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ARTICLE

Coffea arabica var. Borbon Biochemical Response to Chitosan Oligosaccharides Foliar Exposure

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ABSTRACT: The biochemical response of *Coffea arabica* var. Borbon to chitosan and chitosan oligosaccharides (COS) was evaluated in one-year-old plants under greenhouse conditions. COS solutions were synthesized through chemical and physical hydrolysis using acetic acid, hydrogen peroxide, and microwave irradiation. The obtained COS had an average molecular weight (Mw) of 3549.90 ± 0.33 Daltons (Da), a deacetylation degree (DD) of 76.64 ± 1.12%, and a polymerization degree (PD) of 18.91 ± 0.0018. Solutions of chitosan and COS were applied to *C. arabica* var. Borbon at concentrations of 0.25, 0.5, and 1 wt%. The experimental design was conducted using a completely randomized design with four replications. The biochemical responses assessed included soluble protein content, phenylalanine ammonia-lyase (PAL), chitinase, β-1,3-glucanase, peroxidase, catalase, and chlorophyll fluorescence. The application of COS demonstrated significant differences ($\alpha = 0.05$) in protein concentration, with the activity of β-1,3-glucanase, chitinase, and catalase being 1.5, 7.5, and 3.9 times higher, respectively, while showing similar behavior to chitosan in PAL activity, both up to 4.4 times higher than the distilled water control and lower than chitosan in peroxidase activity. Treatments with chitosan yielded a higher photochemical efficiency of Photosystem II (PSII). The application of COS suggests a viable foliar alternative to active plant defense mechanisms without the risk of phytotoxicity.

KEYWORDS: Enzymatic activity; PAL; β-1,3-glucanase; chitinase; chlorophyll fluorescence; photosystem II

1 Introduction

The global coffee trade is one of the most important commodity markets worldwide [1]. In 2023, coffee production reached 171.4 million bags (60 kg), with Brazil as the leading coffee-producing country, followed by Vietnam. Mexico ranked 10th in global coffee production [2], with 81%–82% of its output concentrated in the states of Chiapas, Veracruz, and Puebla [3]. In 2023, approximately 87% of Mexico's total coffee production was *Coffea arabica*, while the remaining percentage was *Coffea canephora*, commonly known as arabica and robusta, respectively [2]. *C. arabica* generates higher income in Mexico due to its cup qualities, but it also presents production challenges due to its susceptibility to pests and diseases [4]. For instance, Coffee Leaf Rust (CLR), caused by *Hemileia vastatrix*, an obligate biotrophic fungus, and Coffee Berry Disease (CBD), caused by *Colletotrichum kahawae* Waller & Bridge [5], are among the most threatening diseases for coffee plantations. *H. vastatrix* infections in *C. arabica* plantations can cause yield losses of up to

70% for farmers. In 2016, Mexico reported a 50% loss in *C. arabica* production due to the severity of the CLR epidemic [6]. Several coffee varieties grown in Mexico, such as Typica, Bourbon, Caturra, Mundo Novo, and Garnica, are known for their susceptibility to CLR [7].

Some alternatives for controlling phytopathogens in coffee plantations involve cultural practices as well as chemical and biological control methods. Cultural practices involve modifying plantation management to create conditions that limit the spread of phytopathogens [6,8]. These measures include reducing plantation density and implementing a canopy crop program to minimize microenvironments and direct contact between plants [9], maintaining a younger population of coffee plants, and ensuring proper plant nutrition with acidified soil to enhance nutrient uptake [8]. Additionally, coffee plantation management could also include establishing physical barriers to protect the plant population from wind, adverse temperature conditions, and the spread of pathogen inoculum [8,10]. Natural barriers also facilitate the implementation of these physical barriers and biological control strategies by enriching of soil microbiota [8,10,11].

Chemical control involves the application of exogenous substances to coffee plants, either soil treatment or foliar spraying. Some pesticides used in coffee plantations belong to chemical families such as carbamates, neonicotinoids, triazoles, and organophosphates [12]. These substances can act as protective or systemic fungicides [13]. Exogenous elicitors are other substances used to control phytopathogens, which do not necessarily possess direct fungicidal activity [14]. Elicitors are molecules recognized by patter recognition receptors (PRRs) that activate the plant's defense mechanisms. Elicitors can be sourced from microbial secretions or from the wound tissues of host plants. Some alternatives for controlling phytopathogens include foliar applications of exogenous elicitors related to the target pathogen as a protective strategy [6].

The primary mechanism in biological and chemical control by elicitors involves activating the plant host defense mechanisms. Plants react to microorganism invasion with biochemical changes associated with biotic stress signaling, which alters their physiology. For instance, available oxygen can be utilized to produce Reactive Oxygen Species (ROS) that inhibit pathogen growth [5,15]. Additional defense strategies include cell wall thickening, oxidative bursts, hypersensitive responses (HR), the synthesis of antimicrobial compounds like phenolics, flavonoids, and lignins, and the expression of antifungal pathogenesis-related proteins (PR) such as chitinases and glucanases, as part of their immune response [5,15–17].

Chitosan is derived from chitin under alkaline conditions through a process known as deacetylation. While both chitosan and chitin are made up of the same monomer units, they differ in their proportions. They consist of N-acetyl-D-glucosamine (GlcNAc) and D-glucosamine (GlcN) linked by a β-1,4-glycosidic bond. Chitin is primarily composed of GlcNAc units, whereas chitosan mainly comprises GlcN units [18,19]. The synthesis of chitosan oligosaccharides (COS) involves breaking glycosidic bonds and depolymerizing chitosan through methods such as acid hydrolysis, oxidative hydrolysis, mechanical hydrolysis, or enzymatic hydrolysis [19]. COS is defined as chitosan with a degree of polymerization (DP) of less than 20 and a molecular weight (Mw) of under 3900 [20]. COS has gained popularity in agricultural applications due to its enhanced water solubility, low viscosity, and stability at neutral pH [19,21].

Chitosan is recognized by PRRs and induces the transcription of defense-related genes, such as β-1,3-glucanases, chitinases, PAL, PR1, and antioxidant enzymes [22]. It also activates the SA pathway and SAR response through the activation of gene NPR1 transcription, a key gene that regulates plant defense mechanisms across wide variety of plants [22].

The molecular and enzymatic effects of chitosan and chitosan derivatives have been studied in various *C. arabica* varieties to mitigate biotic and abiotic stressors [23]. Previous studies have identified key physicochemical parameters that influence the molecular and biochemical responses of *C. arabica* as the source of chitosan, production method, viscosity, molecular weight, and degree of polymerization [22]. In

contrast, fewer studies have been conducted to describe the molecular and biochemical impact of COS on *C. arabica* varieties. This highlights the need for a properly characterization of COS and understanding of how plants respond to its application.

Various biochemical assays can be achieved to properly understand how the exposed plants responded to an exogenous elicitor such as chitosan and derivatives. Beside them can be found gene expression, enzymatic activity, photopigments concentration, and chlorophyll (Chl) fluorescence. Chl fluorescence is widely used in plant studies to examine the effects of biotic and abiotic factors on the plants [24–26]. The relationship between variable and maximum fluorescence is referred to as maximum PSII efficiency and is employed to assess the photosynthetic effectiveness of PSII. The expected range for Fv/Fm is about 0.80 to 0.83. In coffee plants, the optimal value is 0.85; however, this value may decrease to 0.79 under stress conditions [21,27].

Chl fluorescence serves as a sensitive tool used to assess and monitor the physiological and biochemical state of plants. It is employed to observe how external factors (e.g., stressors, application of chemicals, changes in the rhizosphere microbiome) affect the stability and efficacy of PSII [25,26]. This technique can help elucidate and understand potential fluctuations in PSII due to inhibition in the electron transfer chain or physical damage in the chloroplast [25,26].

Previous studies have demostrated that the foliar application of chitosan increases the expression of plant defense genes and enzymatic activity *in C. arabica* [17,22]. The application of COS foliar activated plant defense mechanisms in the *Arabidopsis* ecotype Columbia [28], in *Nicotiana glutinosa* using a Cytosin-peptidemycin and COS system (CytPM-COS) [29], and in *Passiflora* spp. [30]. However, the effects of foliar application of chitosan oligosaccharides have not been fully studied in *C. arabica* varieties, except for the effectiveness of COS against *H. vastatrix*, evaluated in foliar discs [31]. The objective of this study was to evaluate the biochemical responses of *C. arabica* var. Borbon to the foliar application of chitosan oligosaccharide (COS). This research aims to enhance the understanding of COS applications and its potential to strengthen the resistance of *Coffea* spp. against plant pathogens.

2 Materials and Methods

2.1 Synthesis of Chitosan Oligosaccharides (COS)

COS was synthesized according to [19] from low molecular weight chitosan with a deacetylation level greater than 75%, which was dissolved in 1% acetic acid at a concentration of 3 wt% while stirring at 500 rpm and 70°C until the chitosan was fully dissolved. Oxidative and mechanical hydrolysis were performed to break the β-1,4-glycosidic bond by adding 1% hydrogen peroxide dropwise at 25°C while stirring at 300 rpm, followed by microwave application at 700 W for 1.5 min [21].

2.2 Chitosan Oligosaccharides Characterization

2.2.1 Deacetylation Degree (DD)

Deacetylation Degree determination was performed by Fourier Transform Infrared Spectroscopy (FTIR) according to [17,32,33] by FTIR with Attenuated Total Reflectance (ATR) (Agilent 4300 Handheld FTIR, USA) from 4000 to 650 cm^{-1} with 2 cm^{-1} resolution, 10 µL of each sample was dried over ATR. The *DD* was obtained by Eqs. (1) and (2), where A_{1320} and A_{1420} are the absorbance of chitosan at wavelengths 1320 and 1420 cm^{-1}, respectively, and *DA* is the Degree of Acetylation [17].

$$DA\,(\%) = \frac{0.3822 - \frac{A_{1320}}{A_{1420}}}{0.03133} \quad (1)$$

$$DD\,(\%) = 100\% - DA(\%) \quad (2)$$

2.2.2 Molecular Weight Determination

Molecular weight determination was carried out using intrinsic viscosity, as described in [34]. The viscosities of chitosan and COS were measured with an Ostwald capillary viscometer at 25°C, using a solvent system of 0.3 M acetic acid and 0.2 M sodium acetate. The molecular weight was calculated utilizing the Mark-Houwink Eq. (3), where $[\eta]$ represents the intrinsic viscosity, M_V denotes the viscosity average molecular weight, and K and a are constants specific to a given solvent system. The Degree of Polymerization (PD) was determined from the average molecular weight and the proportional contributions of the molecular weights of chitosan monomers N-acetyl-D-glucosamine (GlcNAc) and D-glucosamine (GlcN).

$$[\eta] = KM_v^a \quad (3)$$

2.3 Plant Materials and Experimental Design

A local producer in Angel Albino Corzo, Chiapas, Mexico, provided *Coffea arabica* var Borbon seeds. The seeds were previously disinfected with 20% commercial sodium hypochlorite for 5 min and rinsed with 20 mL of sterile distilled water three times to eliminate traces of sodium hypochlorite. They were then dried at room temperature, sown in sterile sand, and transplanted two months before the analysis to ensure the acclimatization of the plants. Six-month-old transplanted plants were grown in polyethylene bags measuring 6.7 cm × 6 cm × 26 cm (Length × Width × Height) with a substrate mixture of peat moss and agrolite in a 3:1 ratio. Under greenhouse conditions, the plants were watered twice a week with 100 mL of water. Sixty-eight uniform plants were selected to perform the COS foliar application. The selected plants were randomly exposed to different treatments, including a distilled water control and different concentrations of chitosan and synthesized COS (1%, 0.75%, 0.5%, and 0.25%) through foliar application of 2 mL 24 h before each analysis. All treatments were evaluated with four replicates.

2.4 Enzymatic Activity

The second pair of leaves from each plant was collected to perform protein extraction as outlined in [35]. The resulting protein extract was used to assess the soluble protein content (SPC) using the Bradford method. The SPC values obtained were utilized to determine enzyme-specific activity. The enzymatic activities assessed included PAL, β-1,3-glucanases, chitinases, peroxidase (POD), and catalase (CAT). SPC and enzymatic activities were analyzed using UV-Vis spectroscopy (Nanodrop One®, Thermo Fisher Scientific, Waltham, MA, USA).

2.4.1 Enzymatic Extraction

Protein extraction was performed according to [35] by homogenizing 50 mg of collected leaves in liquid nitrogen using pre-chilled mortar and pestles and macerating for 4 h at 4°C in an extraction buffer composed of Tris-HCl 0.1 pH 8, ascorbic acid 0.1 wt%, glycerin 10%, polyvinylpyrrolidone (PVP) 1 wt%, and β-mercaptoethanol 5%. The homogenized samples were centrifuged at 15,000× *g* for 15 min. The supernatant from each sample was then transferred to microcentrifuge tubes and stored at −4°C until further use.

2.4.2 Phenylalanine Ammonia Lyase (PAL) Activity

PAL activity was determined as detailed in [36], with a few modifications. 5 µL of enzyme extract were added to 145 µL of Tris-HCl buffer solution (pH 8.8) at 50 mM, along with 50 µL of 50 mM L-phenylalanine, and incubated in a water bath at 37°C for 20 min. The absorbance was measured at 280 nm.

2.4.3 β-1,3-Glucanases Activity

The activity of β-1,3-glucanases was evaluated using a colorimetric method as described in [35], with some modifications. 8 µL of enzymatic extract were added to 885 µL of a sodium acetate buffer solution (pH 5, 50 mM) along with 7 µL of laminarin at 0.15 wt%. The samples were incubated in a water bath at 40°C for 10 min, then placed in an ice bath for 5 min. Finally, 335 µL of 3,5-Dinitrosalicylic acid at 96 mM were added to the samples and incubated in a water bath at 90°C for 10 min, followed by another ice bath for 5 min. The absorbance was measured at 515 nm.

2.4.4 Chitinases Activity

Chitinase activity was evaluated following the method described by [37], with minor modifications. Briefly, the catalytic activity of chitinases in the presence of chitin as a substrate produces N-acetylglucosamine. To initiate the reaction, 5 µL of the enzymatic extract was added to 445 µL of sodium acetate buffer solution (pH 5) at 0.5 mM, along with 50 µL of chitin at 0.05 wt%. The solution was incubated at 40°C for 30 min, followed by the addition of 1 mL of potassium ferrocyanide, which was then kept in a water bath at 95°C for 15 min. To stop the reaction, the samples were placed in an ice bath. Absorbance was measured at 420 nm.

2.4.5 Peroxidase Activity (POD)

POD activity was conducted as described in [38], with minor modifications. A total of 33 µL of enzymatic extract was added to 1 mL of reaction media consisting of a sodium phosphate buffer solution (10 mM, pH 6), guaiacol (0.25 wt%), and hydrogen peroxide (10 mM). The absorbance was measured at 470 nm every 20 s for 1 min.

2.4.6 Catalase Activity (CAT)

CAT activity was performed according to [38], with minor modifications. An enzymatic extract of 11.11 µL was added to 1 mL of reaction media consisting of 1 M Tris-HCl (pH 8), 5 mM EDTA (ethylenediaminetetraacetic acid), and 30 mM. The absorbance was measured at 240 nm every 10 s for 1 min to monitor the CAT catalytic performance in the presence of H_2O_2.

2.5 Chlorophyll Fluorescence

Chlorophyll fluorescence measurements were recorded according to [39,40]. Using a chlorophyll meter, FluorPen FP 100 (Photon Systems Instruments, Czech Republic), measurements were taken from a dark-adapted second pair of leaves for 5 min. The data were recorded at 24, 48, and 72 h after COS foliar exposure, with four replicates for each treatment. Potential photochemical yield (Fv/Fm) values were extracted from OJIP curves.

2.6 Statistical Analysis

Statistical analysis was performed using a one-way analysis of variance (ANOVA), followed by Tukey's test ($p < 0.05$) with four replicates. Additionally, Principal Component Analysis (PCA) was performed for

groups of COS and chitosan concentrations related to the studied enzymatic activities. R Studio was utilized for statistical analysis and to generate graphical representations.

3 Results and Discussion

Varieties of *Coffea arabica* and *Coffea canephora* have the highest economic impact on the global coffee indusdry. However, *Coffea arabica* plantations have faced biotic and abiotic stresses, such as climate change and disease advances concerning agricultural communities and coffee markets [41]. In response to these issues, it is essential to develop new sustainable technologies to alleviate the effects of biotic and abiotic stress factors in coffee plantations. This includes the synthesis and foliar application of chitosan oligosaccharides (COS) [21]. Chitosan and its derivatives are widely studied as elicitors of plant immune responses. Unfortunately, there is limited research and information regarding the potential application of COS in *Coffea arabica* plants.

3.1 Characterization of Chitosan Oligosaccharides

In this study, the microwave-assisted synthesis of COS was successfully conducted in the presence of acetic acid and hydrogen peroxide, according to the COS characterization results [20]. The synthesized COS exhibited an average molecular weight of 3547.90 ± 0.33 Da based on intrinsic viscosity values ([η] = 23.3 ± 0.18 cP), a polymerization degree (PD) of 18.91 ± 0.0018, and a degree of deacetylation (DD%) of 76.64 ± 1.12, compared to chitosan's original maximum supplier values of 190,000 Da based on viscosity (300 cP) and a measured DD% of 76.67 ± 2.32. This illustrates the effect of chemical and physical hydrolysis on the viscosity and molecular weight of oligosaccharides, resulting in reductions of 92.23% ± 0.12 and 98.13% ± 0.00017, respectively, without influencing the degree of deacetylation (DD%) of 0.91% ± 0.97, which aligns with previously reported findings [19] due to the synthesis conditions that only favored the glycosidic bond rupture.

COS physicochemical properties define its bioactivity and impact on exposed plants [21,42]. Completely deacetylated COS is less capable of being recognized by plant receptors and activating the plant immune system [21]. The elicitor function of COS in activating plant defense mechanisms closely relates to PD. To enhance COS bioactivity, applied COS should have a PD of at least 4 [42].

Additionally, molecular weight and the method of application are key factors influencing the bioassimilation of chitosan and its derivatives. The molecular weight of the used chitin derivative influences its water solubility and viscosity, making it a vital factor in the mobilization and availability of COS within plant tissues [43,44].

The FTIR spectra of chitosan and chitosan oligosaccharides (Fig. 1) exhibit similar behavior, with changes in transmittance intensity corresponding to glycosidic bond breakage [19]. Both polysaccharides display chatacteristic peaks related to saccharide structures at 1151.3, 1060.7, 1018.5, and 895.81 cm^{-1} associated with C-O and β-1,4-glycosidic bond stretching vibrations [45–47]. The peak at 1660.2 cm^{-1} is linked to C-N vibration, while those at 1626, 1453, and 1420 cm^{-1} relate to amide I (C=O); the peak at 1320 cm^{-1} pertains to amide III [32,45,46,48]. Sharp bands at 1400.7 and 1376.5 cm^{-1} are assigned to C-H bending and stretching vibrations [49], and the absorption at 1251.8 cm^{-1} corresponds to C-O-C stretching vibrations. The peaks at 3200.6, 2861, and 2921 cm^{-1} are associated with -OH vibration and C-H stretching [48,49].

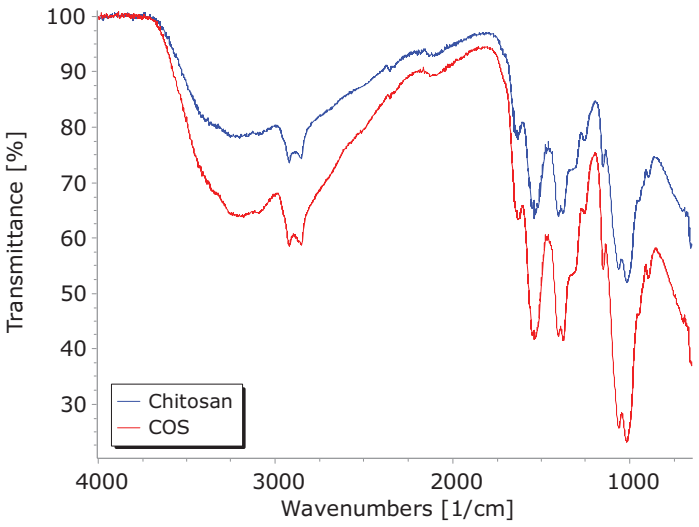

Figure 1: FTIR spectrum of chitosan and chitosan oligosaccharides (COS)

3.2 Enzymatic Activity

The primary mechanism involving elicitor molecules, such as chitosan and COS entails activating the plant's defense responses. In reaction to microbial invasion, plants undergo biochemical changes associated with biotic stress signaling, which modifies their physiology. For instance, available oxygen can be utilized to generate Reactive Oxygen Species (ROS), which inhibit pathogen growth [5,15]. Additional defense strategies encompass cell wall thickening, oxidative bursts, hypersensitive responses (HR), the synthesis of antimicrobial compounds like phenolics, flavonoids, and lignins, and the expression of antifungal pathogenesis-related proteins (PR) such as chitinases and glucanases [5,15–17].

The foliar application of chitosan has been well-documented to activate defense mechanisms associated with Systemic Acquired Resistance (SAR) responses. Chitin and its derivatives, such as chitosan and chitosan oligosaccharides, are recognized by plants as Microbial Associated Molecular Patterns (MAMPs) via transmembrane proteins called Pattern Recognition Receptors (PRRs) and the CERK1 receptor (Chitin Elicitor Receptor Kinase 1) [50]. The activation of defense mechanisms by exogenous elicitors like chitosan and chitosan oligosaccharides, which function as MAMPs, is linked to the expression of pathogenesis-related proteins (PR proteins), including β-1,3-glucanases and chitinases. These enzymes play a key rol fall under the category of PR proteins with catabolic activity; these enzymes degrade β-1,3-glucan and chitin in the fungal cell walls of pathogens, respectively [51].

Chitinases and β-1,3-glucanase activity lead to the release of low molecular weight saccharides, including oligosaccharides and polysaccharides, which are recognized by PRRs as elicitors or MAMPs. This recognition activates immune signaling pathways, triggering Pattern Triggered Immunity (PTI) [52–55]. The activation of PTI is associated with plant defense mechanisms, involving enzymatic pathways such as Phenylalanine ammonia-lyase (PAL), polyphenol oxidase (PPO), superoxide dismutase (SOD), catalase (CAT), and peroxidases (POX) [15]. The overall plant defense response (both enzymatic and non-enzymatic) near the wound site includes the SAR mechanism [56].

Systemic Acquired Resitance SAR mechanisms can be triggered by external compounds that function as signaling molecules, MAMPs, or Damage-Associated Molecular Patterns (DAMPs). These include salicylic acid, jasmonic acid, polysaccharides, and oligosaccharides such as chitosan, alginate, and carrageenan, along with their oligosaccharide derivatives, to induce the expression of defense genes [17,18,57,58].

The foliar application of COS increases the total soluble protein content related to various metabolic processes induced by COS application; in contrast, chitosan decreases protein content compared to the control treatment. Additionally, chitosan increases total soluble protein content depending on the applied concentration. The accumulation of total soluble protein can be linked to cell redox homeostasis under induced stress for Reactive Oxygen Species (ROS) scavenging [59]. Notable, COS applications exhibits higher 1,3-glucanase activity than the control and chitosan treatments.

The differences in β-1,3-glucanase and chitinase activity suggest improved assimilation of the applied exogenous elicitor, COS, compared to other chitosan derivatives. PR proteins accumulate in the apoplast to inhibit the extracellular growth of pathogens. For instance, β-1,3-glucanase (PR2) and chitinases (PR3) break down penetration structures such as the intercellular hyphae and haustoria walls of phytopathogens by catalyzing the hydrolysis of chitin and β-glucans [16,53,58].

The results (Fig. 2) indicate a significant increase in soluble protein content (SPC) in *C. arabica* var. Borbon treated with COS at 0.25%, 0.75%, and 1%, compared to control plants treated with distilled water. In contrast, a notable reduction in SPC was observed in plants treated with Chitosan at 0.25% and 0.5%. Plants treated with COS at 0.5% and Chitosan at 0.5% did not display a significant difference compared to control plants ($p < 0.05$). All enzymatic activity results (Fig. 2) are expressed as specific activity based on SPC results.

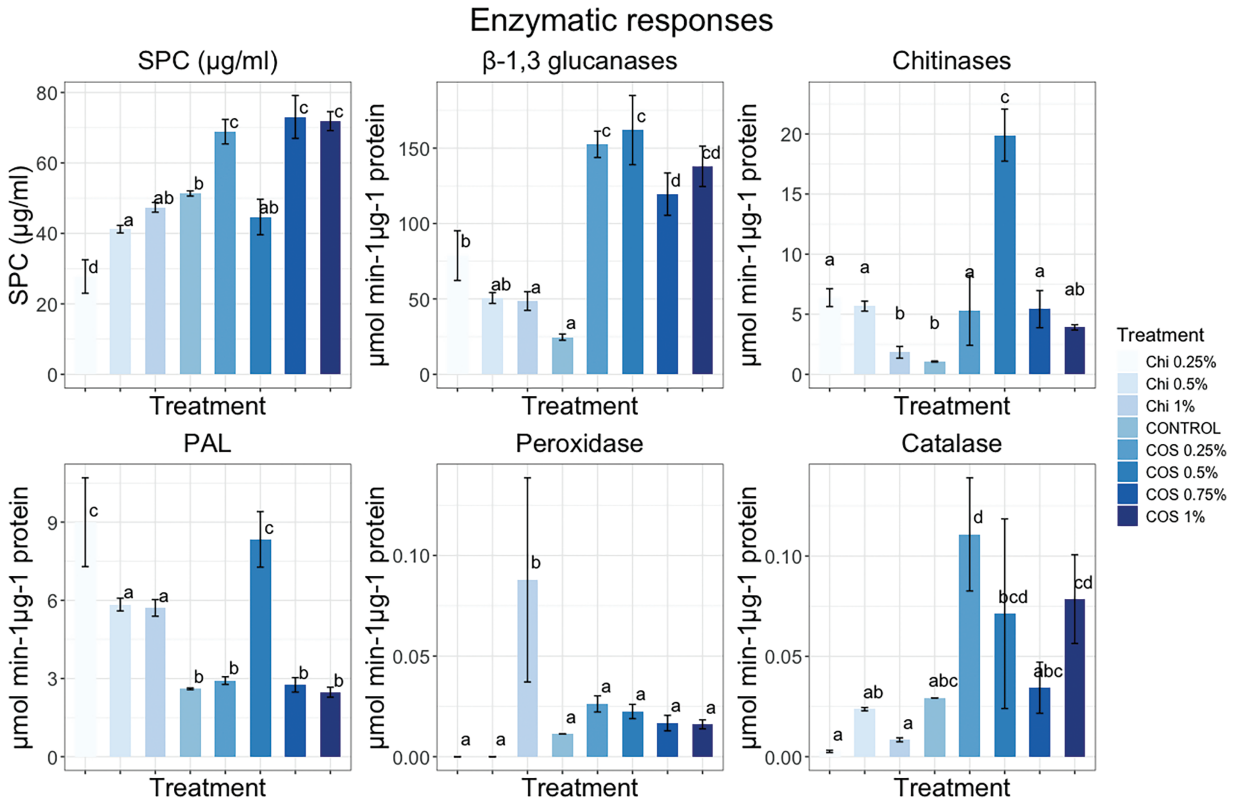

Figure 2: Enzymatic responses of *Coffea arabica* var. Bourbon to foliar exposure of chitosan, chitosan oligosaccharides, and distilled water used as a control. The evaluated specific responses were soluble protein content by Bradford methodology, β-1,3-glucanase, chitinases, PAL, peroxidases, and catalase

C. arabica var. Borbon plants treated with COS at concentrations of 0.25%, 0.5%, 0.75%, and 1%, along with chitosan at 0.25%, exhibit an increase in β-1,3-glucanase activity (Fig. 2) compared to control plants

($p < 0.05$). Conversely, plants treated with chitosan at 0.5% and 1%, which did not show a significant difference ($p < 0.05$). The plants treated with COS at 0.25% and 0.5% demonstrate the highest β-1,3-glucanase activity, as well as those treated with chitosan at 1%. The chitinase activity results (Fig. 2) indicate a significant increase ($p < 0.05$) in plants treated with COS at 0.25%, 0.5%, 0.75%, and 1% and chitosan at 0.25%, 0.5%, and 1% compared to control plants. The plants treated with COS at 0.5% display the highest chitinase activity.

Treatments with COS 0.5% and Chitosan 0.25% exhibited higher PAL activity (Fig. 2), followed by Chitosan 0.5% and 1%. In contrast, COS at 0.25%, 0.75%, and 1% did not show significant differences ($p < 0.05$) when compared to control plants. Moreover, only Chitosan 1% demonstrated a significant increase ($p < 0.05$) in POD activity (Fig. 2) compared to the control and plants treated with COS 0.25%, 0.5%, 0.75%, 1%, and Chitosan 0.25% and 0.5%. Higher CAT (Catalase) activity (Fig. 2) was observed in plants treated with COS 0.25%, while no significant differences ($p < 0.05$) were observed in plants treated with COS at 0.5%, 0.75%, 1%, or Chitosan at 0.25%, 0.5%, and 1%.

Phenylalanine ammonia-lyase (PAL) plays regulatory rol in the synthesis of phenylpropanoid compounds [21]. The increase in β-1,3-glucanase and chitinase activity has been associated with PAL activity; while, the rise of reactive oxygen species (ROS) at low concentrations, such as hydrogen peroxide (H_2O_2), acts as secondary messengers in the phenylpropanoid pathway [21,51]. The increase in PAL activity is linked to lignin synthesis, which strengthens plant cell walls. By engaging PAL in the synthesis of cinnamic acid from phenylalanine, the production of cinnamic acid contributes to the synthesis of phenolic compounds like lignin and flavonoids [21,43]. The results suggest a correlation between β-1,3-glucanases, chitinases, and antioxidant enzymes such as catalase (CAT) and peroxidase (POD) with PAL activity in plants treated with COS 0.5 wt%. The observed enzymatic activities suggest that COS 0.5 wt% treatment holds promise for enhancing SAR and ISR responses (Induced Systemic Resistance). Coffee plants treated with COS 0.5 wt% exhibit higher enzyme activity levels of β-1,3-glucanases, chitinases, POD, CAT, and PAL, which may correlate with a potentially better response and greater efficacy in controlling biotrophic pathogens [21,43,51].

Principal Component Analysis (PCA) results show that PC1 accounts for 41.3% of the variance, while PC2 explains 34.25% (Fig. 3). It demonstrates how the chitosan groups at 0.25%, 0.5%, and 1% are clustered together near the control (C) group, suggesting a certain similarity among them. The dispersed and clustered groups of COS at 0.25%, 0.75%, and 1% reveal specific differences between COS and chitosan. The enzyme activity vectors indicate that antioxidant enzymes, such as POD, for chitosan groups compared to COS, but show slight differences when contrasted with the control group. Furthermore, PAL activity exhibits more influence for chitosan groups as well as the COS 0.5% group. In contrast, the COS 0.25%, 0.75%, and 1% groups demonstrate higher influences in chitinases (CHI), β-1,3-glucanase (GLU), and catalase (CAT).

Overall, β-1,3-glucanase, chitinases, PAL, and CAT activities were significantly higher in COS plants treated compared to those treated with chitosan. These differences may be attributed to the variations in bioavailability between the molecules. The bioavailability of these molecules could be enhanced by the increased hydrophilicity of COS along with the substantial reduction in the viscosity of chitosan to COS, from 300 cP to 23.3 ± 0.18 cP. These changes may improve the mobility of the molecules and potentially enhance their bioavailability in coffee plants. Additionally, the reduction in the molecular weight of chitosan compared to COS improves the bioassimilation of the molecules, as demonstrated by the increase in enzymatic activity [43,44]. The variability in defense enzyme activity in coffee plants resulting from foliar exposure to COS and Chi treatments can be attributed to differences in molecular weight, demonstrating improved bioassimilation for COS treatments, particularly for COS 0.5 wt% with Mw of 3549.90 ± 0.33 Da compared with the Mw of Chi of 190,000 Da.

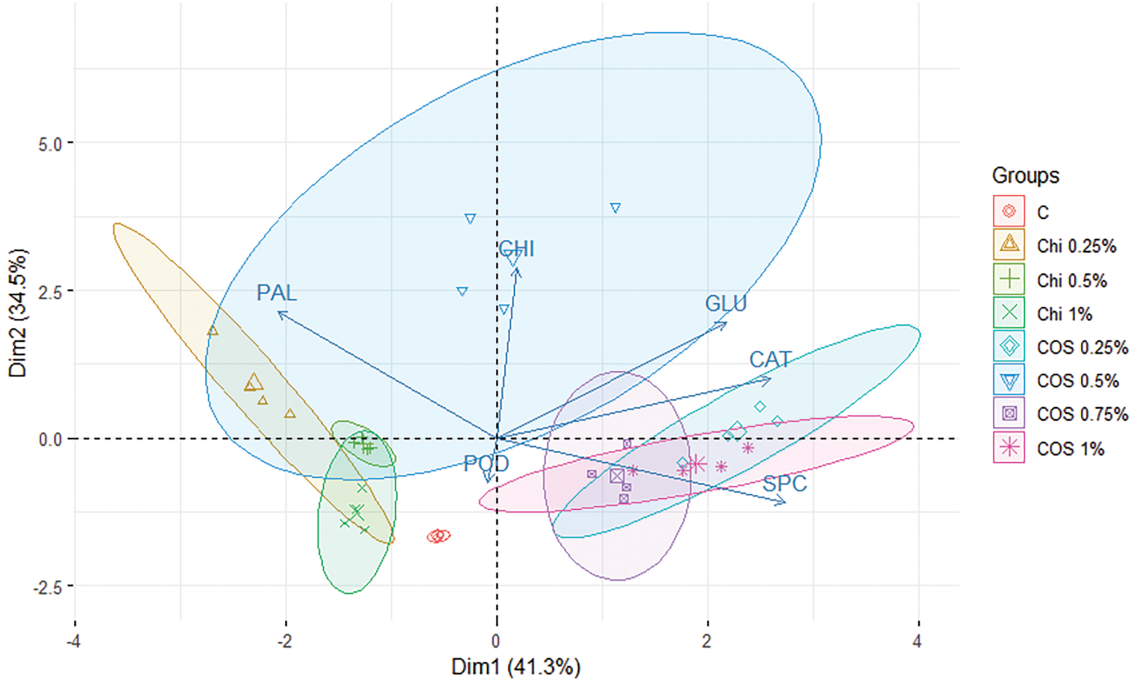

Figure 3: Principal Component Analysis (PCA) for enzymatic responses of *Coffea arabica* var. Borbon by exposure to chitosan (Chi) and chitosan oligosaccharides (COS) at different concentrations, compared to control C plants treated with distilled water

3.3 Chlorophyll Fluorescence

Chlorophyll fluorescence (Chl fluorescence) is a non-invasive technique used to measure the activity of PSII. It measures the fraction of absorbed light energy that is not utilized in photochemical reactions and is instead re-emitted as fluorescence. When a plant is exposed to photosynthetically active radiation (PAR), approximately 97% of this energy is utilized in photochemical reactions, while the remainder is released as fluorescence and heat [59,60]. Photochemical potential yield (Fv/Fm) values (Fig. 4) demonstrate slight differences between treatments at 24, 48, and 72 h after exposure. Twenty-four hours post-exposure, the Fv/Fm values indicate a minor decrease in COS 0.5%, but no treatment exhibited a significant difference ($p < 0.05$) from the control treatment. At 48 and 72 h after exposure, neither treatment displayed a significant difference ($p < 0.05$).

Fig. 4 suggests that COS and chitosan (Chi) exert a transient effect on the photochemical efficiency of PSII within the first 24 h following foliar application. A slight increase in Fv/Fm of PSII in the 0.75% and 1% treatments. This can be related to the increase in photopigments (chlorophyll a, b, and carotenoids) observed in the *Coffea canephora* var. Robusta assay with the foliar application of chitosan with 80% DD and 750,000 Da, along with COS with 2000 Da and DP 8–16 [61]. The increase in Fv/Fm may also be connected to photopigment concentrations and the impact of nutrient uptake from the treatments on the plants. This was suggested in an assay conducted on *Coffea canephora* Pierre Var. Robusta treated with chitosan nanoparticles (600 kDa, 85% DD) and chitosan oligomers, which could be linked to the property of chitosan derivatives to enhance magnesium and nitrogen levels in the leaves [23].

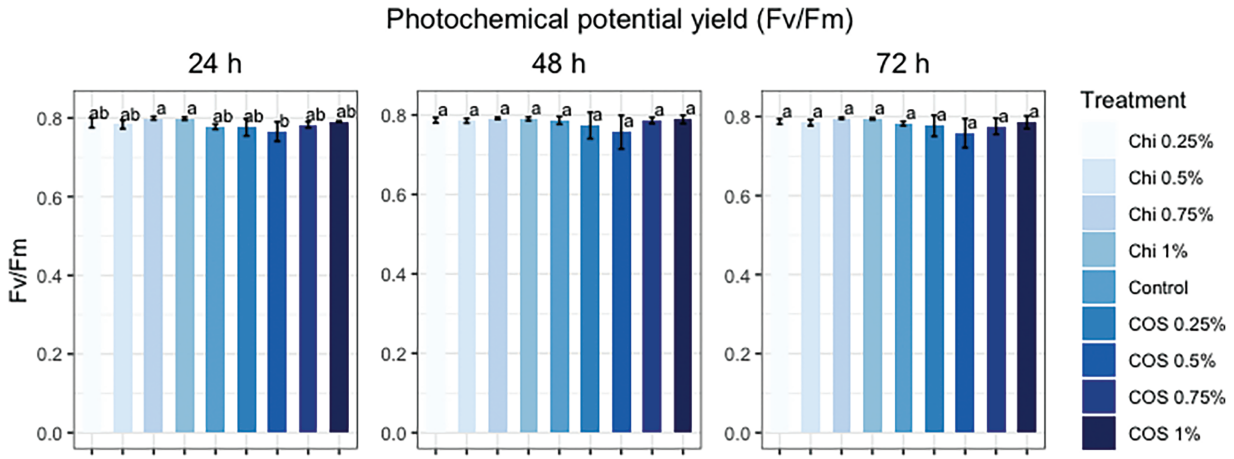

Figure 4: Photochemical potential yield (Fv/Fm) in *Coffea arabica* var. Borbon exposed to foliar applications of chitosan (Chi) and chitosan oligosaccharides (COS) at various concentrations compared to control plants treated with distilled water

4 Conclusions

The conducted study suggests the application of exogenous chitosan oligosaccharides (COS) is safe for *Coffea arabica* var. Borbon plants. Foliar application of COS with an average molecular weight of 3549.90 ± 0.33 and a Deacetylation Degree (DD%) of 76.64 ± 1.12 demonstrates a higher availability of amide I and III in FTIR spectrograms compared to chitosan samples. Thus, this can be associated with the increased specific enzymatic activity (β-1,3-glucanases, chitinases, PAL, and CAT) observed in coffee plants treated with COS. The COS treatment that yielded better responses was COS 0.5 wt%. However, coffee plants treated with chitosan exhibited a higher potential photochemical yield 24 h after application in comparison to both the control and COS treatments. Further research are needed to clarify the effect of foliar application of COS in coffee plants and its potential to mitigate the development of certain diseases like Coffee Leaf Rust (CLR).

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Abbreviations

CAT	Catalase
CERK1	Chitin Elicitor Receptor Kinase 1

CHI	Chitosan
Chl	Chlorophyll
CLR	Coffee Leaf Rust
COS	Chitosan oligosaccharides
CytPM-COS	Cytosinpeptidemycin and COS
DA	Degree of Acetylation
DAMPS	Damage Associated Molecular Patterns
DD	Deacetylation Degree
Fv/Fm	Potential photochemical yield
GlcN	D-glucosamine
GlcNAc	N-acetyl-D-glucosamine
HR	Hypersensitive Response
ISR	Induced Systemic Resistance
M_W	Molecular Weight average
PAL	Phenylalanine Ammonium Lyase
PD	Polymerization Degree
POX	Peroxidases
PPO	Polyphenol oxidase
PR	Pathogenesis Related proteins
PRRS	Pattern Recognition Receptors
PSII	Photosystem II
PTI	PAMPs or DAMPs triggered immunity
ROS	Reactive Oxygen Species
SAR	Systemic Acquired Resistance
SOD	Superoxide Dismutase
SPC	Soluble Protein Content

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