *Citri (Xac).*, which spreads through the innocuous of bacteria in natural lesions or other openings as stomates of leaves, fruits, and branches. Its symptoms include necrotic lesions on leaves, fruits, and branches. This disease can lead to defoliation, fruit deformation, and premature fruit drop (BARBIERI ET AL., 2023; BRUNINGS & GABRIEL, 2003; DAS, 2003; LI & WANG, 2014; YAN & WANG, 2012).

Huanglongbing (greening) is caused by the gram-negative bacteria Candidatus Liberibacter spp. It is primarily disseminated by an insect vector. Characteristic symptoms include yellowish spots, fruit deformation, seed abortion, premature leaf and fruit drop, and sprout death (BARBIERI ET AL., 2023; BATOOL & IFTIKHAR, 2007; DO CARMO TEIXEIRA ET AL., 2005; WETTERICH et al., 2017).

A fungal pathogen, *Phyllosticta citricarpa* causes the black spots. The pathogen disseminates through ascospores or/conidia. The main symptoms are reddish-brown lesions on leaves and fruits. Although black spots do not degrade fruit quality, it restrict its exportation, causing

significant economic losses. This disease is responsible for up to 40% of production losses in countries like South Africa, Brazil, Australia, and the United States (Barbieri et al., 2023; Franco, Goes & Pereira, 2020; Savi et al., 2019; Tran et al., 2019).

To reduce the impact caused by those diseases it is necessary to apply measures to reduce incidence and control the damage. For canker, the most expressive form of decreasing the impact is controlling the pathogen since there are no treatments to cure the disease. The most optimized way to prevent the infection is copper spraying. The copper acts reducing the Xac population in citrus leaves, although efficiently, this method requires multiple applications which causes negative impacts on the environment (BARBIERI et al., 2023; BEHLAU et al., 2017; FERREIRA et al., 2022; FRANCIS et al., 2009; ISLAM et al., 2019). For greening, control is achieved by managing the vector population using insecticides and cutting infected plants to prevent the spread (ALQUÉZAR et al., 2022; BARBIERI et al., 2023; DORTA; MACHADO; FREITAS-ASTÚA, 2019; JR. et al., 2010). The treatment of black spots symptoms is done by applying antifungal in the crop, especially in the most susceptible seasons (KUPPER; MORETTO; FUJIMOTO, 2019).

Even though these methods are effective, they pose serious environmental problems, copper is a bioaccumulative heavy metal that can contaminate the environment, as well as soil and water, causing the death of microorganisms and other animals. Prolongate exposure can result in health damage (KAZEMINEZHAD; MOSIVAND, 2017). In the case of insecticides, the continuous application of these substances can increase the vector's natural resistance to this substance or negatively affect other susceptible animals (ALQUÉZAR et al., 2022; TIAN et al., 2018). The antifungal case is similar, and constant application can produce fungal strains that are resistant to these substances (KUPPER; MORETTO; FUJIMOTO, 2019). To reduce those problems, innovative approaches have been studied, and natural compounds and other molecules derived from those compounds have been highlighted, the great advantage of those natural compounds is their bactericidal ability, low toxicity, and specificity against pathogens, reducing the selection or induction of more resistant organisms (CAVALCA et al., 2020, 2021; MORÃO et al., 2019; POLAQUINI et al., 2019).

Despite their effectiveness, the application of those compounds is challenging. Those natural compounds, especially essential oils, usually presents low solubility on water, easily oxidation besides its volatilities, which makes its usages limited. To overcome those constraints, encapsulation strategies have been developed (AL-MAQTARI et al., 2022). For this purpose, biopolymers are studied, with the ability to encapsulate those natural compounds, reducing their

degradation. Several bio-based polymers are under evaluation, including gelatin, natural gums, modified chitosan, and neat. Among these, chitosan is particularly noteworthy due to its extensive use in creating films, hydrogels, fibers, and nanoparticles. Its deacetylated structures make it highly modifiable for various applications. Based nanomaterials have the propriety of controlled release of active ingredients such as pesticides, plant hormones, and micronutrients (DENG et al., 2020; MALERBA; CERANA, 2016).

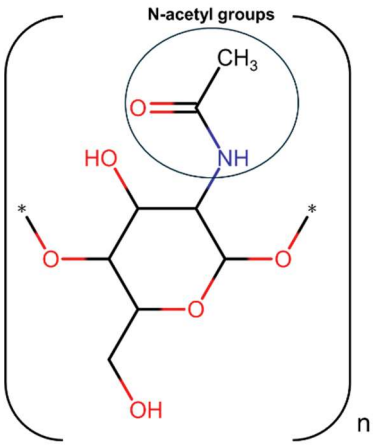
International organizations such as the UN World Health Organization (WHO), the Food and Agriculture Organization (FAO), and the European Union (EU) have established acceptable maximum residual levels for pesticides to protect the level of food quality. In this context, biodegradable materials such as chitosan-based nanomaterials have an advantage over conventional synthetic pesticides, as they can avoid regulations of nanomaterials applications. Chitosan itself has antimicrobial and stimulative properties, the hydroxyl and amine groups along the chemical backbone of chitosan molecules allow the bounding of organic and inorganic compounds. All those characteristics combined make chitosan a very promising material for carrying natural compounds (MUJTABA et al., 2020). Even though chitosan and its by-products have already shown positive effects when applied in other plants it is necessary to investigate the interaction of this polymer with sweet orange tree leaves and its film-forming ability in different environmental conditions to assess its real value as a carrier of natural compounds. This study aims to explore the impact of drying temperature variations, as an environmental parameter, on the mechanical properties of chitosan gel films. Additionally, plan to assess the potential of using the gel as a carrier for foliar spray on citrus leaves to prevent citrus canker.

2.Chitosan and Chitin

2.1.Structure and composition of polymer.

Chitosan is a polymer derived from chitin through a deacetylation process of the molecule. Chitin itself is a natural occurring polymer composed of D-glucosamine monomers with N-acetyl groups attached (Figure 1). It is the second most abundant polymer on Earth, second only to cellulose (IBER et al., 2022; MOHAN et al., 2022).

Figure 1 - Chitin monomer.

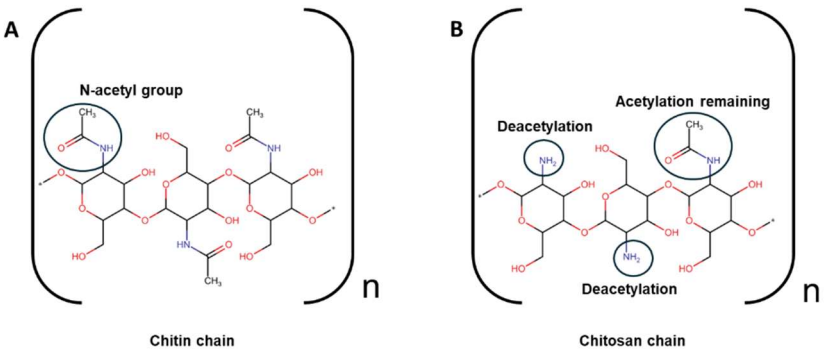


Source: Author.

Chitin is found in various sources such as shrimp, crabs, mollusk shells, insect exoskeletons, and fungal cell walls (MOHAN et al., 2022). The main commercial chitin source is seafood industry waste, the marine crustaceans. This by-product is considered a low-cost residue of fishing activities (LICHTFOUSE et al., 2019).

Chitosan is a derivative product of chitin. The transformation of chitin into chitosan involves the deacetylation of D-glucosamine monomers, as illustrated in Figure 2.

Figure 2 - Chitin deacetylation.



Source: Author.

In the process, the chitin is partially deacetylated (Figure 2 - B), and acetyl groups remain in the polymer. The deacetylation degree is a reference to distinguishing chitin from chitosan.

When the material is richest in N-acetyl glucosamines is chitin while the material with more abundance in glucosamines deacetylated is chitosan (IBER et al., 2022).

2.2.Chitosan productive process

Chitosan is a by-product obtained through the deacetylation of chitin, a biopolymer found in various sources such as shells, carapaces, and structures like fungal cell walls (MOHAN et al., 2022). The most common source of chitin for chitosan extraction is marine crustaceans due to their low cost (LICHTFOUSE et al., 2019). The extraction of chitosan involves a four-step process: deproteinization, demineralization, decolorization, and deacetylation. During deproteinization, strong alkaline chemicals combined with high temperatures are used to remove the proteins present in the shells and exoskeletons of marine crustaceans (IBER et al., 2022; MOHAN et al., 2022).

After removing the proteins, the material is washed with strong acids to remove calcium carbonates and calcium phosphates. With those two processes, the raw material is transformed into chitin. For converting the chitin in chitosan is necessary to remove the pigments, usually using ethanol to dilute them, and perform the deacetylation by applying strong alkaline as NaOH highly concentrated (>40 wt%) (IBER et al., 2022; MOHAN et al., 2022).

2.3.Chitosan in the emulsification process

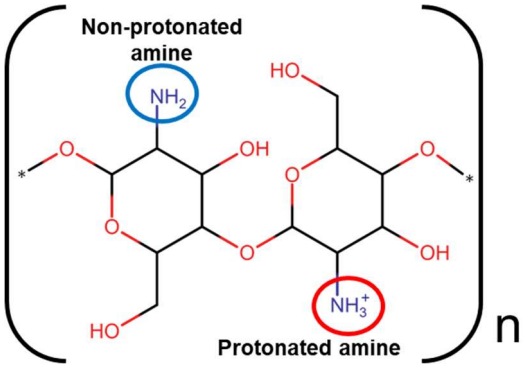
Chitosan is an interesting material widely used to carrier compounds due to its polycationic tendency. Below pH 6 the amines present in the chitosan tend to protonate (NH_2 to NH_3^+), and with the abundance of OH hydrophilic groups, the chitosan can form covalent bonds with functional groups from different chemical compounds, improving its applicability. One of those applications is the controlled release of chemicals bonded in chitosan by the interaction of those complexes with different pH (LI et al., 2020b; LINGAIT et al., 2023).

Naturally, the chitosan molecule is neutral, without protonation of its amine group (Figure 2 - B). The protonate process consists of solubilizing the chitosan in solutions with low pH (below 6), in this environment the amines will react with the H^+ available creating NH_3^+ complexes. The protonation degree is controlled by the acid concentration, in low-concentrated acid

solutions some amines won't protonate, resulting in non-solubilized chitosan molecules (RINAUDO; PAVLOV; DESBRIÈRES, 1999).

Figure 3 shows the two possibilities of protonation. In red, the protonated amine has a positive charge and can create covalent bonds with other molecules while the non-pronated amine, in blue, there is no charge reducing its ability to have interactions with other molecules. The higher the concentration of protons (H^+) in the solution more amines are protonated, increasing the solubility of the chitosan molecules.

Figure 3 - Partially protonated chitosan.



Source: Author.

The most common solution used to dilute chitosan is low-concentration acetic acid. Although this method is efficient, it is not suitable for biological applications because acetic acid has corrosive properties that can damage living tissues (LI et al., 2020a). Neutralizing the acid is necessary to use alkaline products and water washing. Those products are expensive and leave residues in chitosan, increasing negative biological effects and interfering with the solubility of chitosan. To avoid those problems is possible to use other methods such as ionic liquids to solubilize chitosan (LI et al., 2020a).

Ionic liquids are salts that behave like liquids at room temperature. These liquids have interesting properties like high thermal stability and low volatility. Besides those two properties, the complexes formed by ionic liquids and chitosan can be customized to present properties like hydrophobicity or hydrophilicity. Combined with the ability to solubilize other

biopolymers as cellulose, the usage of ionic liquids eases the application of chitosan in various fields (ALQAHTANI, 2024).

2.4.Chitosan in agriculture

The growing demand for food and the accumulation of pollution have created the need to develop new techniques and products that promote accelerated but sustainable development. (FAQIR; MA; CHAI, 2021). Several researchers started to look for biodegradable materials that could carry compounds such as fertilizers and pesticides with slow liberation (FAQIR; MA; CHAI, 2021; WANI et al., 2019). Chitosan fits in those parameters, it is a polycationic polymer extracted from the deacetylation of chitin and has low solubility in water when non-protonated (FAQIR; MA; CHAI, 2021; NGUYEN; WANG, 2017; ZARGAR; ASGHARI; DASHTI, 2015), besides shows a great ability to control the release of agrochemicals, reducing the needed amount of those substances, making its application more efficient, and reducing the risks of environmental contamination (SHARIF et al., 2018). Chitosan can improves plant growth and self-defense mechanisms by interacting with cascade reactions, and can be used as a carrier for other compounds (FAQIR; MA; CHAI, 2021; SABERI RISEH et al., 2023).

The blend of chitosan with other biopolymers like gum (ABREU et al., 2012), starch (TAN et al., 2022), or alginate (NAIR et al., 2020) causes changes in its properties affecting the liberation of chemical compounds, which can be slowed, enabling better release. Further the control of agrochemical liberation, the chitosan itself has a biocide capacity favoring its use for this purpose (MORIN-CRINI et al., 2019).

2.5.Versatility of chitosan in agriculture

In the field of fertilizers, chitosan has been explored for nutrient encapsulation. The high demand for fertilizers in modern agriculture caused an imbalance in the environment. Nutrients such as nitrogen and phosphorous can lead to eutrophication and have an inefficient conversion application of about 10% (URSO; GILBERTSON, 2018). To reduce the dosage and mitigate the environmental damage strategies such as nanotechnology have been adopted (KAH; TUFENKJI; WHITE, 2019). This innovative approach can achieve the desired effect with the lowest dosages possible, resulting in efficient production with low environmental impact. In

this context, the binding properties of chitosan with essential nutrients such as nitrogen, phosphorous, and potassium through inter and intramolecular entrapment, superficial adhesion, and antimicrobial behavior are examples of potential uses in this new kind of technology (CYRIAC et al., 2023).

In the post-harvest phase of agricultural products, one of the main challenges is the loss caused by microbial activity (BIST & BIST, 2021). During storage or on shelves, products such as seeds are susceptible to microorganisms that degrade their quality, rendering them unfit for consumption (SOZBILEN; YEMENICIOĞLU, 2020). Chitosan gels and films have antimicrobial properties; the application of a layer of this biopolymer on seeds and fruits can reduce the microbial load, extend shelf life, and preserve germination and growth parameters. (MORIN-CRINI et al., 2019; SOZBILEN; YEMENICIOĞLU, 2020).

After harvesting, the citrus fruits are susceptible to molds (blue and green) that can degrade the fruits (PANEBIANCO et al., 2014). Similar to seeds, a chitosan coating has a significant effect against these molds. Even at low concentrations (0.02% to 0.5%), chitosan has demonstrated inhibitory activity against these pathogens. (PANEBIANCO et al., 2014). Besides microbiological issues, citrus fruits are susceptible to natural self-degradation, after harvest, tangerine parameters such as mass, vitamins, and flavor are degraded, reducing the shelf time of this product. A chitosan layer can significantly reduce the yellowing of the tangerines and their mass loss (PLÁCIDO et al., 2016).

Applying chitosan biofilms to pomelos has positive effects on preserving the quality of the fruit for a longer period. One of the issues with this fruit is juice sac granulation, also known as "dry juice sac disorder," which is usually related to juice crystallization and lignin deposition. This phenomenon occurs in other citrus fruits as well, leading to tasteless juice, significant fruit loss, and postharvest challenges. The application of a chitosan film can help by improving shelf life and reducing quality loss (CHEN et al., 2021; THEANJUMPOL et al., 2019).

In addition, chitosan can also be used as a foliar spray for nutritional purposes or to treat diseases. Tadayon, Safaiefarahani e Sadeghi (2023) studied alternatives for treating citrus greening and found that alternative treatments show promising results, substances such as salicylic acid and potassium salicylate improve the plant's natural resistance to *Candidatus Liberibacter asiaticus*. These substances are effective in enhancing plant resistance, but their application is challenging due to environmental sensitivity. When applied, these substances can be easily degraded by sunlight or temperature, reducing their efficacy, increasing the dosages

can solve the persistence problem but can lead to toxicity issues (TADAYON; SAFAIEFARAHANI; SADEGHI, 2023). Chitosan encapsulation is an alternative for high dosages, the application of chitosan coating nanoparticles charged with salicylic acid as foliar spray showed a significant suppression capacity against *Candidatus Liberibacter* (TADAYON; SAFAIEFARAHANI; SADEGHI, 2023).

The same encapsulation technique can also be used to deliver microorganisms. *Bacillus subtilis* induces interesting metabolic responses in citrus plants that may interfere with infections caused by *C. Liberibacter*. Competition for nutrients with *C. Liberibacter*, along with plant immune responses, can help suppress citrus greening. Studies have shown that applying microorganisms in combination with chitosan yields better results in promoting plant growth and providing systemic protection against pathogens. (TADAYON; SAFAIEFARAHANI; SADEGHI, 2023).

When in the leaves, chitosan shows interesting interactions, especially when the plant is under stress. The application in the early stages of development (V1 and V2) of soy is beneficial for nitrogen metabolism stimulating vegetative growth (ZENÓN et al., 2020).

Chitosan can be explored for sustainable water management. In drought conditions, antitranspirants can be applied in the foliage to limit water loss through film-forming and stomata-closing compounds (TAMBUSSI; BORT; ARAUS, 2007). The reduction of water availability can cause significant impacts on production, resulting in food issues given that those kinds of plants are essential for feeding (IRITI et al., 2009). In this case, antitranspirant materials can be used to reduce the loss of humidity to the environment, making the water more efficient. The application of chitosan enhances the natural responses from drought without compromising the maximal photosynthesis activity while other antitranspirants have better results for water efficiency but have a significant impact on photosynthesis activity (IRITI et al., 2009).

In *Zea mays L.* (corn), the application of chitosan foliar spray 140 mg/L in plants suffering from drought stress reduced the negative impacts of these plants, showing similar parameters with plants with regular hydration (ALMEIDA et al., 2020). For sorghum, the improvement was 19% in the production in drought stress if compared with the plants without treatment. The production improvement can be attributed to the better parameters of rates of photosynthesis, transpiration, and stomatal conductance. Besides those parameters, the chitosan showed positive interactions with enzymes like ascorbate peroxidase and catalase, which manage biological responses linked to stress situations (ÁVILA et al., 2023). In grapes, the drought

stress revealed a systemic process of degradation of the plants. In severe drought stress, about 40% of normal irrigation, parameters such as dry weight, foliar area, chlorophyll, and carbohydrates were affected negatively while proline had a substantial increase (KHALIL; BADR ELDIN, 2021). In grapes, the accumulation of proline indicates suggests drought stress given that this substance reduces the water potential of the tissues, reducing the water loss. With the application of 0.1 gram of chitosan for each plant as foliar spray three times a week the drought impacts were reduced compared to non-treated plants. Under usual irrigation, chitosan as foliar spray affected positively the plant's growth (KHALIL; BADR ELDIN, 2021)

In *Calendula officinalis* L. (Marigold) chitosan as foliar spray has mitigating properties against excessive salinity. In the Mediterranean Sea region, the salinity of water and soil negatively affect the Marigold, disturbing metabolic activities such as hormone production, photosynthesis, and respiration balance. A foliar spray of chitosan can reduce the negative impact of salinization. The plants with chitosan foliar spray had significantly improved in vegetative growth, flowering yield, and chemical constituent if compared with plants with conventional irrigation (ABDEL-MOLA; AYYAT, 2020).

Chitosan's antimicrobial properties can be synergized with other compounds for better results. The leaf spots, a fungal disease that infects kiwi, cause severe economic losses to producers of this fruit, the usual treatment is the application of artificial fungicides. This treatment is effective but can lead to environmental contamination and stimulate pathogen resistance (ZHANG et al., 2022). Natural fungicides are less effective than artificial ones limiting their usage, to improve their effectiveness it is possible to appeal to the co-application of compounds. For leaf spots, the application of chitosan and tetramicyn, a natural fungicide, have 56.61% and 79.80% of efficiency, respectively, when applied individually, when combined the efficiency is 89.44% (ZHANG et al., 2022).

Apple trees suffer with fungal diseases which are a major problem, too. The disease known as Glomerella leaf spot (GLS) infects apple plant leaves, causing black spots that causes the reduce of chlorophyll amounts, affecting photosynthesis. Chitosan, besides its natural antimicrobial properties, has a significant impact on plant's defensive mechanisms, since it can induce the synthesis of enzymes and other marks related to defense against external agents. The application of 0,5 g/L of chitosan on leaves can reduce the impact of the GLS by 89%, which is an interesting prospect for chitosan usage in agriculture (LIU et al., 2023).

2.6.Gel formulation

In the literature, gels are described as being classified into two distinguished groups: chemical gels and physical gels. The main characteristic used to identify the type of gel produced is the nature of the bonds formed by different molecules. In chemical gels, the molecules form covalent bonds, making the gel permanent and irreversible. In contrast, physical gels are held together by weaker interactions, such as hydrogen bonds, which make them reversible and non-permanent. Chitosan gel is a representative example of a physical gel (HONG et al., 2024). Chitosan molecules are inherently insoluble in water, despite the presence of hydroxyl –OH groups. To enhance solubility, molecular modifications are required. One approach is to dissolve chitosan in acidified solutions. Under these conditions, the amine groups of chitosan become protonated, increasing the overall molecular charge. This increase in charge enhances the polymer's ability to form intermolecular bonds, thereby improving its solubility.

3.Materials and Methods

3.1.Biogel formulation and drying profile

To perform the experiments, different concentrations of chitosan were evaluated from 3 g/L to 10 g/L, maintaining the proportion of glycerol of 20% (g of glycerol per g of chitosan), that was used as a plasticizer, and the concentration of the solvent (acetic acid) in 2% (v/v). The chitosan used to produce the film-forming solutions was acquired from Sigma-Aldrich (C3636) at >75% degree of deacetylation produced from shrimp shells. The chitosan powder and glycerol were solubilized in acetic acid for 5 hours, using mechanical stirrer at room temperature (22 °C to 25 °C). After stirring, the solution was filtered through a 180-mesh sieve to remove any non-solubilized particles and was stored in Schott flasks at room temperature (25°C).

The films were prepared in petri dishes with a density of 3.75 mg of chitosan per cm² and they were dried inside desiccators (Figure 4 - A), with lithium chloride, for humidity stabilization. The drying temperature was adjusted between 6 °C to 50 °C, and those parameters were observed, inside the desiccator, with a thermohygrometer (Figure 4 - B). The films were weighed every hour, and the remaining water was calculated using Equation 1.

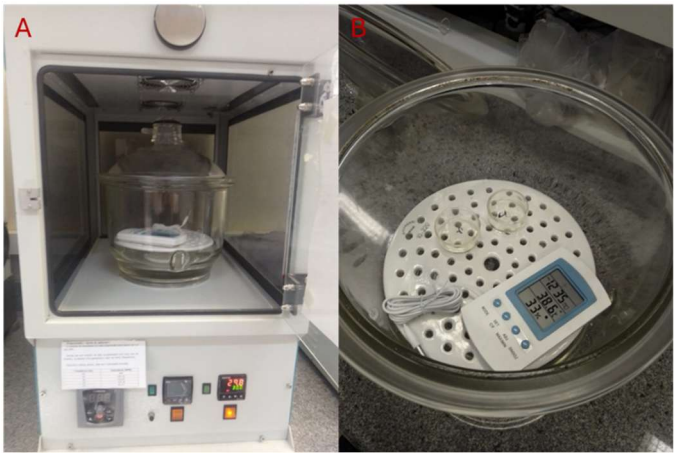
The data was analyzed using Origin (2024b). The water loss rate was estimated using linear models and the relation of drying time and temperature was estimated using an exponential model.

$$Water\ loss\ (\%) = \left[1 - \left(\frac{Actual\ weig}{Water\ weight}\right)\right] * 100 \quad (1)$$

Actual weight = Weight of the gel in at the measure

Water weight = Weight corresponding to the amount of water in the gel

Figure 4 - Chitosan film drying system. A is the desiccator placed inside the oven and B is the desiccator opened to collect the petri dishes with the gel for weighing.



Source: Author.

3.2.Physical properties

The physical properties of chitosan films were analyzed in preconditioned samples at a stable relative humidity of about 20% at room temperature (25 ºC). The samples were conditioned for 3 days before the test.

3.2.1.Thickness

Seven samples sized 30mm x 4mm were taken from the center of the film and measured using a micrometer (Mitutoyo) with an accuracy of 1 µm. 5 random measurements by sample were taken.

3.2.2.Opacity

The opacity was calculated by the ratio of absorbance by thickness (Equation 2) in samples of 30mm x 4mm (height x width). The thickness was measured 5 times for each sample and used the average thickness for calculating opacity. The absorbance was measured by scanning the visible spectrum (wavelengths from 400 to 800 nm).

$$Opacity = \frac{Abs}{Th} \quad (2)$$

Opacity = mm^{-1}

Abs = Absorbance

Th = Average thickness (mm)

3.2.3.Moisture content and solubility

The moisture content was measured by comparing the initial weight of the plastics and the final weight after drying for 24 h at 105 °C. Samples of 20 mm x 20 mm were cut and placed in a desiccator with lithium chloride, to regulate the relative humidity to 20%, at room temperature (~25 °C) for 3 days to stabilize the moisture content in the films. After that, the material was weighed and put to dry at 105 °C for 24 hours. After 24 hours, the material was weighed again, and the moisture content was inferred by the percentage of weight lost during the drying (Equation 3).

$$MC\ (\%) = \left(\frac{W_{hi} - W_{hf}}{W_{hi}}\right) * 100 \quad (3)$$

MC = moisture content in the plastic

W_{hi} = Initial weight after preconditioning the samples

W_{hf} = Weight after drying at 105 °C

After the humidity experiment, the samples dried at 105 °C were placed in flasks with 50 mL of distilled water. The flasks were gently shaken in a shaker at room temperature for about 24 hours. After the shaking, the samples were dried again at 105 °C for 24 hours and their weight

was measured. The solubility of the material was measured by comparing the initial and final weight using Equation 4.

$$S(\%) = \left(\frac{W_{si} - W_{sf}}{W_{si}}\right) * 100 \quad (4)$$

S = Solubilized film weight (%)

W_{si} = Initial dry weight

W_{sf} = Final dry weight

3.2.4.Rupture tension

The rupture tension experiment was conducted in a Dynamic Mechanical Analyzer - DMA (TA Instruments - Discovery DMA 850). The samples used were cut to a size of 30 mm (length) to 4 mm (width). Before the tests, the samples were preconditioned for 5 days at room temperature (~25 °C) at relative moisture of 25%. Its thickness was measured with a 1 μm precision micrometer, taking 5 measurements for each sample using the average for calculating the tension. For each drying temperature, 7 samples were used. The dislocation ramp used was 1 mm/min with 0 N of preload with an initial opening of the clamps was 7 mm. The experiments were conducted at room temperature (~25 °C).

3.2.5.Sensitivity assays

In literature it was reported that chitosan has antimicrobial potential against Gram-positive and Gram-negative bacteria and fungi (GAO et al., 2022; LI; ZHUANG, 2020) and it was expected that this property would be valid for Xac. Then a sensitivity test against chitosan gel was performed as was described in literature by counting colony forming unit (CFU) (ZAMUNER et al., 2020). The strain used in the sensitivity assays was the isolate 306 (IBSBF 1594) (SCHAAD et al., 2006).

Before the tests, the bacteria were cultivated for about 16 hours in NYG/NYG-agar medium (nitrogen-yeast-glycerol 5 g/L of peptone, 3 g/L of yeast extract, 2% glycerol (w/v); for solid medium bacterial agar was added to 20 g/L) at 29 °C. After the initial cultivation, the optical

density of the aqueous media was observed and adjusted to 0.3 using fresh NYG media. This cell suspension then was diluted 100x with fresh media in falcon tubes to obtain 5mL cultures with an approximate density of 10^6 CFU/mL.

The tests were conducted using 4 different compounds. For the positive and negative controls, kanamycin at 20 µg/mL and water were used, respectively, as described by Zamuner et al. (2020). The third test was done by the addition of chitosan gel to the media, resulting in a final chitosan concentration of 8 g/L. In the fourth test, acetic acid was added to the medium at a final concentration of 2% (v/v). Each treatment was performed in triplicate.

After preparing the inoculum with the compounds, the tubes were kept at 29 °C at 200 rpm for 4 hours. After this period, samples of 100 uL of each tube were diluted (from 10^1 to 10^6) as necessary, then 60 uL of the dilutions were spread on NYG-agar plates for CFU counting. The sensitivity of Xac was evaluated from the emergence of CFU after 72 hours of incubation at 29 °C. This experiment was repeated twice.

3.3.Plant acclimatation

The physiological tests were made with nursery trees of Citrus sinensis Osbeck cultivar Pera (grafted on Citrus limon Osbeck) at 50 cm height bought from Citrograph (Ipeuna – SP). These plants were acclimated as described on literature (ZAMUNER et al., 2023) in the vegetation house for 2 weeks and were pruned (Figure 5) removing their leaves to encourage the sprouting of new leaves, which are susceptible to Xac. Automated fertigation was used with a mixed mineral fertilizer (Amazon AgroSciences Citrus 100) in a volume of approximately 200 mL per plant/day. Temperature was controlled using an extractor fan and Pad cooling system, temperature and humidity were monitored using a digital thermohygrometer (HOBO MX2305). The experiments were conducted about 3 weeks after pruning, the time needed to sprout new expanded leaves (~4 cm in width and ~10 cm in length).

Figure 5 - Pruning of seedlings after acclimatization.

Source: Author.

3.4.Experimental model

Three application conditions were evaluated (C1, C2, C3): two with excessive chitosan and one with a regular dose (expected dosage with spray), the applications were represented on Figure 6. All applications were compared to acetic acid alone, proportional to the chitosan solution used (S1, S2, S3). The regular application (C1) used a 0.8% chitosan gel (0.8 g/100 mL solvent) without glycerol, applied with a sprayer until dripping point on both leaf surfaces. For other applications, a 3.2% chitosan gel (3.2 g/100 mL solvent) was used, which increased the gel's viscosity. This thicker gel required application and spreading with a syringe instead of a sprayer. The amount of gel applied in the second application (C2) was sufficient to partially cover the leaves (around 2.5 mL of gel per fully expanded leaf), whereas in the third application (C3), the amount of gel used was sufficient to completely cover the leaf (around 5 mL of gel per fully expanded leaf). To isolate the effect of acetic acid on the leaves, different groups of control plants (S1, S2, and S3) were sprayed with acetic acid 2% proportional to the amount applied to C1, C2, and C3.

Figure 6 – Gel application on orange tree leaves. In C1 drops were formed, in C2 the leaf was partially covered and C3 the leaf was totally covered.

Source: Author.

3.5.Physiological parameters

3.5.1.Gas exchange

Gas exchange parameters were measured using an infrared gas analyzer - IRGA device (LCpro-T) under artificial light (Figure 7), with measurements taken from the expanded leaves (~4 cm in width and ~10 cm in length), from November 2023 to May 2024. The photosynthetically active radiation (PAR) was set at 800 µmol $m^{-2}s^{-1}$, a value chosen because it corresponds to the

peak of carbon consumption in the radiation-response curve. An external source of light was applied owing to the use of shade nets to reduce sunlight on the nursery trees. The parameters were collected from five plants per application, with two leaves per plant, in a vegetation house, from 8:30 am to 11:30 am. Four measures were taken, the first one was taken 2 days after gel application, the other three were taken approximately a week apart from each other, with a focus on collecting data on days with similar environmental conditions.

Figure 7 - Data collection using IRGA device.

Souce: Author.

3.5.2.Antioxidant enzymes

The process of energy production on plants depends on photosynthetic apparatus, transforming physical energy, carbon gas and oxygen into chemical energy in the form of carbohydrates. This process occurs in the chloroplasts where chlorophyll uses light energy to boost electrons to higher energy levels through an electron carrier chain (SOUSA, 2024). As subproducts of the electron carrier chain occur the formation of reactive oxygen species (ROS). The production of those substances can be harmful to the plant because they can damage nucleic acids (oxidation of deoxyribose, breaking of strands, modification of nitrogenous bases) and other enzymes essential to plant development. Usually, those substances levels are kept controlled by producing enzymes such as ascorbate peroxidase (APX), superoxide dismutase (SOD), catalase (CAT) and other mechanisms (ZAINY et al., 2023). Under biotic stress the plants use many mechanisms to protect themselves, one of them is stomata enclosure (SOFO et al., 2015;

ZANDALINAS et al., 2017). By closing the stomata, the amount of carbon gas available for photosynthesis is reduced, causing a disturbance on the electron carrier chain, increasing the accumulation of ROS. This accumulation induces hypersensitive response which can lead to necrosis, to avoid major consequences, is necessary to produce more enzymes capable of dismutating the ROS in less dangerous substances (ZAINY et al., 2023). The amount of antioxidant enzymes can be used as markers to identify possible biotic stresses on the plants.

To measure the antioxidant enzymes was necessary to collect and prepare the leaves as described by (BONACINA et al., 2017). Fresh leaves were macerated in liquid nitrogen and approximately 0.2 g of each sample was homogenized in an extraction solution. The leaf samples were collected and stored in an ultra-freezer (−80 °C) until analyzed for the antioxidant enzymes: SOD, CAT, and APX. All tests were performed with five samples, with three replicates per test.

3.5.2.1.Superoxide dismutase activity (SOD)

The SOD is responsible for dismutating O_2^- into H_2O_2. The enzyme activity (U) was determined and described by GIANNOPOLITIS & RIES (1977), and was defined as the amount of enzyme needed to inhibit 50% of the reduction of nitro blue tetrazolium (NBT). The samples were analyzed at 560 nm and U SOD was expressed as g FW^{-1} min^{-1}.

3.5.2.2.Catalase activity (CAT)

Catalase is responsible for transforming H_2O_2 into H_2O and O_2 and its activity in the leaf samples was determined following the methodology of HAVIR & MCHALE (1987) and evaluated at an absorbance of 260 nm. The enzyme activity was quantified using the molar extinction coefficient of 36 M^{-1} cm^{-1} (ANDERSON; PRASAD; STEWART, 1995). Catalase activity was expressed in μmol H_2O_2 g FW^{-1} min^{-1}.

3.5.2.3.Ascorbate peroxidase activity (APX)

Ascorbate peroxidase is responsible for transforming H_2O_2 into H_2O and O_2 in presence of ascorbic acid and its activity was determined using the methodology described by Nakano &

Asada (1981). The activity was evaluated by the amount of H_2O_2 degradation in 3 min at 290 nm. The APX activity was quantified using a molar extinction coefficient of 2.8 mM^{-1} cm^{-1} and was expressed in μmol ascorbic acid g FW^{-1} min^{-1}.

3.5.2.4.Leaf damage

After 21 days of gel application, it was evaluated the damage caused on leaves. The damage was measured by visual identification of the lesions in all leaves of the samples that received the gel (C1, C2 and C3) and their respective controls. The severity of the damages was classified as D0 for leaves without any significant damage, D1 for leaves with damages less than a quarter of the leaf area, D2 for damages less than half of the leaf area and D3 for damages more than half of the leaf area.

3.6.Adhesion test

To evaluate fixation of the chitosan films on the leaves an adhesion test was conducted with distinct kinds of application (C1, C2 and C3). The test was conducted in nursery trees recently pruned with fully expanded leaves. The gel was applied on the trees and was waited 3 days before conducting the test for complete dry.

A system (Figure 8) was built to simulate falling drops as an attempt to simulate a rain event. The system was built 2 meters high with a valve (Figure 8 – Yellow arrow) to regulate the water flow. The flow used in this experiment was 1 liter of water per minute, and the trees were exposed to water drops for 3 minutes, this time was chosen following parameters of similar tests described previously in literature (SYMONDS et al., 2016).

The fixation of the chitosan films was measured by the difference of foliar area covered by the gel after and before the exposition to the flowing water, the software Image J 1.54m was used to process the photos of the leaves. Was analyzed 2 leaves of each tree and the test was done in triplicates.

Figure 8 - Washing system used on adhesion test experiments. In red the droplet disperser, in yellow the valve used to regulate the water flow.

Source: Author.

4.Results and discussion

4.1.Drying profile

The drying temperature has a significant impact on the water loss rate (Figure 9, 10 and 11), especially at higher temperatures. From 6 °C to 30 °C the increment of 1 °C increases the water loss rate by 0.06%. In the interval from 35 °C to 45 °C this increment is higher than the first interval, each degree means an increment of 0.9% in the water loss rate. The most significant increase occurs from 45 °C to 50 °C, in this interval each Celsius degree increases the water loss rate by 0.9%. At 6 °C the film takes 22.7 times longer to dry if compared to 50 °C.

The drying data was converted into relative loss using Equation 1 and plotted in Figure 9. It was noted that the data exhibited linear behavior, allowing for the adjustment of a model of the form y = ax, as shown in Figures 10 and 11, where y represents the percentage of water weight loss, a is the drying rate, and x is the drying time. The drying rates, presented in Table 1, were derived from linear models with a fit greater than 0.993. These values were used to estimate the time required to dry the films, which was then adjusted using an exponential model of the form y = exp(a*x). This model correlates the drying temperature with the drying rate and weight loss

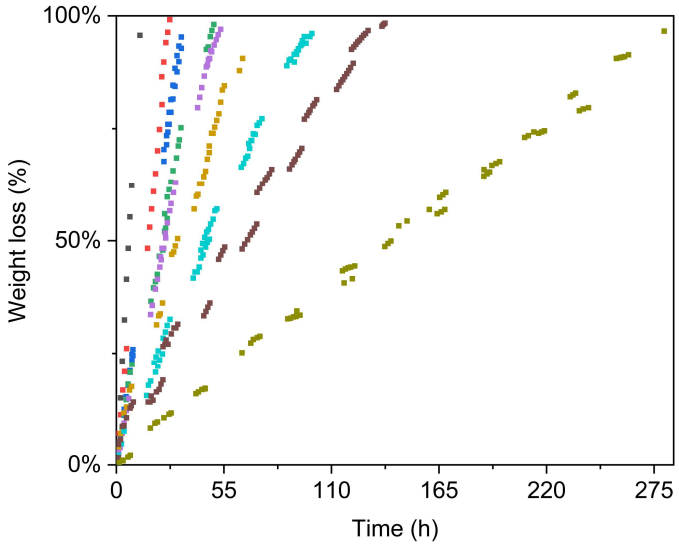
rate (Figure 12). These models are applicable for predicting gel drying patterns after application to plants.

Table 1 - Film's drying rate and estimated drying time.

Temperature (°C)	Drying rate (%/h)	Estimated drying time (h)	R^2
50	0.08	12.5	0.999
45	0.035	28.6	0.993
40	0.029	34.5	0.999
35	0.021	47.5	0.995
30	0.019	55.1	0.998
25	0.015	67.1	0.997
20	0.0102	97.9	0.998
15	0.0077	129.8	0.995
6	0.0035	285.6	0.999

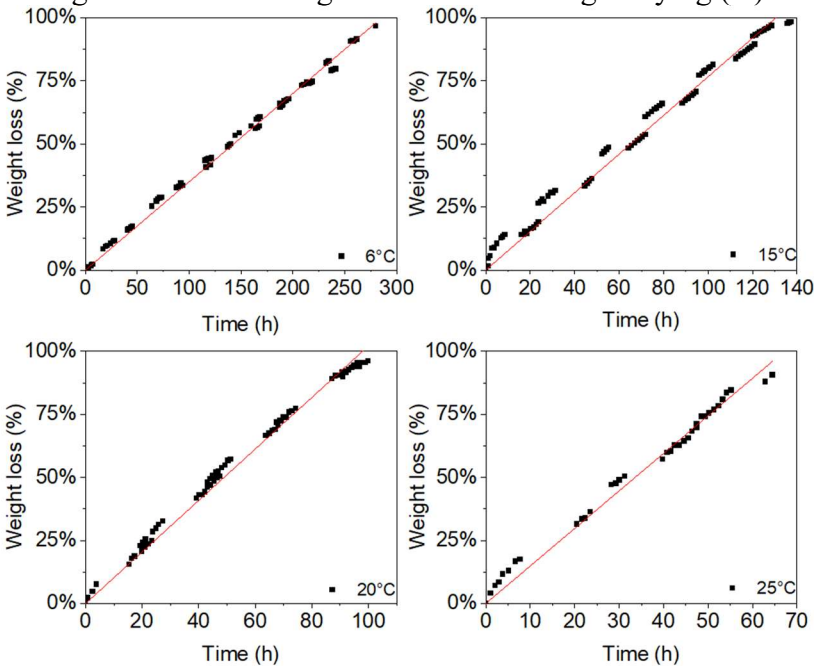
Source: Author.

Figure 9 – Correlation between weight loss and drying temperature. ■ 50 °C, ■ 45 ºC, ■ 40 °C, ■ 35°C, ■ 30 °C, ■ 25 °C, ■ 20 °C, ■ 15 °C, ■ 6 °C.



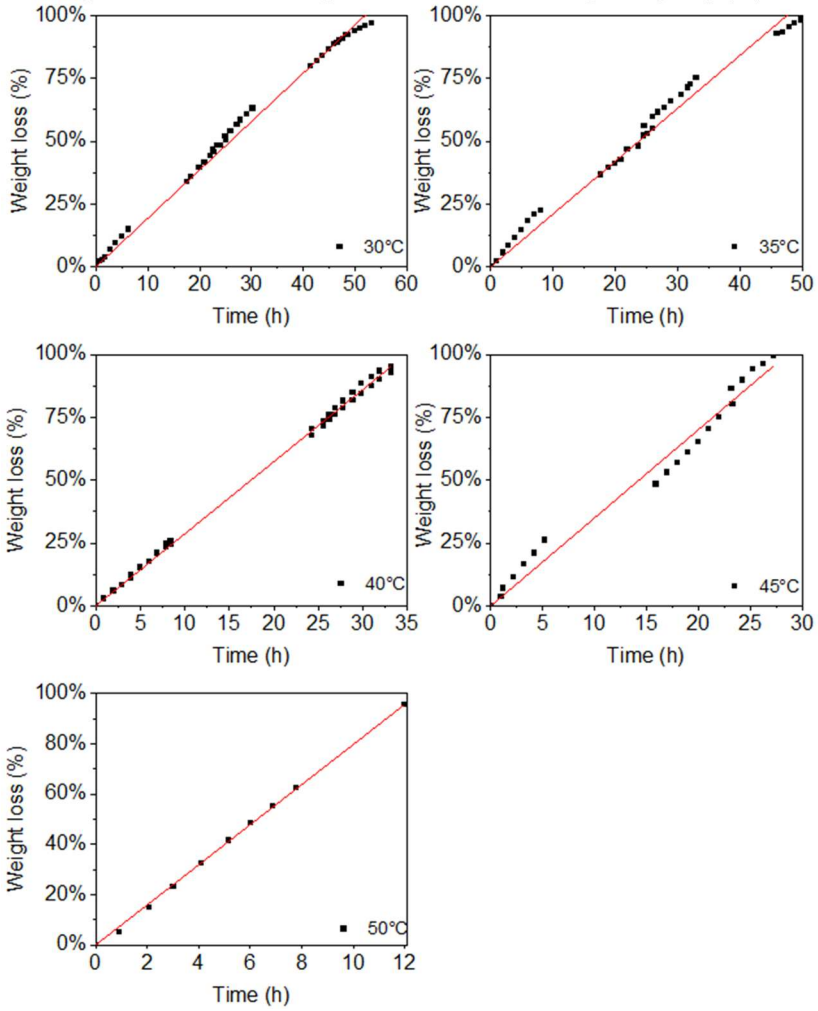
Source: Author

Figure 10 – Linear regression of chitosan gel drying (A)



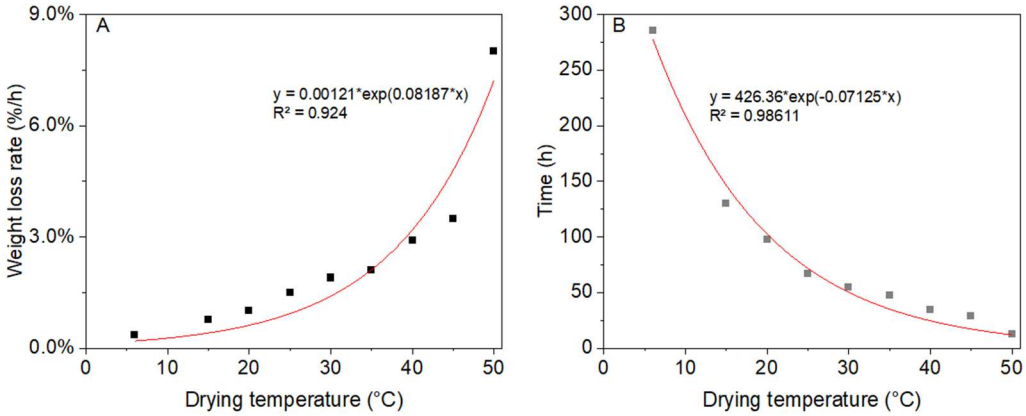
Source: Author.

Figure 11 - Linear regression of chitosan gel drying (B)



Source: Author.

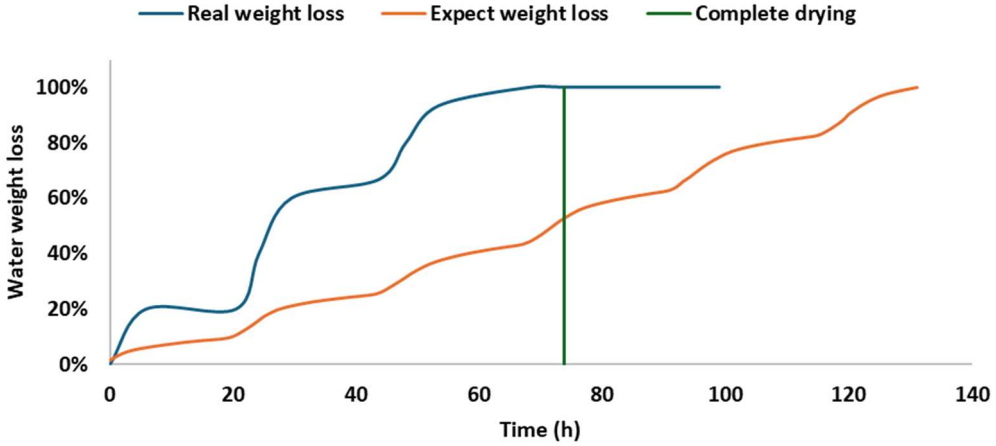
Figure 12 - Weight loss models. A – Weight loss rate x Temperature, B – Drying time x Temperature. ▪ Drying rate, ▪ Time, -- Exponential Fit.



Source: Author.

In order to evaluate the model's capability to predict the drying pattern in an environmental situation, a drying test was conducted in a vegetation house. In this test, the chamber used to control humidity was not employed, and humidity and temperature parameters were measured using a thermohygrometer. A model comparing both parameters was developed based on the measurement of actual weight loss and temperature in the vegetation house (Figure 13).

Figure 13 – Comparative of the model with environmental situation.



Source: Author

Based on the observed weight loss, it can be concluded that the model significantly underestimates the weight loss. Under environmental conditions, the gel took approximately 72 hours to dry completely, whereas the model predicted that it would take twice as long. This discrepancy could be attributed to differences between the experimental model and the actual environmental conditions. The model was developed using data from films dried at various temperatures with humidity control in closed chambers with salt, which was not the case in the

environment. In the vegetation house where the experiment was conducted, exhausted fans were used to control the temperature, creating a forced airflow that accelerated the drying rate of the gel.

Under controlled conditions, the models demonstrated a good fit; the correlation between the drying rate and temperature (Figure 12 – A) had a fit of 0.92, and the correlation between drying time and temperature (Figure 12 – B) had a fit of approximately 0.99. Despite the good fit, the models do not accurately represent environmental conditions due to the omission of airflow considerations. To develop a more accurate model, additional parameters such as airflow and variations in humidity should be analyzed.

4.2.Mechanical tests results

The results of mechanical tests were analyzed and organized in Table 2. The data represents 3 replications for moisture and humidity, 5 replications for thickness and 7 replications for rupture tension and elongation; Means with the same letter are not significantly different ($p>0.05$).

Table 2 - Chitosan film's physical properties.

Drying temperature	Moisture content (%)	Solubility (%)	Film Thickness (um)	Rupture tension (MPa)	Elongation at rupture (%)
6	16.1 ± 5.3^{a}	19.1 ± 1.5^{a}	54.7 ± 9.1^{a}	51.6 ± 5.3^{a}	36.0 ± 6.0^{a}
15	12.9 ± 0.8^{a}	17.7 ± 4.9^{a}	54.0 ± 3.5^{a}	50.1 ± 9.8^{a}	43.5 ± 7.5^{a}
20	11.9 ± 3.1^{a}	16.0 ± 3.4^{a}	52.6 ± 7.5^{a}	48.3 ± 4.4^{ab}	47.5 ± 5.5^{a}
25	11.4 ± 5.0^{a}	15.4 ± 3.8^{a}	52.6 ± 5.1^{a}	47.6 ± 5.5^{ab}	43.3 ± 6.0^{a}
30	10.6 ± 1.4^{a}	13.8 ± 0.7^{a}	52.5 ± 6.8^{a}	42.8 ± 2.8^{ab}	9.8 ± 4.9^{b}
35	8.9 ± 0.1^{a}	13.6 ± 1.8^{a}	52.4 ± 7.5^{a}	-	-
40	8.7 ± 5.1^{a}	13.4 ± 5.6^{a}	52.3 ± 8.7^{a}	40.9 ± 4.0^{ab}	15.4 ± 4.7^{b}
45	8.6 ± 3.2^{a}	12.4 ± 1.6^{a}	51.8 ± 7.4^{a}	37.1 ± 6.1^{b}	17.5 ± 6.7^{b}
50	6.4 ± 4.1^{a}	11.3 ± 7.8^{a}	48.6 ± 3.0^{a}	36.3 ± 2.3^{b}	11.7 ± 4.2^{b}

Source: Author.

4.2.1.Thickness

Analyzing the data presented in Table 2, it was found that the average thickness was 52.4 μm, with no significant difference ($p>0.05$). This result was expected, as the film-forming method

remained the same, producing the films using the same gel at 0.8% (0.8 g of Ch per 100 g of solution), in the same Petri dish, with the same amount of gel for all the films, the only changing parameter was the drying temperature. As discussed earlier, the drying temperature significantly influences the drying rate. Literature indicates that this parameter is closely related to the formation of amorphous zones in the films; the faster the film dries, the less organized the chitosan becomes. This organization affects parameters such as humidity, which in turn can influence thickness. However, this relationship was not observed in the tests conducted. Although there appeared to be a tendency when examining the means, statistical analysis (p>0.05) revealed that the difference was not significant. This confirms that the drying temperature does not correlate with thickness, a behavior previously documented in literature (LIU et al., 2019).

4.2.2.Film moisture and solubility

The results of the humidity experiment (Table 2) show that the films had an average gain of 10.65% of their weight in moisture in an environment with a low relative humidity of approximately 20%, which indicates that the chitosan film has a good capacity to absorb moisture. This characteristic has already been observed in blends with other polymers such as agar (AGUSMAN et al., 2022), hordein (CHENG; LI; MEI, 2022), and starch (HAO et al., 2023), the proportion of chitosan in the films has a significant relation with the moisture content. Comparing the humidity data in Table 2 from different films, the values suggest that there is a tendency of reduction in the moisture content with an increase in drying temperature. However, when the means were analyzed by statistical similarities test, no significant statistical differences ($p < 0.05$) were found, suggesting that the temperature did not affect the moisture content of the films.

The average solubility results (Table 2) did not show a significant correlation with the drying temperature when analyzed by Tukey's test ($p < 0.05$). Although the values suggest a tendency for reduction, the average weight loss was 14.79%. Literature suggests various parameters that can affect the solubility of chitosan films, with the most relevant being the polymer itself, chitosan concentration is the most relevant parameter (SINGH; CHATLI; SAHOO, 2015), the second parameter is the plasticizer, the presence of a plasticizer as glycerol decreases the polymer network interaction density and associated increase in solubility properties (SINGH; CHATLI; SAHOO, 2015). The least important parameter is the drying temperature. At low

levels of plasticizer, the variation in solubility was not significative when correlated with the drying temperature (SINGH; CHATLI; SAHOO, 2015).

For agricultural purposes, the aspects of humidity and solubility are essential. The solubility of chitosan is important because the films will be loaded with chemical compounds for controlled release. If the chitosan has low solubility, the compounds loaded in the chitosan matrix may not be released properly into the environment. Another important aspect correlated with solubility is biodegradability. Materials with high solubility are susceptible to film biodegradability (SOUZA et al., 2017), and the low persistence of the material in the environment is an important characteristic of biomaterials in agricultural applications.

4.2.3.Rupture tension

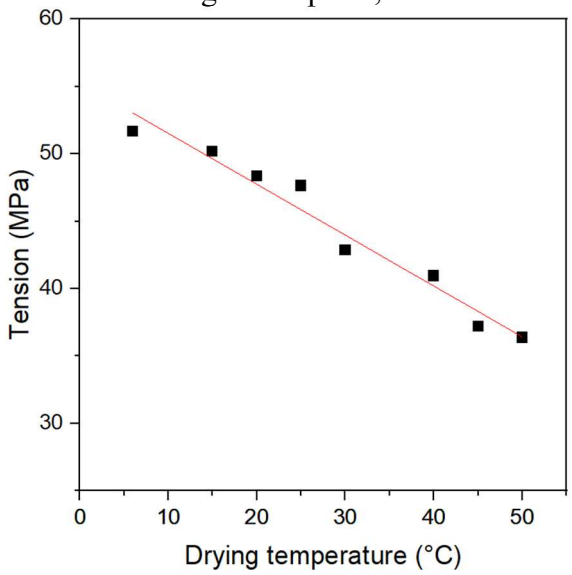
The results of the tensile strength experiment (Table 2) show that the temperature significantly impacts the rupture tension. The increase in temperature shows a tendency to decrease the rupture tension that can be adjusted on a first-degree equation (Figure 14) that reveals a reduction of 0.3774 MPa for each 1 °C increase in the drying temperature. The difference in rupture tension between the extremities is 15.3 MPa.

The values for rupture tension in chitosan films vary in literature. Chitosan is versatile, films with different solvents (QIAO et al., 2021), compositions (REN et al., 2017), concentrations, and plasticizers (SUN et al., 2020) can be formulated. Parameters such as chitosan molecular weight (PARK; MARSH; RHIM, 2002), and moisture content (RACHTANAPUN; WONGCHAIYA, 2012) have a significant impact on rupture tension and elongation.

It produced films in different temperatures: room temperature (21 °C), 40 °C, and 50 °C and the results found showed only small differences between the films dried at room temperature and the ones at higher temperatures (40 and 50 °C) (SUTHARSAN; BOYER; ZHAO, 2023). In other experiments, drying the gel at higher temperatures (45 °C to 85 °C) similarly resulted in reduced rupture tension (LIU et al., 2019b). One interesting data described in this second research was the crystallinity of the material; this parameter has the same behavior of rupture tension strength. This parameter can be used to explain the correlation between drying temperature and rupture tension strength. Elevated temperatures promote the mobility of the chitosan but restrict the bound formation of the chitosan chains, this resulted in a decrease in intermolecular interaction, enlarging the amorphous zones in the film (ZHU et al., 2016).

In order to confirm the pattern, it would be interesting to expand the variables testing chitosan prepared in different conditions of solvent, glycerol concentration, and drying temperatures.

Figure 14 – Correlation between tensile strength at break (MPa) and dry temperature. ▪ Tensile strength at rupture, - Linear fit.

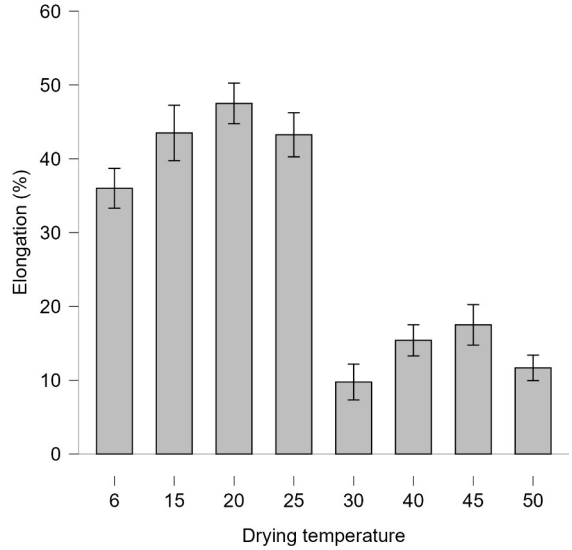


Source: Author.

4.2.4.Elongation

The results found for elongation at rupture (Table 2) of the chitosan films are interesting, with 2 distinguished groups (Figure 15).

Figure 15 - Means of elongation at rupture of films dried at different temperatures



Source: Author.

At higher drying temperatures (30 - 50 °C), the films were much less flexible, with a small deformation before its rupture, about 13.6%. Those results are compatible with the ones expressed previously in literature; Liu et al. (2019) and Gomes de Menezes et al. (2021) reported and deformation of about 5% using different concentrations of glycerol, 10% and 40% respectively, while Suyatma et al. (2005) reported an elongation of 19.1% using similar parameters with this work. This degree of deformation is one of the restrictions found in chitosan. When applied to food packaging or similar applications, elongation is an important property because it is intrinsically linked to flexibility and allows for better manipulation of the material without damaging the package. Several methods have been explored to increase elongation, such as increasing the density of plasticizers (SUYATMA et al., 2005), the better homogenization of chitosan through extreme agitation, pressure, and heat (THAKHIEW et al., 2015) and blending it with other polymers like starch (FAN et al., 2023). By using different techniques, this study enhanced the mechanical properties of chitosan. Unlike common practices of drying chitosan films at room temperature or above 30°C, this research dried films at lower temperatures (6°C to 25°C), achieving similar elongation properties. This may offer a new way to enhance chitosan film properties.

4.2.5.Films opacity

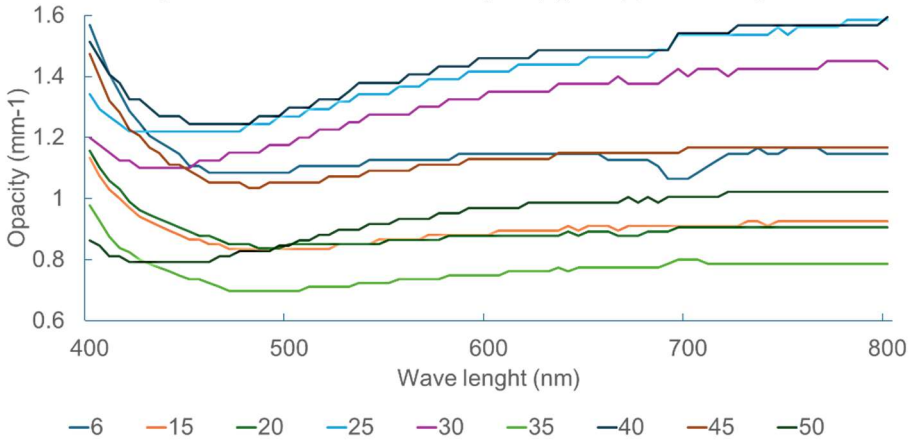
The opacity scanning of the films, shown in Figure 16, exhibited a similar pattern, at 400 nm there was a peak, which rapidly declined and formed a valley at 460 nm. The opacity then increased until it reached 800 nm. The distributions were consistent in shape and showed no significant correlation with the drying temperature. When isolated the opacity values at 400 nm and 800 nm (Figure 17), it was not possible to identify any tendency correlating the film drying temperature with the opacity.

Opacity is linked to a material's crystallinity; more crystalline materials allow less light through, making them opaque. Crystallinity depends on factors like the material and drying temperature. Higher drying temperatures speed up solvent removal, which affects how well molecules organize into crystalline structures (GUZMAN-PUYOL; BENÍTEZ; HEREDIA-GUERRERO, 2022). This experiment aimed to see if higher drying temperatures impacted opacity by altering crystallinity, but no such effect was observed.

For photosynthesis, the most important wavelength ranges are red (600 to 700 nm), blue (400 to 500 nm), and then green (500 to 600 nm), respectively (HOGEWONING et al., 2012). High

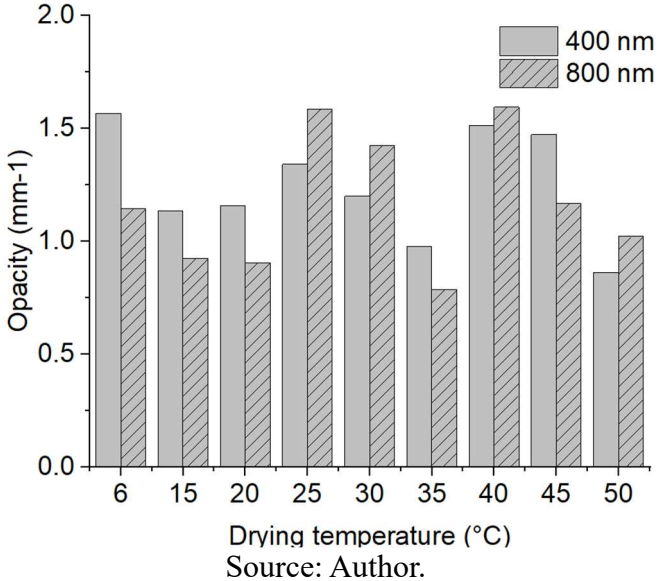
opacity means a reduction of the light that will be available for the leaves to photosynthesis, and this reduction can negatively affect the plant by reducing the photosynthetic efficiency. On the other hand, a barrier against sunlight can have beneficial aspects, during extreme weather conditions, citrus plants can be negatively affected by unfit environmental conditions and the application of barriers against excessive irradiance can mitigate those harmful effects (BERNARDI et al., 2023).

Figure 16 – Film's wavelength (opacity) scanning.



Source: Author.

Figure 17 -Film opacity comparison



Source: Author.

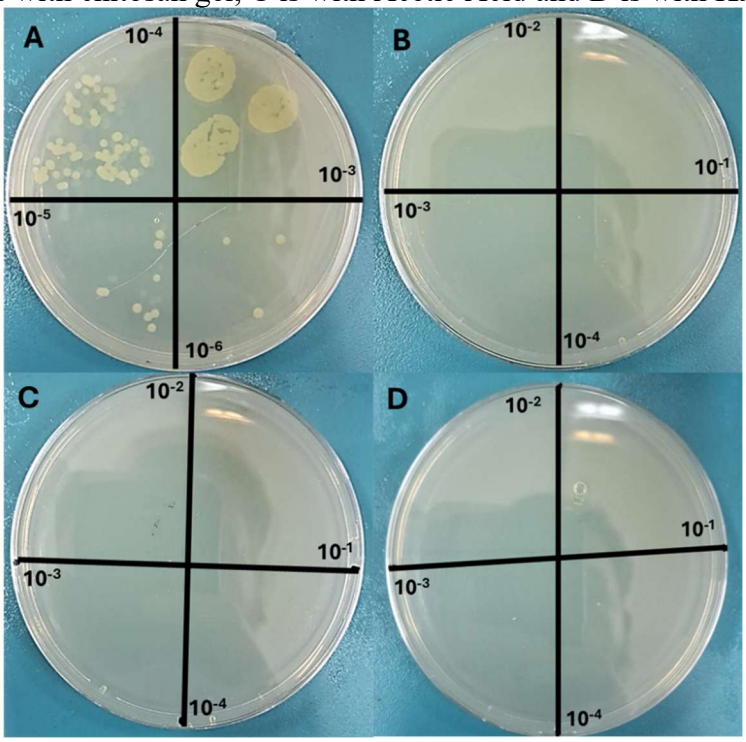
4.3.Sensitivity of *X. citri* against chitosan gel

The biological effect of the gel on Xac was tested by applying it directly to the NYG culture medium. As shown in Figure 18, CFU growth occurred only in the inoculum treated with water.

The initial inoculum had a CFU density of 10^6, which increased to 1.39 ± 0.53 x 10^7 CFU/mL after 4 hours, indicating active growth of the bacteria. No growth was observed in the other three inoculums, even at lower dilutions, showing the compounds' effectiveness against Xac. In literature, it was reported that chitosan is effective against Gram-positive and Gram-negative bacteria and fungi (GAO et al., 2022; LI; ZHUANG, 2020) and was expected that this property would be found against *X. citri* as well.

These results were interesting because the initial purpose of using chitosan gel was as a carrier for compounds with biological activity, such as essential oils. However, the chitosan gel also has the capacity to control Xac on its own. This property could be further explored to enhance the protective effect of the compounds against infection by *X. citri* by using chitosan as a adjuvant.

Figure 18 - Sensibility assays of X. citri against chitosan gel. A is the serial dilution with water, B is with chitosan gel, C is with Acetic Acid and D is with Kanamycin.



Source: Author.

Although the mechanism of action of chitosan against microorganisms is not yet fully understood, evidence suggests that chitosan interacts with the cellular surface of bacteria. This interaction causes permeabilization of the cell walls, primarily through electrostatic interactions between the positively charged amino groups of chitosan and the negatively charged components of the cell wall. This destabilization leads to leakage of intracellular substances,

ultimately resulting in cell death (LI; ZHUANG, 2020). Other hypotheses suggest that low molecular weight chitosan can penetrate the cell and inhibit bacterial RNA, protein synthesis, and other pathogen metabolites (GAO et al., 2022). In fungi, the main mechanism reported is the capacity to penetrate the luidity-fungal cell membrane and trigger the production of intracellular reactive oxygen species (ROS) and suppress DNA, RNA, and protein synthesis (GAO et al., 2022). Chitosan fibers of different manufacturers with different molecular weight were tested against *Escherichia coli*, *Staphylococcus aureus* and *Candida albican* and it was reported that chitosan fibers have the capacity to inhibit those microorganisms in more than 95% against *S. aureus* and more than 90% against *E. coli* and *C. albican* (LI et al., 2022). Those studies suggest this property could extend to *X. citri*. As detected in the experiment, the chitosan gel inhibits bacterial growth *in vitro* and shows promise for agricultural use against microbiological infections.

4.4.Physiological tests

4.4.1.Gel application on leaves

The three applications were conducted from November 2023 to April 2024, in the first experiment was used the most aggressive application, with a complete cover of the axial and abaxial faces of the leaf with gel (C3), in the second experiment was used the intermediate application, with partial cover of the leaf with gel (C2), on the third application was used the foliar spray (C1). Besides the gel application, the control plants (S1, S2 and S3) received the acetic acid simultaneously with their respective application comparative.

After dying, was observed that the gel formed 2 types of superficial structures on the leaves, a more homogenic covering, forming a pellicle on the leaves (Figure 19 – A), and a fragmented structure, with individual pools spread across the leaves (Figure 19 – B). The film structure was commonly found on the C3 and C2 applications while the fragmented structure was more expressive on C1.

In all applications, damage to the leaves was observed, indicating a level of phytotoxicity in the gel. The extent of damage was categorized into four groups based on the affected foliar area, correlating the number of damaged leaves with the total number of leaves on each plant. Leaves without significant damage were classified as D0, leaves with damage less than a quarter of the foliar area were classified as D1, leaves with damage less than half of the foliar area were

classified as D2, and leaves with more than half of the foliar area damaged were classified as D3. Examples of leaf damage after application of chitosan gel are shown in Figure 20. It was observed that plants treated only with acetic acid showed no leaf damage.

Figure 19 - Leaves covered by dried chitosan gel. A is a leave with a pellicle covering its surface, and B is a leave with fragmented films pools.

Source: Author.

Figure 20 – Damages caused on leaves by the chitosan gel application.

Source: Author.

The frequency of the damage varied with the load of gel applied. In Table 3, the results reported for the application of foliar spray (C1) and in the intermediate application (C2), show that the amount of leaves without any damage was about 97%, while the damage on leaves found was not higher than 25% of the foliar area. In the more extreme application (C3), only about 73% of the leaves were found without any significant damage, 25% of the leaves had damages in less than 1/4 of the foliar area but were found more severe damages of less than 1/2 of the foliar area (1,44%) and more than 1/2 of the foliar area (0.44%). Those results suggested that the

plants can handle the chitosan gel in moderate concentrations suffering minor damages, at high dosages such as C3, the gel presented high phytotoxicity increasing the impact on the leaves, causing significant damage, which could implicate a reduction of vital functions, impacting negatively on the plant.

Table 3 - Relative damage caused by the application of different charges of gel on citrus leaves.

Application	Leaf area damaged (%)			
	D0	D1	D2	D3
C1	97.2 ± 4.8	2.8 ± 4.8	-	-
C2	97.6 ± 4.7	2.4 ± 4.7	-	-
C3	72.9 ± 9.9	25.2 ± 10.0	1.5	0.4

Source: Author.

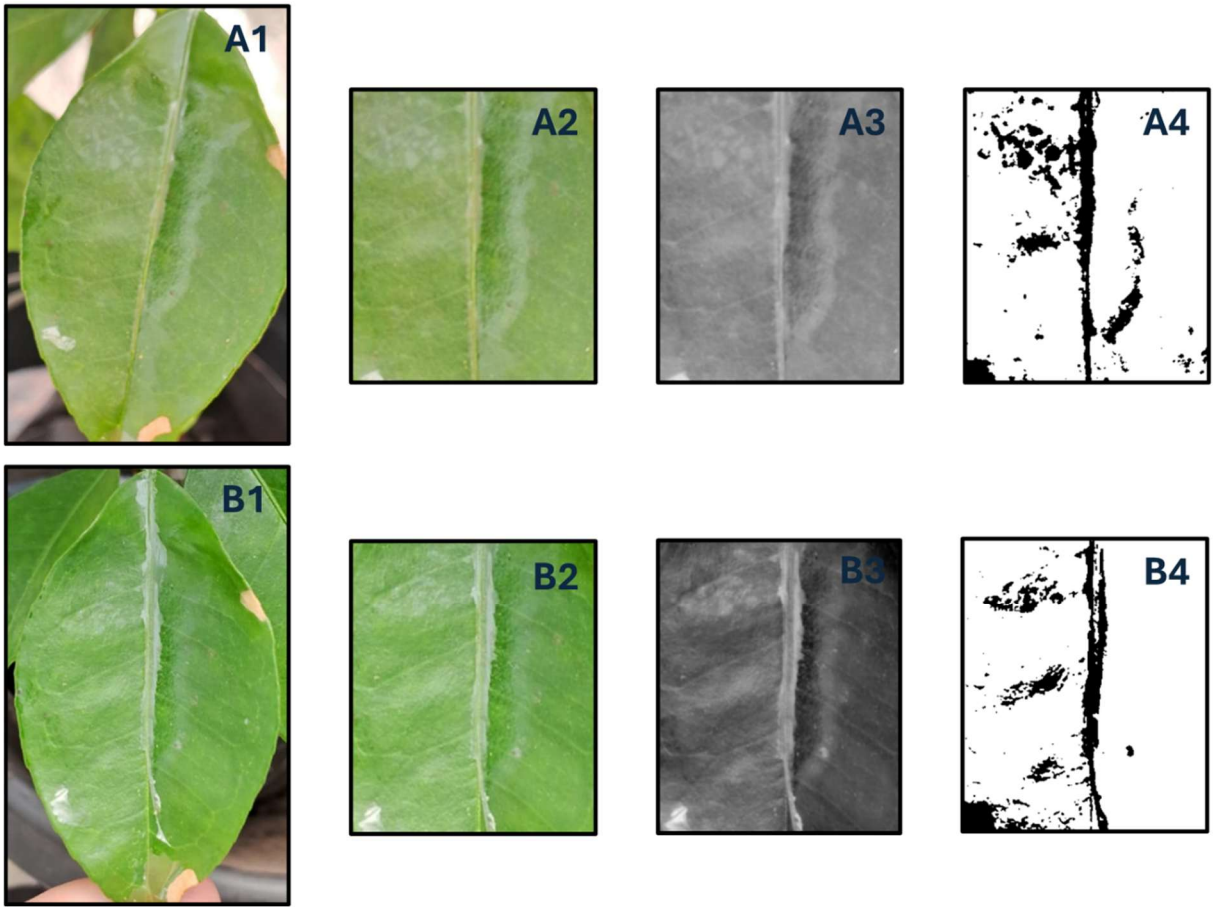
Before applying, it was expected some damage to the leaves, especially to young ones that have not developed their natural resistance and were more susceptible. The results showed that, even in the young leaves, the solvent without chitosan does not cause any visible damage. The damage was caused by the interaction between solvent and chitosan. A hypothesis to explain this reaction is the interaction time between the gel and the leaves. The solvent is liquid and volatile, quickly evaporating or running down the leaf when applied alone, but when it is applied as a gel it gets viscous and takes more time to volatilize, increasing the contact time with the leaves. Another possibility for the damage is the interaction of chitosan and the solvent. In acid, the chitosan has its amines protonated, this functional group gets more reactive, and this reactivity might be the cause of damage in the leaves. Further experiments could be conducted to understand the mechanism that causes damage to the leaves.

4.4.2.Adhesion test (washing/rain simulation)

Chitosan gel adhesion was assessed by exposing the dry gel on leaves to a stream of droplet water to simulate the effect of falling raindrops. The chosen leaves were photographed before the test started, the water flow was adjusted to 1 liter per minute and the trees were positioned under the water spreader. After 3 minutes under the water flow, the plants were left 3 hours to dry. Once completely dried, the previously chosen leaves were photographed again and the software ImageJ (version 1.54m) was used to determine the foliar area covered by chitosan film. The process used to determine the foliar area covered by chitosan was shown in Figure 21.

After exposing the trees to water flow it was possible to identify a considerable reduction in the area covered by chitosan (Figure 22). In application C1, the area covered decreased from 21.6% to approximately 10%; in application C2, the area covered by chitosan was 16% and 7.3%, respectively. The same pattern was observed in C3, and washing decreased the area covered from 42.4% to 23.8. After washing, the area covered by the chitosan decreased by approximately 50%. An interesting result was the relative area covered by the chitosan gel in different applications. In application C3, the greatest film coverage was found, which was expected because most of the chitosan was used. An unexpected result was obtained for C2, using approximately half of the gel used in C3, and the total coverage was inferior to that of C1, where the application was just a spray with a lower concentrated gel. The hypothesis for this result was that the gel applied in C2 was considerably heavier than that applied in C1, causing part of the gel to flow out of the leaf, which could explain why the covered area was smaller than in C1.

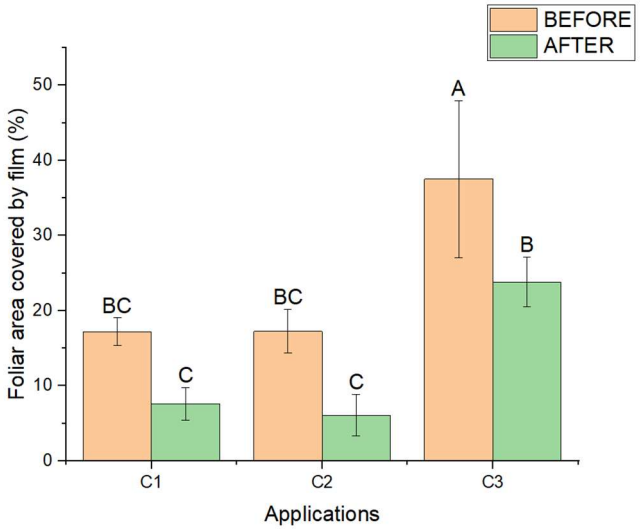
Figure 21 - Evaluation of chitosan gel adhesion to the leaves. A, B, C and D are respectively the photo of the leaf, the section analyzed, the RGB band used and the gel coverage on the leaf. 1 is the leaf before the test and 2 is the leaf after the test.



Source: Author.

Regarding the average 50% reduction in foliar coverage, it is important to highlight the water flow used in this experiment. The minimum water flow was approximately 1 L/min, corresponding to 60 liters per hour. In meteorological terms, this water flow was equivalent to 60 mm of rain, which is considered heavy rainfall over 1 m², as determined by the World Meteorological Organization (WORLD METEOROLOGICAL ORGANIZATION, 2020). The area of the water spreader was much smaller than 1 m² (approximately 0.042 m²), which further intensified the severity of the event. Despite this, approximately 50% of the chitosan gel was washed off the leaves, indicating that the gel adhered well to the leaf surface. The literature reports that chitosan, under milder conditions (10 mm to 30 mm of rain), can resist washing from *Vicia faba* leaves, with minimal losses after 3 minutes of water exposure. These results suggest that chitosan has strong adhesion to leaves during typical events, making it a reliable carrier for active compounds.

Figure 22 - Foliar area covered by chitosan film before and after the washing test.



Source: Author.

4.4.3.Gas exchange parameters

The gas exchange parameters suffered no significant alteration with the application of the products when comparing the application and its control on the same day (Table 4). Using the Tukey test (p<0.05) to evaluate the statistical differences of the results, it was observed that there was no significant difference between the plants with chitosan and acetic acid only, indicating that the chitosan was not prejudicial to the plant gas exchange. In Figure 20 it was possible to observe a homogeneous film covering a significant portion of the leaf, in this

situation (applications C2 and C3) was expected to occur some reduction in the gas exchange parameters in function of the mechanical barrier formed by the chitosan. In the literature the chitosan was explored for its capacity to reduce the transpiration rate, reducing the degradation of fruits at post-harvest (JIN et al., 2024; TOKATLI; DEMIRDÖVEN, 2020), even with those properties, the results found in the present study suggest that the layer of chitosan applied did not interfere significantly with gas exchange.

Besides its capabilities of reducing the respiration rate, chitosan has already been explored in literature. It showed interesting properties such as antitranspirant agent, reducing the water loss in *Phaseolus vulgaris L*. (IRITI et al., 2009) and *Vicia faba L.* (FOUDA et al., 2022), improving the growth and productivity of these crops, especially under situations of drought stress. Zong et al. (2017) reported that chitosan increased the photosynthetic rate in *Brassica rapa L.* under stress conditions. ALMEIDA et al., (2020) found related results in corn. In kiwi fruits, the application of chitosan as a adjuvant increases the resistance against fungal pathogens and the photosynthesis rate (ZHANG et al., 2022), in monocotyledons and dicotyledons the application of chitosan and chitin as foliar spray induced responses in plants innate defenses, these compounds activate genes responsible for expression of chitinases, glucanase, phytoalexin, jasmonic acid, and other unique early responsive and defense-related genes (RISEH et al., 2022).

Table 4 – Gas exchange parameters of different treatments measured in different days.

Experiment	Days after application	Carbon assimilation rate (µmol CO_2 / m² * s)		Transpiration rate (µmol H_20 / m² * s)		Stomatic conductance (mol / m² * s)	
		Gel	Solvent	Gel	Solvent	Gel	Solvent
C1	2	4.262^{a}	5.769^{a}	0.626^{ab}	0.755^{ab}	0.026^{a}	0.026^{a}
	8	4.640^{a}	4.807^{a}	0.917^{b}	0.44^{ab}	0.028^{a}	0.019^{a}
	15	5.096^{a}	5.849^{a}	0.633^{ab}	0.725^{ab}	0.03^{a}	0.031^{a}
	22	4.961^{a}	6.895^{a}	0.400^{a}	0.776^{ab}	0.018^{a}	0.036^{a}
C2	2	6.386^{b}	6.560^{b}	2.333^{ab}	3.487^{b}	0.072^{bc}	0.088^{c}
	8	6.778^{b}	6.377^{b}	2.010^{a}	3.492^{b}	0.066^{abc}	0.068^{abc}
	13	3.757^{a}	2.461^{a}	2.457^{ab}	1.686^{a}	0.04^{ab}	0.036^{a}
	19	4.635^{ab}	3.053^{a}	1.484^{a}	2.442^{ab}	0.037^{ab}	0.040^{ab}
C3	2	6.437^{bc}	5.004^{ab}	2.248^{ab}	2.046^{ab}	0.104^{b}	0.07^{ab}
	8	7.901^{c}	6.045^{bc}	1.887^{ab}	1.535^{ab}	0.094^{b}	0.061^{ab}
	12	5.474^{abc}	3.645^{ab}	2.884^{b}	1.999^{ab}	0.067^{ab}	0.034^{ab}
	18	5.707^{abc}	3.363^{a}	2.018^{ab}	0.758^{a}	0.064^{ab}	0.019^{a}

Data are means of five replications; means of the same experiment with the same letter are not significantly different (p>0.05).

Source: Author.

Chitosan gel has a wide range of benefits, when applied alone can decrease the water loss by transpiration, reduce the impact of drought stress, increase the photosynthetic rate, improve the plant development and works as an inducer of natural defense system of plants. In addition to its own natural antimicrobial capacity, chitosan enhances protection against diseases. When applied as a coadjutant chitosan can carry, release and improve the properties of other compounds making its use beneficial in agriculture.

4.4.4.Oxidative stress (Biochemical parameters)

The oxidative stress was observed by measuring the activity of the enzymes Ascorbate peroxidase (APX), Superoxide dismutase (SOD) and Catalase (CAT) on application C1 only, 2 days and 21 days after gel application. The activity of APX and SOD (Figures 23 and 24, respectively) showed no statistical differences when comparing different plants at different periods of time. On the other hand, CAT showed significant peaks 2 days after application (Figure 25), on the second measurement levels of catalase activity decreased to usual levels, like plants without any kind of treatment.

During the process of electron carrier chain, essential to the photosynthesis, some Residual Oxygen Species (ROS) radicals and non-radicals are liberated (O_2^-, O_2, H_2O_2, OH^-), those molecules, in especially H_2O_2, at low concentrations act as a signalizing pathway and regulate the cells responses for environmental factors (SOFO et al., 2015). When the plant faces biotic or abiotic stresses such as pathogen attack, wounding, UV irradiation, exposure to intense light, drought, salinity, or chilling, one of the natural mechanisms of self-protection is stomatal enclosure. With the reduction of CO_2 available to photosynthesis the amount of ROS increases, in high amounts those molecules can lead to oxidative stress, degrading proteins, DNA and lipids (SOFO et al., 2015; ZANDALINAS et al., 2017). To prevent the outburst of ROS inside the leaves, enzymes like SOD, CAT and APX work together through different pathways to reduce H_2O_2 to non-toxic levels, SOD has the function of catalyzing the removal of O_2^- by dismutating in O_2 and H_2O_2. CAT and APX works to degrade H_2O_2 in water and oxygen, APX uses ascorbate as a specific electron donor to scavenge H_2O_2 into water, being more active in sub-cellular locations, CAT converts H_2O_2 into water and molecular oxygen (O_2) without any specific electron donor, being more active at higher concentrations of H_2O_2 (RAJPUT et al., 2021).

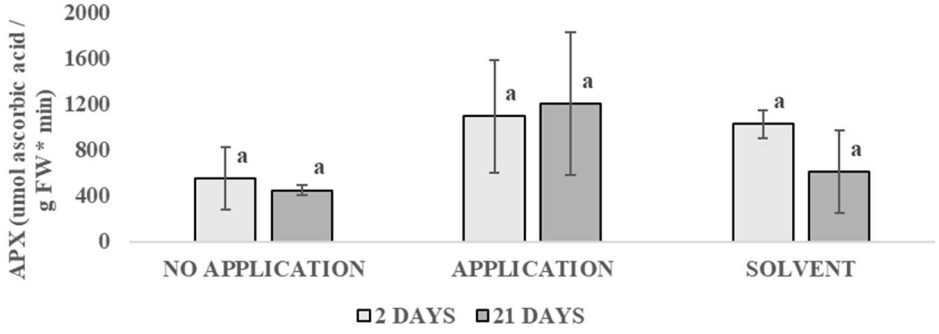
The results found in Figure 25 suggest an immediate response of the plant caused by the application of the gel, the values for catalase activity 2 days after application in plants with treatment (gel or solvent) were significantly higher than the plants without any treatment. The high catalase activity suggested an acute reaction against the gel and solvent application. The effect was not persistent, 21 days after the application the values for catalase were back at normal levels for plants without treatment, indicating that chitosan does not cause lasting stress effects.

These results were positive for the chitosan application, while in SOD and APX the levels of enzymatic activity did not suffer significant variations, in CAT the peak of activity occurred after the application. However, the stress caused by gel and solvent had no chronic effect, after 21 days the levels of CAT returned to normal levels, indicating that the Long-term use of chitosan was not harmful to the plant.

In literature, studies have reported that chitosan can enhance the activity of enzymes such as CAT, SOD or APX. Liu et al. (2021) described that the application of exogenous chitosan as spray foliar in Triticum aestivum L. increased the concentration of CAT and APX what leaded to a decrease in ROS, comparable results were found by Attia et al. (2021) with tomato. It has also been reported that chitosan functions in cell signaling, and the chemical constitution of chitosan includes uridine diphosphate N-acetyl-d-glucosamine (UDP-GlcNAc) as a nucleotide sugar that can be recognized by cells through chitin synthases or chitin deacetylases (ATTIA et al., 2021). These enzymes that interact with chitosan can generate oligomers that can interact with the nucleus of the cells and initiate cascade reactions related to the expression of antioxidant enzymes (RABÊLO et al., 2019).

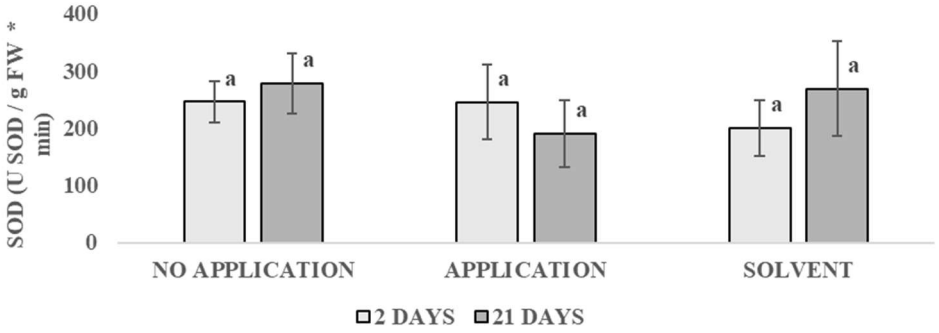
According to what is discussed in the literature, it is necessary to carry out more physiological tests to verify whether the catalase peak is related to stress or due to the natural ability of chitosan to act as a signaling agent for the expression of genes related to antioxidant enzymes.

Figure 23 – Ascorbic acid activity 2 days and 21 days after C1 application.



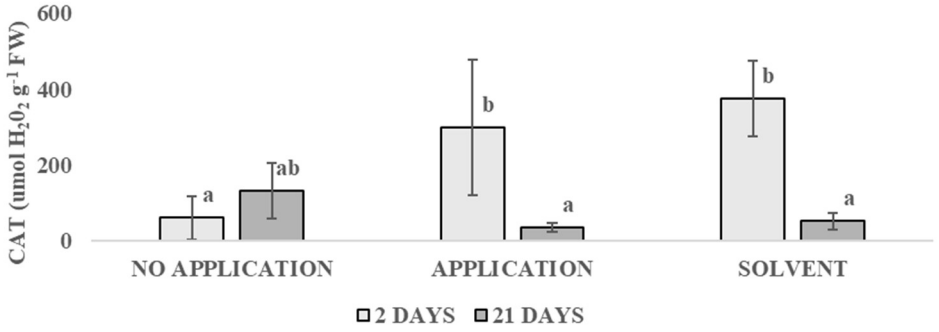
Source: Author.

Figure 24 - Superoxide dismutase activity 2 days and 21 days after C1 application.



Source: Author.

Figure 25 - Catalase activity 2 days and 21 days after C1 application.



Source: Author.

5.Conclusion

As conclusions of this work, it was noted that the drying temperature is relevant on mechanical properties of chitosan gel, particularly elongation and rupture tension. Additionally, it was also observed that the chitosan gel, when applied on citrus leaves, showed interesting properties such as good adhesion and did not interfere with the plant physiology in vegetation house. *In Vitro*, chitosan gel was effective against *X. citri*. All those characteristics suggest that chitosan gel formulations could be explored for controlling the citric canker as an alternative to the use of copper.

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