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Hyperbaric Pressures Enhance Antioxidant Enzyme Activity and Suppress Grey Mould in Débora Tomato Fruits

Vanessa Voigt Palaretti[1] | Carlos Inestroza-Lizardo[2] | José Sidnaldo Pinzetta Junior[1] | Ben-Hur Mattiuzzo[3]

[1]Department of Technology, College of Agriculture and Veterinary Sciences, São Paulo State University, UNESP, Jaboticabal, Brazil | [2]College of Technological Sciences, National University of Agriculture, Catacamas, Honduras | [3]Institute of Biosciences, São Paulo State University, UNESP, Rio Claro, Brazil

Correspondence: Ben-Hur Mattiuzzo (b.mattiuzzo@unesp.br)

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ABSTRACT

Hyperbaric pressures have been shown to be an efficient technique to inhibit physiological mechanisms of ripening, microbiological development and senescence in vegetables, prolonging their shelf life. However, scarce information is available regarding the effect of hyperbaric pressures on the control of postharvest diseases. This study aimed to evaluate the effect of hyperbaric pressures of 100 (control), 200, 400, 600 and 800 kPa on growth in vitro and in vivo of grey mould (*Botrytis cinerea*), as well as the effects on the synthesis of bioactive compounds and defence- and antioxidant-related enzymatic activity of cv. Débora tomatoes. We evaluated mycelial growth and germination of conidia in vitro, the incidence and severity of grey mould in vivo, lycopene content, total phenolic compounds, and the activity of defence and antioxidant enzymes. The higher hyperbaric pressures inhibited mycelial growth and conidial germination. Tomatoes stored at 800 kPa for 3 days presented a lower incidence and severity of grey mould than the control. In addition, tomatoes showed a reduction in lycopene synthesis, indicating delayed fruit ripening. They also showed a higher content of phenolic compounds and greater activity of defence enzymes phenylalanine ammonia-lyase (PAL), β-1,3-glucanase (GLU) and catalase (CAT). This study demonstrated that hyperbaric pressures at 600 and 800 kPa at room temperature for 3 days retarded grey mould growth in Débora tomatoes and showed the potential of hyperbaric pressures in the control and induction of disease resistance in postharvest conservation.

1 | Introduction

Tomato (*Solanum lycopersicum*) is one of the most widely cultivated, consumed and industrialised vegetables in the world (Azabou et al. 2016). Fresh and processed tomatoes are extremely beneficial to human health, preventing cardiovascular diseases, different types of cancer and neurodegenerative disorders (Figueira et al. 2017; Thies et al. 2017). The protective effect of tomatoes is attributed to the presence of bioactive compounds with antioxidant properties, such as vitamins A and C, flavonoids, carotenoids (lycopene, β-carotene and lutein), and phenolic acids (Del Giudice et al. 2015; Niranjana et al. 2015).

Because tomatoes are climacteric fruits, they are subject to short postharvest conservation periods due to their high physiological activity and susceptibility to physical damage and injury caused by pathogens during transport and storage (Arah et al. 2015; Ochida et al. 2018).

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Among the pathogenic fungi, *Botrytis cinerea*—known as grey mould—stands out as the cause one of the most severe postharvest diseases of tomatoes, resulting in significant economic losses worldwide (Ahmed et al. 2016; Dean et al. 2012). This fungus is considered universal because it is widely distributed and pathogenic to several species, and can cause disease at any developmental stage of the tomato plant (Carisse and van der Heyden 2015). Although the application of chemical fungicides is the most common method to control grey mould in preharvest, their use in postharvest is not allowed in many countries (Mari et al. 2014). Also, the increase in fungal resistance and the growing concern of consumers about the harmful effects on health, environment and presence of fungicide residues in fruits has driven the pursuit of alternative postharvest disease control (Abbey et al. 2019; Romanazzi et al. 2016).

Simone et al. (2020) have explored physical, chemical and biological control treatments to combat the development of *B. cinerea* in postharvest scenarios. One of the alternatives to chemical postharvest protection is through the induction of the fruit's natural resistance or using environmentally safe techniques. Abiotic stresses may activate natural resistance in plants by induction of the biosynthesis of bioactive compounds and resistance pathways (Cisneros-Zevallos and Jacobo-Velázquez 2020).

Among abiotic stresses, the use of hyperbaric pressures has been shown to be a promising technique for the short-term storage of several plant products, in which plants are exposed to pressures of 100 to 1000 kPa using constant compressed airflow, acting instantly and uniformly (Goyette et al. 2011; Vigneault et al. 2012). Thus, by acting as an abiotic stressor, hyperbaric pressure may induce resistance to pathogens in an ecofriendly and chemical-free way, meeting the demands of the current consumer market.

Hyperbaric pressures have already been demonstrated to have beneficial effects on tomatoes by slowing fruit ripening, maintaining firmness and reducing weight loss, respiratory rate and ethylene production (Goyette, Vigneault, Charles, et al. 2012; Goyette, Vigneault, Raghavan, et al. 2012; Inestroza-Lizardo et al. 2021; Liplap 2013). Also, the flavour-influencing characteristics remained stable, such as total soluble solids and acidity, improving postharvest quality and extending the shelf life of tomatoes (Inestroza-Lizardo et al. 2018).

Tomatoes previously exposed to hyperbaric pressure had an increase in content of bioactive compounds by up to 28%, especially in lycopene, after being stored for 12 days at 20°C (Goyette 2010). Recent research by Inestroza-Lizardo et al. (2019, 2021) suggests that the increase in postharvest conservation of tomatoes may be caused by hyperbaric stress activation of antioxidant enzymes. The authors verified an increase in catalase activity (CAT) and a reduction in peroxidase (POD), acting on cell protection and fruit senescence delay. Similarly, in broccoli, hyperbaric treatments have also been shown to increase CAT enzymatic activity and reduce oxidative stress, thereby delaying senescence (Pereira et al. 2021).

In addition to postharvest conservation of fruits and vegetables, hyperbaric pressure has been reported to reduce the incidence and severity of fungal and bacterial diseases. Romanazzi et al. (2008) showed that a hyperbaric pressure of 150 kPa at 20°C for 4 h reduced the natural incidence of brown rot (*Monilinia laxa*), grey mould (*B. cinerea*) and blue mould (*Penicillium expansum*) in sweet cherries (*Prunus avium*), as well as total rot, including rot by *Rhizopus stolonifer, Alternaria alternata* and *Cladosporium* spp. The authors also subjected table grape berries to a pressure of 150 kPa for 24 h and subsequently inoculated them with *B. cinerea*, obtaining a reduction in the percentage of infected fruit and disease severity. Liplap et al. (2014) found that hyperbaric pressure treatment had a direct impact on the behaviour of rot-causing bacteria in fruits and vegetables. The treatment with 850 kPa pressure reduced bacterial growth by 71% in *Pseudomonas cichorii*, 56% in *Pectobacterium carotovorum* and 43% in *Pseudomonas marginalis*.

Studies have suggested that hyperbaric pressures may affect the growth of pathogens by the direct impact of elevated pressure (Segovia-Bravo et al. 2012) and/or by the increase in synthesis of compounds related to fruit defence induced by slight stress, as observed during heat and UV light treatments (Romanazzi et al. 2016; Usall et al. 2016). However, research related to these hypotheses is limited and the possible mechanisms linked to pathogenic microorganism inactivation are unknown.

This study aimed to evaluate the effect of hyperbaric pressure on the control of grey mould (*B. cinerea*) in vitro and in inoculated tomatoes of cultivar Débora, as well as its effects on the synthesis of bioactive compounds and enzymes related to defence and antioxidant activity in fruit.

2 | Materials and Methods

2.1 | Plant Material

Tomatoes of cultivar Débora were harvested at the green-mature stage, according to López-Camelo and Gómez (2004). The fruits were obtained from a commercial producer in the region of Ribeirão Preto, State of São Paulo, Brazil (21°20′18″ S 47°43′58″ W, 793 m a.s.l.). The cultivation was carried out in open fields during the autumn–winter season with a minimum temperature of 13.3°C ± 2.0°C and a maximum of 28.3°C ± 2.7°C. The fruits were transported to the laboratory and washed with potable water and neutral detergent. They were sanitised in sodium dichloroisocyanurate (Sumaveg) at 200 mg/L for 5 min and dried at room temperature. To ensure homogeneity, they were selected according to their size, shape, colouration and absence of injuries and diseases.

2.2 | Pathogen

Botrytis cinerea isolate MMBF 47/13 from tomato was obtained from the fungal collection of the Biological Institute of São Paulo (IB). The fungus was grown in potato dextrose agar (PDA; Difco) and incubated in a biological oxygen demand incubator at 20°C ± 1°C with a 12 h photoperiod.

A conidial suspension was obtained from a 10-day-old *B. cinerea* culture by scraping the surface of the mycelium in 10 mL of sterile distilled water containing 0.01% (vol/vol) of Tween 80,

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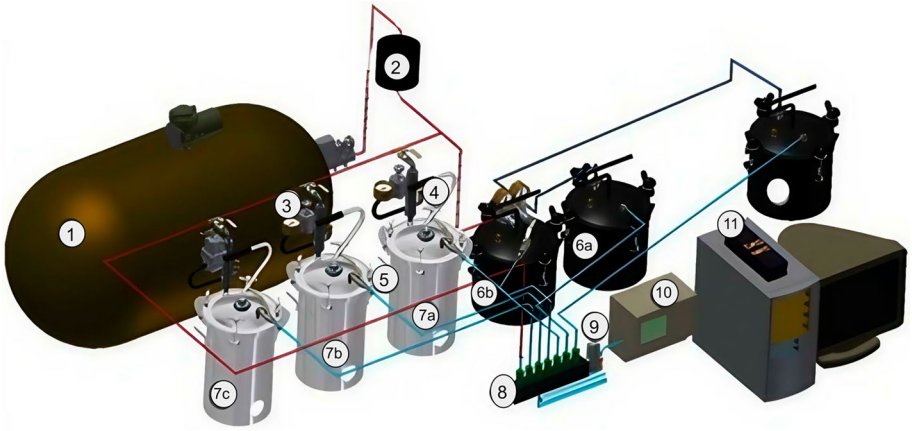


FIGURE 1 | Schematic of the hyperbaric pressure system used in the experiments: 1. Compressed air tank; 2. CO_2 scrubber; 3. Pressure regulator; 4. Safety valve; 5. Flow control valve; 6a. Chamber for ambient pressure (100 kPa); 6b. Pressure chamber of 200 kPa; 7a, b, c. Pressure chamber of 400, 600, and 800 kPa, respectively; 8. Valve-manifold assembly; 9. Airflow meter; 10. Infrared gas analyser; 11. Data acquisition board and computer. [Colour figure can be viewed at wileyonlinelibrary.com]

followed by gauze filtration. The conidial count was performed in a Neubauer chamber and the concentration was adjusted to 2×10^5 conidia/mL.

2.3 | Hyperbaric System

Experiments in vitro and in vivo were conducted using hyperbaric pressure equipment as described by Inestroza-Lizardo et al. (2018) and represented in Figure 1. This equipment consists of six stainless steel chambers, with 10.75 L of internal volume, connected to a continuous flow system of compressed air, which maintains constant concentrations of O_2 (21%) and N_2 (78%). CO_2 was adsorbed by the previous passage of the flow through an appropriate chamber containing calcium oxide. Each chamber was sealed with a screwed steel cap, and at the inlet and outlet of each chamber, a pressure regulator and an airflow control valve were installed, respectively, as well as a safety valve to prevent excessive pressure. The chamber inlet was connected to a compressed air tank with a capacity of 250 L (Schulz, mod. MSV 20 MAX), for air supply. A flow meter was used to measure airflow (Bronkhorst) with an output range of 5 to 2000 mL/min. The CO_2 concentration was measured through an infrared gas analyser (Guardian Plus) with a measurement range of 0 to 2000 ppm.

The airflow meter, control valve and CO_2 gas analyser were connected to a control and data acquisition board (Personnel DAQ 3000) of a laptop computer.

2.4 | Hyperbaric Treatment of *B. cinerea* In Vitro

The effect of hyperbaric pressure on the mycelial growth of *B. cinerea* mycelium was assessed using discs of 5 mm diameter taken from the edge of *B. cinerea* colonies after 10 days of culture and transferred to the centre of Petri dishes containing PDA. Fifteen plates containing the fungus were placed in a sealed vessel and subjected to one of the following hyperbaric pressure (*hp*) treatments: 100 (control), 200, 400, 600 and 800 kPa. The treatment periods were 3 and 6 days, which are referred to as 3 *hp* and 6 *hp*, respectively.

The internal conditions of the chambers were 23°C ± 1°C and 95% ± 2.5% RH. At the end of each period, the chambers were depressurised automatically for 2 h, and the mycelial growth of the plates was determined. Each treatment, at each time, was repeated three times.

After hyperbaric treatments, the plates were stored under ambient conditions (23°C ± 1°C, 50% ± 2% RH, and 12 h photoperiod) for 7 days (3 *hp* + 7d) or for 4 days (6 *hp* + 4d) to assess the fungal growth after treatments. At the end of the storage periods (10 days after inoculation), mycelial growth and conidial germination were evaluated.

2.5 | Hyperbaric Treatments in Tomatoes Inoculated With *B. cinerea* (In Vivo)

For fungal inoculation, a perforation of 1 mm diameter and 2 mm depth was made in the equatorial region of tomato fruits. Using a chromatography syringe, tomatoes were inoculated with 10 μL of a conidial suspension (2×10^5 conidia/mL). The fruits were kept at 23°C ± 1°C for 24 h to establish the pathogen.

The same procedure described in section 2.4 was used to apply the treatments, placing 25 inoculated tomatoes in each chamber. The hyperbaric treatments applied were 100 (control), 200, 400, 600 and 800 kPa, under the same conditions of temperature and relative humidity as the experiment in vitro. The treatment periods were 3 and 6 days, and at the end of each treatment period, the chambers were depressurised according to section 2.4.

Five tomatoes (experimental unit) of each treatment were evaluated immediately, and the others were stored at ambient conditions (23°C ± 1°C, 50% RH, 100 kPa) for another 4 days to assess the growth of the fungus in the fruit. Each treatment, at each time, was repeated three times.

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In each evaluation period (treatment and storage), five fruits were randomly selected, the seeds removed, and 50 mm of healthy tissue around the injured area was cut, macerated in liquid nitrogen and stored at −80°C for further evaluation of enzymatic activity.

2.6 | Evaluation of Mycelial Growth and Spore Germination In Vitro

Mycelial growth was determined by the mean of two perpendicular diameters (mm) of the colonies, with the aid of a digital calliper (Mitutoyo, model CD-6).

Conidial germination was evaluated at the end of the incubation period ($3\,hp + 7$d and $6\,hp + 4$d) by depositing 120 μL of the conidial suspension (2×10^5 conidia/mL) on microscope slides containing 1% water agar (Kasvi). After 24 h of incubation at 23°C ± 1°C, 200 conidia were observed per microscope slide of each treatment, under an optical microscope (CX41; Olympus). A conidium with a germ tube equal to or longer than the spore length was considered germinated (Mercier et al. 2001). Three microscope slides were prepared for each treatment.

2.7 | Evaluation of the Incidence and Severity of Grey Mould in Tomatoes

The incidence and severity of grey mould were evaluated in fruit presenting an injured area greater than 10 mm wide, both after treatment and storage periods. The incidence of fruit with symptoms of grey mould was determined visually and expressed as a percentage of infected fruits. The severity was determined as the mean lesion diameter (mm) of the grey mould in the fruit, measured in two orthogonal directions of the fruit, with the aid of a digital calliper (model CD-6; Mitutoyo).

2.8 | Physicochemical and Enzymatic Analyses

Extracts were taken at the end of treatments and stored at −80°C as described in section 2.5. These extracts were used for the following analyses.

2.8.1 | Lycopene Content

The lycopene content was determined following Nagata and Yamashita (1992). The absorbance of samples was read at wavelengths of 663 nm (chlorophyll *a*), 645 nm (chlorophyll *b*), 505 nm (lycopene) and 453 nm (β-carotene). Equation (1) quantifies the lycopene content.

$$\text{Lycopene} = -0.0458\,A_{663} + 0.204\,A_{645} + 0.372\,A_{505} - 0.0806\,A_{453} \quad (1)$$

in which A_{XXX} = absorbance at specific wavelength. The results were expressed in mg/100 g of fresh weight (FW).

2.8.2 | Total Phenolic Content

Total phenolic content was determined using the Folin–Ciocalteu reagent, according to Singleton and Rossi (1965). The absorbance reading was performed at 725 nm, and the results were expressed in mg of gallic acid equivalent per 100 g of fresh weight (mg gallic acid equivalents [GAE]/100 g FW).

2.8.3 | Peroxidase Activity (EC 1.11.1.7)

Peroxidase (POD) activity was determined according to Lima et al. (1999). The reaction consisted of 1 mL of enzymatic extract with 0.5 mL of 30% hydrogen peroxide in 0.2 M potassium phosphate buffer (pH 6.7) and 0.5 mL of phenol and aminoantipyrine solution. The reaction mixtures were placed in a water bath at 30°C for 5 min. Two millilitres of ethanol was added to stop the reactions, and readings were taken on a spectrophotometer. The absorbance was read at 505 nm, and specific activity was expressed in μmol of H_2O_2 decomposed/min/g protein.

2.8.4 | Phenylalanine Ammonia-Lyase Activity (EC 4.3.1.5)

Phenylalanine ammonia-lyase (PAL) activity was determined according to Peixoto et al. (1999). The extract was obtained by mixing 1 g of the sample with 10 mL of 0.1 M borate buffer solution and centrifuging for 20 min at 4°C. Subsequently, 1 mL of extract (supernatant) was incubated with 2 mL of 0.2 M borate buffer at 36°C for 5 min before adding 1 mL of phenylalanine and incubating for a further 1 h at 36°C. After this period, 0.1 mL of 6 M hydrochloric acid was added to stop enzymatic activity. Absorbance readings were performed in a UV–visible spectrophotometer (700 plus; Femto) at 290 nm, and the results were expressed in nmol/min/mg of protein.

2.8.5 | β-1,3 Glucanase Activity (EC 3.2.1.39)

β-1,3-Glucanase (GLU) activity was determined according to Lever (1972). The quantification was determined by colourimetric measurement of glucose released by laminarin, using *p*-hydroxybenzoic acid hydrazide (PAHBAH). Samples were dissolved in 600 μL of 100 mM potassium phosphate buffer (pH 7.5), and the enzymatic extract was obtained by centrifugation at 20,000 *g* for 25 min at 4°C. The reaction mixture, containing 150 μL of the enzymatic extract and 150 μL of laminarin (0.2%), was incubated at 37°C for 1 h. Subsequently, 1.5 mL of PAHBAH solution (0.5 g dissolved in 20 mL of 0.5 M HCl, added to 80 mL of 0.5 M NaOH) was added, and the mixture was heated to 100°C for 10 min. After cooling to 25°C, readings were performed at 410 nm, and results were expressed in glucose mg/μg protein.

2.8.6 | Catalase Activity (EC 1.11.1.6)

Catalase (CAT) activity was determined according to Kar and Mishra (1976). The reaction used 150 μL of the sample extracted

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in potassium phosphate buffer + ethylenediaminetetraacetic acid (EDTA) + dithiothreitol (DTT) + polyvinylpolypyrrolidone (PVPP) 100 mM (pH 7.5). An aliquot of 1950 μL of 100 mM potassium phosphate buffer (pH 7.5) was used as the determination buffer, 150 μL of the sample in extraction buffer was the enzymatic extract, and 750 μL of 50 mM hydrogen peroxide solution was the enzymatic substrate. The readings were performed at 240 nm, and results were expressed in μmol H_2O_2 consumed/min/g protein.

2.9 | Experimental Design and Statistical Analysis

Randomised block design (RBD) was used with five treatments and three replicates, where each pressure condition was considered a treatment, and each harvest was a block. ANOVA was performed on the results, and the means were compared at 5% probability using Tukey's test.

3 | Results

3.1 | Mycelial Growth and Conidial Germination

The effect of hyperbaric pressure on the mycelial growth of *B. cinerea* mycelium was assessed in vitro using mycelial discs exposed to varying pressures.

The lower pressures (control and 200 kPa) presented the highest mean growth, followed by the other pressures that provided a gradual reduction of mycelial growth with increase in applied pressure, both at 3 *hp* and 6 *hp*. The pressure of 800 kPa caused the least mycelial growth of *B. cinerea* in both periods of treatment under hyperbaric pressure, with an average reduction of 44% (3 *hp*) and 35% (6 *hp*) compared to the control.

After ambient storage, the maximum mycelial growth for the shortest treatment period (3 *hp* + 7d) occurred in the control treatment and at 200 kPa (Figures 2B and 3). With increasing pressure treatment, the mycelial growth after ambient storage was reduced, especially at 800 kPa pressure, which was 42% lower than control. For the longer treatment period (6 *hp* + 4d), mycelial growth was the highest in treatments at 200, 400 and 600 kPa (Figures 2B and 3).

The conidial germination rate of *B. cinerea* followed a similar trend to mycelial growth, being inversely proportional to the pressures applied, both after 3 *hp* + 7d and 6 *hp* + 4d (Figure 2C). The lowest germination rate of conidia was 54%, at 800 kPa pressure after 3 *hp*, compared to 100% in the control treatment.

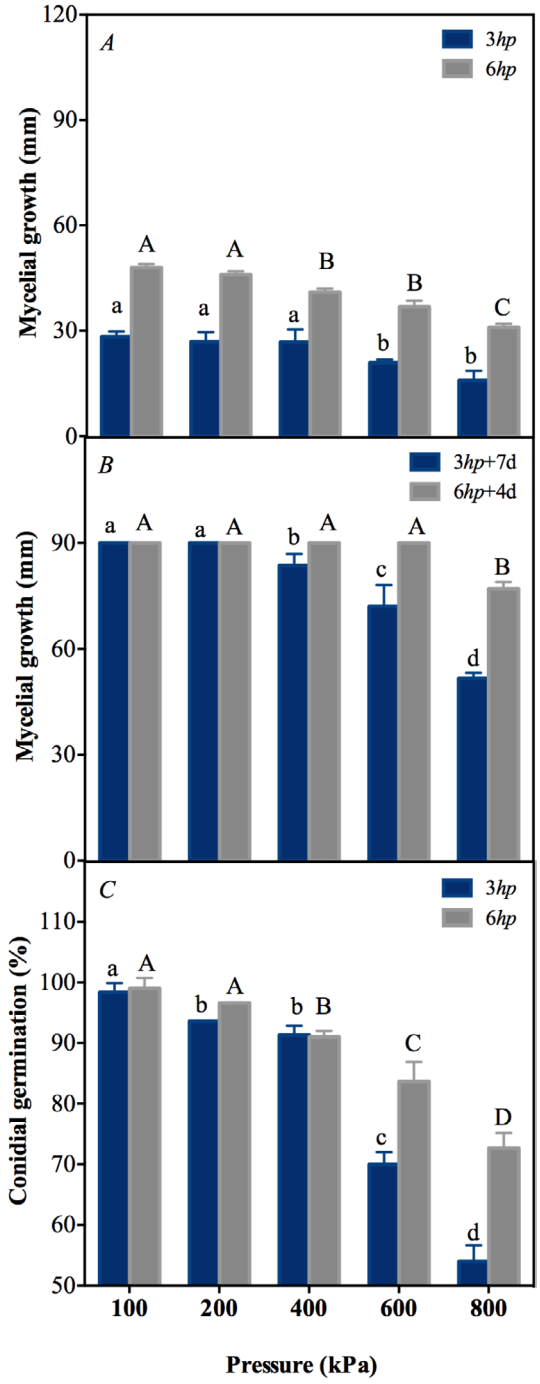


FIGURE 2 | Mycelial growth (A, B) and conidial germination (C) of *Botrytis cinerea* treated with hyperbaric pressures (*hp*) for 3 or 6 days at 23°C and 95% RH, without (A, C) or with (B) 7 or 4 days, respectively, at ambient conditions (23°C, 50% RH and 100 kPa). Means followed by different letters indicate significant differences between pressures within the treatment period by Tukey test ($p < 0.05$). Bars indicate standard error ($n = 3$). [Colour figure can be viewed at wileyonlinelibrary.com]

3.2 | Disease Incidence and Disease Severity

The effect of hyperbaric pressure on disease incidence and severity was assessed using tomatoes inoculated with *B. cinerea* and exposed to different pressures.

Pressures of 600 and 800 kPa were the most efficient in reducing grey mould incidence after 3 or 6 days of treatment. In particular, the pressure of 800 kPa for a 3-day period completely inhibited fungal development. In addition, for the *3 hp* treatment, a progressive reduction in disease incidence occurred as the applied pressure increased. However, with 6 *hp*, only the highest pressures (600 and 800 kPa) were effective (Figure 4A).

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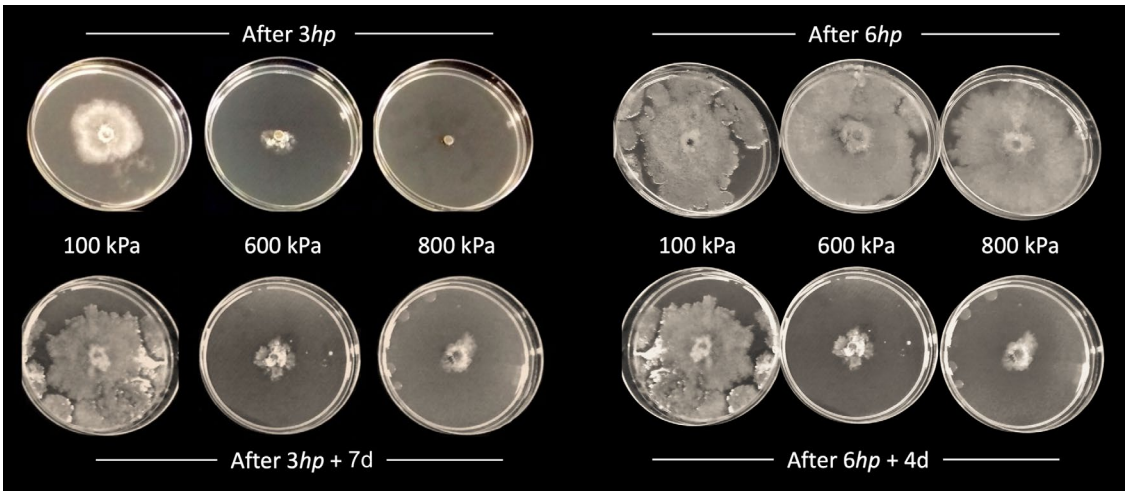


FIGURE 3 | Mycelial growth of *Botrytis cinerea* treated with hyperbaric pressures (*hp*) for 3 or 6 days at 23°C and 95% RH, followed by 7 days (3 *hp* + 7d) or for 4 days (6 *hp* + 4d) at ambient conditions at 23°C, 50% RH and 100 kPa. [Colour figure can be viewed at wileyonlinelibrary.com]

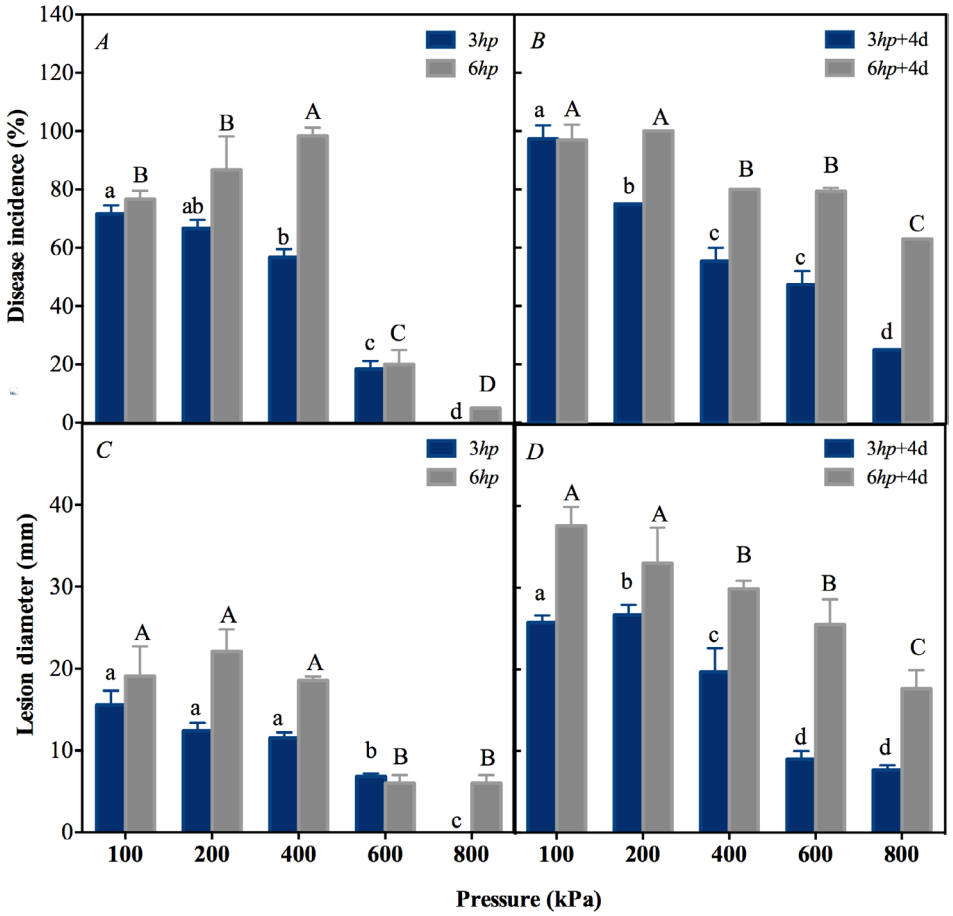


FIGURE 4 | Disease incidence (A, B) and disease severity (lesion diameter) (C, D) of tomatoes treated with hyperbaric pressures (*hp*) for 3 or 6 days at 23°C and 95% RH, without (A, C) or with (B, D) a following 4 days at ambient conditions of 23°C, 50% RH and 100 kPa. Means followed by different letters indicate significant differences between pressures within the treatment period by Tukey test ($p < 0.05$). Bars indicate standard error ($n = 3$). [Colour figure can be viewed at wileyonlinelibrary.com]

Symptoms increased in all treatments after 4 days in ambient storage, especially in the 6 *hp* + 4d treatments (Figure 4B). The treatment at 800 kPa for 3 *hp* + 4d presented disease incidence in only 25% of fruit. In contrast, disease incidence was 63% in tomatoes with 6 *hp* + 4d.

Similar to the findings for disease incidence, tomatoes subjected to pressures at 600 and 800 kPa showed decreased disease severity ($p < 0.05$) after both periods of *hp* (Figures 4C, 5). In particular, after 3 *hp*, disease severity decreased ($p < 0.05$) to 0 in tomatoes treated at 800 kPa, with no fungal development (Figures 4C and 5).

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After 4 days at ambient conditions, tomatoes previously treated with 600 and 800 kPa for 3 days showed a reduction ($p < 0.05$) in grey mould severity compared to other pressures, with average lesion diameters of 8.4 mm, which was an average 68% lower than the control (26 mm) (Figure 4D). For fruit treated for 6 days of *hp* (+4d ambient), the 800 kPa pressure treatment gave the most efficient results, reducing the severity of grey mould by 52% (18 mm diameter) compared to control (38 mm diameter).

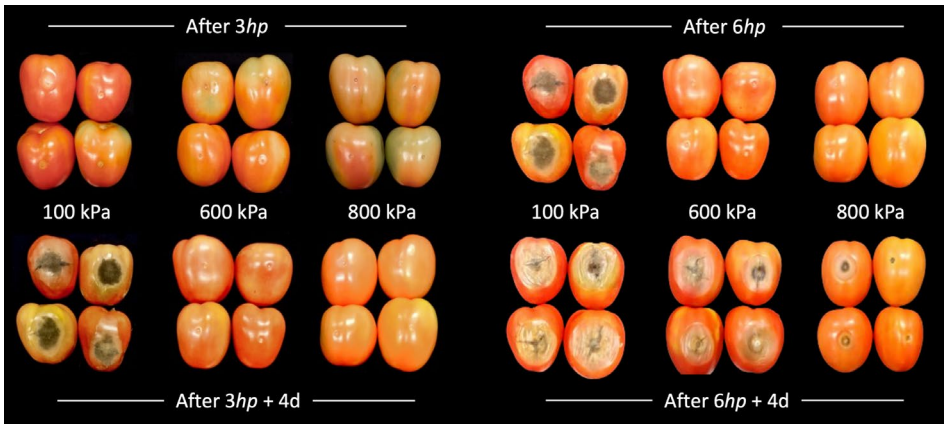


FIGURE 5 | Disease severity of tomatoes inoculated with *Botrytis cinerea* and treated with hyperbaric pressures (*hp*) for 3 or 6 days at 23°C and 95% RH, followed by 4 days at ambient conditions at 23°C, 50% RH and 100 kPa. [Colour figure can be viewed at wileyonlinelibrary.com]

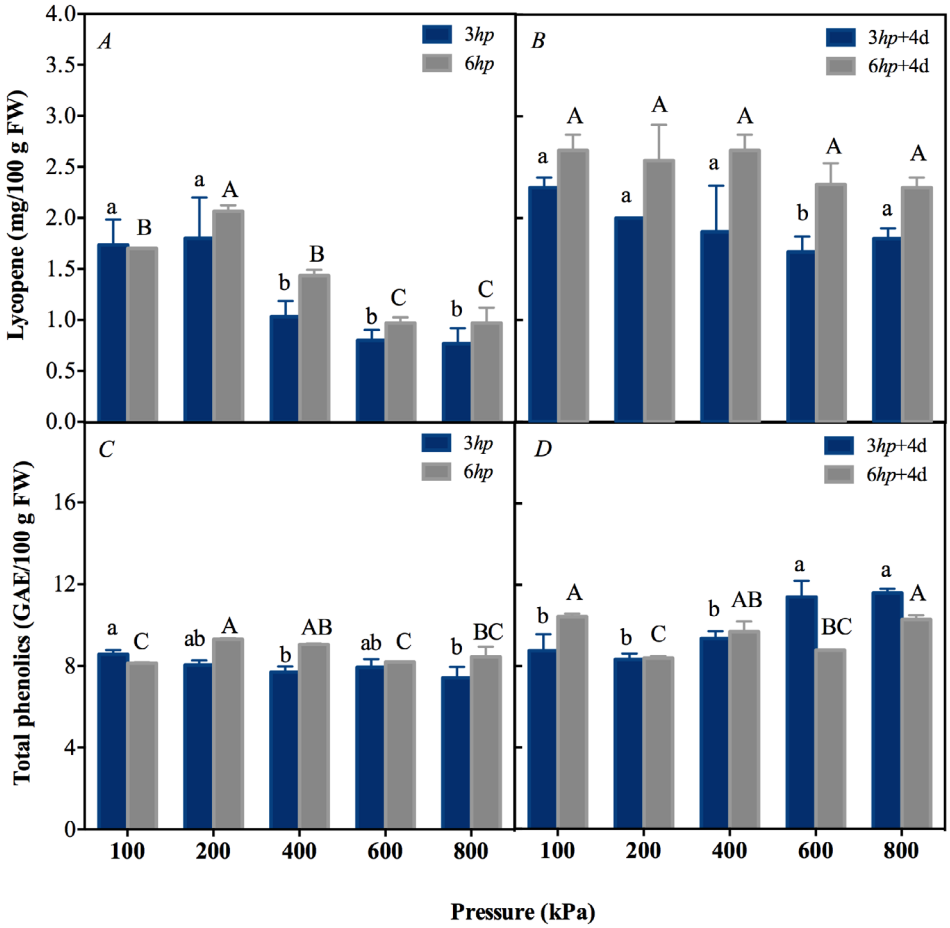


FIGURE 6 | Lycopene (A, B) and total phenolics content (C, D) of tomatoes treated with hyperbaric pressures (*hp*) for 3 or 6 days at 23°C and 95% RH, without (A, C) or with (B, D) a following 4 days at ambient conditions of 23°C, 50% RH and 100 kPa. Means followed by different letters indicate significant differences between pressures within the treatment period by Tukey test ($p < 0.05$). Bars indicate standard error ($n = 3$). FW, fresh weight. [Colour figure can be viewed at wileyonlinelibrary.com]

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3.3 | Lycopene and Total Phenolics Content

Lycopene and total phenolic content of inoculated fruit exposed to pressure treatment were measured. The pressures of 400, 600 and 800 kPa applied for 3 days resulted in lower lycopene content in the fruit, showing an average of 0.9 mg/100 g FW; a 47% reduction compared to the control (1.9 mg/100 g FW). The fruit treated for 6 days at 600 and 800 kPa presented the lowest lycopene levels, with an average of 0.8 mg/100 g FW (Figure 6A).

At the end of the 4-day storage period, the treatments showed no difference in lycopene content compared to the control, except for the fruit treated with 600 kPa pressure for 3 days, which had lower ($p < 0.05$) levels than the fruit exposed to other pressures (Figure 6B).

The total phenolic content remained stable in tomatoes treated for 6 days and the control (Figure 6C,D). However, the 3 *hp* + 4 days treatment increased total phenolic content by 32% compared to the control at 600 and 800 kPa (Figure 6D).

3.4 | POD and PAL Activities

POD activity was significantly higher in tomatoes treated at 600 kPa for 3 days and 800 kPa for 6 days, compared to the control, with an average increase of 28% and 31%, respectively (Figure 7A).

At the end of the period of 3 *hp* + 4d, tomatoes subjected to pressures of 100 (control), 200 and 400 kPa had 15% higher enzymatic activity ($p < 0.05$; average 2.0 nmol H_2O_2 decomposed/min/g of protein), relative to tomatoes treated with 600 and 800 kPa (average 1.7 nmol H_2O_2 decomposed/min/g of protein; Figure 7B). However, in the 6 *hp* + 4d treatment, exposure to hyperbaric pressures did not alter the POD activity.

The PAL activity of tomatoes increased at 600 and 800 kPa after 4 days in ambient conditions (Figure 7D), in both 3 *hp* + 4d and 6 *hp* + 4d treatments.

At the end of the storage period of 3 *hp* + 4d, the highest PAL activity was recorded in tomatoes subjected to 800 kPa, with an average of 0.24 μmol/min/mg of protein, representing a 74%

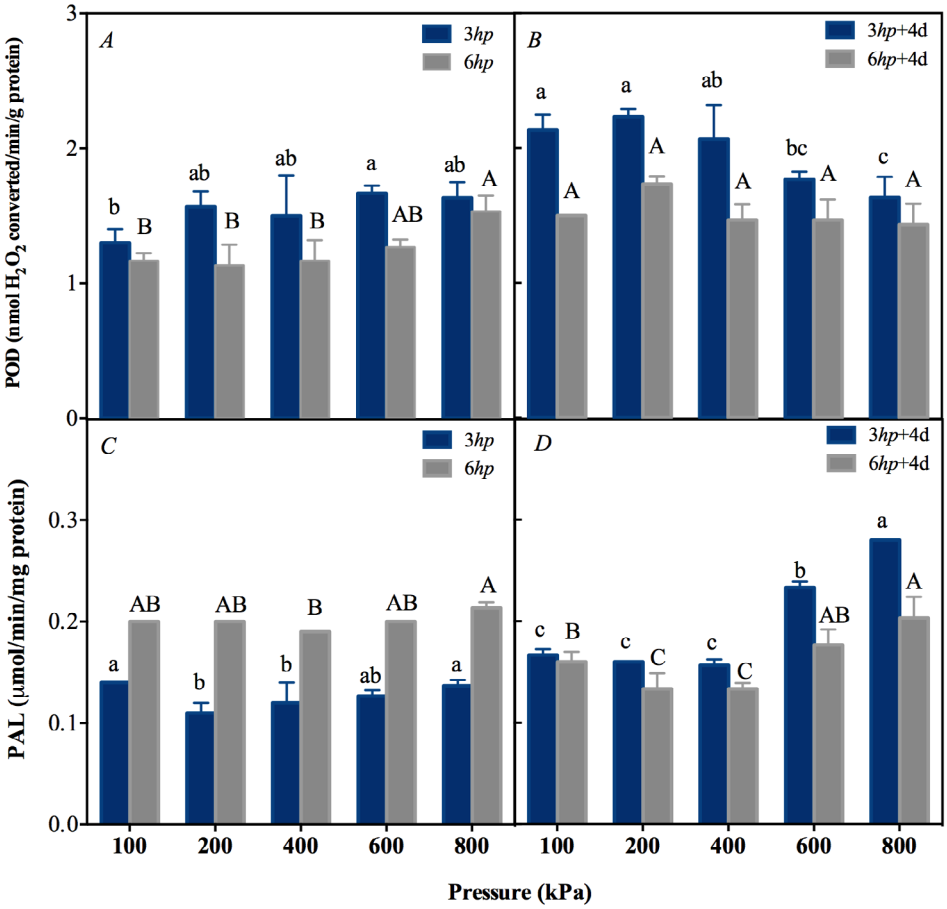


FIGURE 7 | Peroxidase (POD) activity (A, B) and Phenylalanine ammonia-lyase (PAL) activity (C, D) of tomatoes treated with hyperbaric pressures (*hp*) for 3 or 6 days at 23°C and 95% RH, without (A, C) or with (B, D) a following 4 days at ambient conditions of 23°C, 50% RH and 100 kPa. Means followed by different letters indicate significant differences between pressures within the treatment period by Tukey test ($p < 0.05$). Bars indicate standard error ($n = 3$). [Colour figure can be viewed at wileyonlinelibrary.com]

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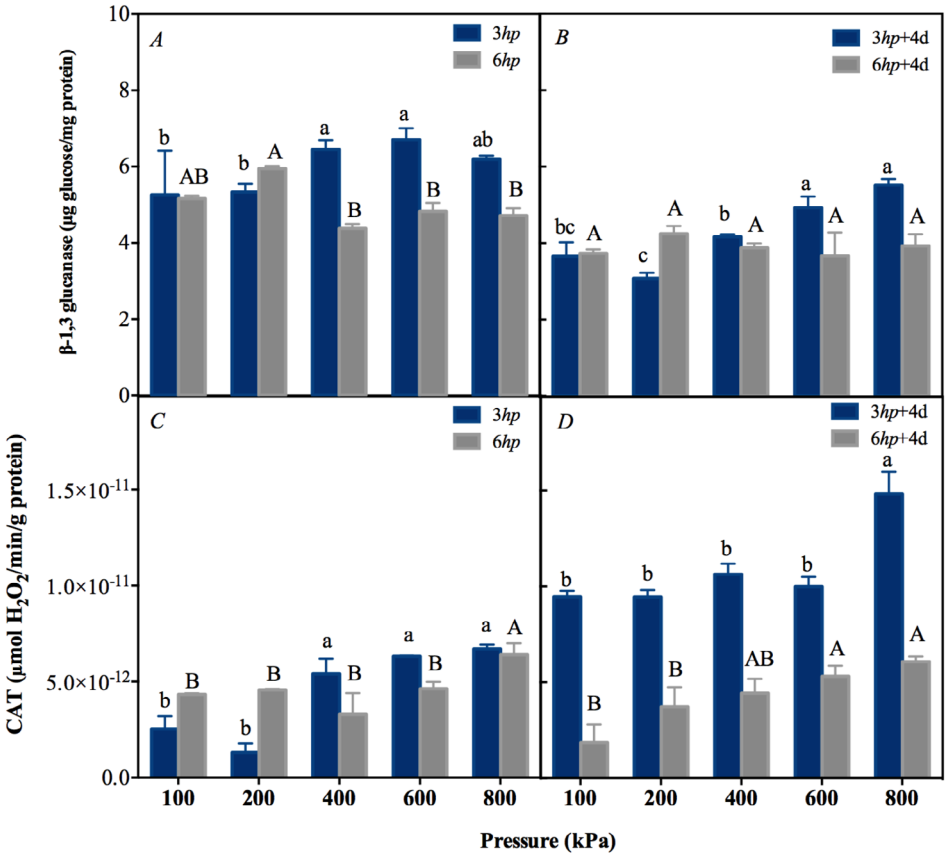


FIGURE 8 | β-1,3 glucanase (GLU) (A,B) and catalase (CAT) (C, D) activity of tomatoes treated with hyperbaric pressures (*hp*) for 3 or 6 days at 23°C and 95% RH, without (A, C) or with (B, D) a following 4 days at ambient conditions of 23°C, 50% RH and 100 kPa. Means followed by different letters indicate significant differences between pressures within the treatment period by Tukey test ($p < 0.05$). Bars indicate standard error ($n = 3$). [Colour figure can be viewed at wileyonlinelibrary.com]

increase compared to 100, 200 and 400 kPa treatments, with an average of 0.15 μmol/min/mg of protein (Figure 7D). The tomatoes treated at 800 kPa for 6 *hp* + 4d showed an average of 0.20 μmol/min/mg of protein, which represents a 26% increase over 100, 200 and 400 kPa (average of 161.3 nmol/min/mg of protein) (Figure 7D).

3.5 | GLU and CAT Activities

The application of pressures of 400 and 600 kPa for 3 days induced GLU activity by up to 27% compared to the 100 and 200 kPa pressures. An exposure for 6 days to 400, 600 and 800 kPa inhibited GLU enzyme activity by up to 26% compared to 100 and 200 kPa treatments, although the difference from the control 100 kPa was not statistically significant (Figure 8A).

In tomatoes stored at ambient for 4 days after pressure treatment, GLU activity was higher in fruit treated at 600 and 800 kPa for 3 *hp* + 4 days compared to the control; however, in tomatoes treated with pressure for 6 days, GLU activity remained at levels seen in the control after 4 days of storage (Figure 8B).

CAT activity in tomatoes exposed to 400, 600 and 800 kPa for 3 days increased up to three times compared to control (100 kPa). For the treatment period of 6 days, the increase in CAT activity at 800 kPa was 47% higher than the control, although treatment at other pressures did not lead to significantly different activity (Figure 8C). In tomatoes stored for 4 days at ambient after pressure treatment, those treated for 3 days at 800 kPa showed an increase in CAT activity of 56% compared to the control, and fruit exposed to 600 and 800 kPa demonstrated CAT activity of up to 4 times higher than the control (Figure 8D).

4 | Discussion

In this study, we obtained promising results for the use of hyperbaric pressures as an alternative method for postharvest control of *B. cinerea* in tomatoes.

Exposure to high pressures (600 and 800 kPa) for 3 days controlled mycelial growth and conidial germination of the pathogen in the experiments in vitro, as well as reducing disease incidence and severity in tomatoes. Previously, Liplap et al. (2014) found that a pressure of 850 kPa for 7 days significantly reduced the growth of bacteria *Pseudomonas cichorii*, *Pectobacterium carotovorum* and *Pseudomonas marginalis* by 71%, 56% and 43%, respectively. Similarly, hypobaric treatments of 3 to 6 kPa for 5 days inhibited the mycelial growth of *B. cinerea;* however, growth resumed after exposure to ambient conditions (100 kPa, 20°C) (Barkai-Golan 2001). Our research results are promising as they show that 800 kPa pressure for 3 days reduced the mycelial growth and conidial germination rate of *B.*

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cinerea. However, the pressure only had a fungistatic effect, as the fungus resumed mycelial growth after storage under ambient conditions. We observed that a pressure of 800 kPa affected the reproductive system of the fungus, because after treatment, almost half of the conidia produced by the mycelium did not germinate. Similar results were observed in a study conducted with the fungus *Metharrizhium anisoplie* where mycelium grown in culture medium with different enzymes led to sterile spores (Mustafa and Kaur 2009). Further research is needed to clarify how hyperbaric pressures affect rot-causing microorganisms during the postharvest process.

When investigating disease incidence, we found that pressures of 600 and 800 kPa were efficient in reducing grey mould incidence after 3 or 6 days of treatment, in some cases preventing any disease development. Our results contrast with those of Romanazzi et al. (2008) whose grape berries subjected to pressure of 150 kPa for a short period (24 h) and then inoculated with *B. cinerea*, showed only a 31% reduction in grey mould incidence. Nonetheless, this behaviour may be explained by the inoculation of fungi in the post-hyperbaric period.

Although the hyperbaric pressures used in this study did not show a fungicidal effect on tomato grey mould, promising results were found when simulating market conditions. This was especially evident for the 3 *hp* + 4 days treatment, in which we observed a significant reduction in disease incidence and disease severity of tomatoes treated at 800 kPa for 3 days.

During the ripening process, tomatoes change from green to red as chlorophyll is degraded; carotenoids (β-carotenes and lycopene) and anthocyanins (xanthophylls) accumulate (Smita et al. 2013; Su et al. 2015). Because lycopene is the major carotenoid in tomatoes, representing 71% of the total content in ripe fruits, 9- to 25-fold increments are reported during the ripening process, giving the fruits a red colour (Del Giudice et al. 2015).

According to Inestroza-Lizardo et al. (2018), hyperbaric treatment of 600 or 800 kPa for 4 days at 23°C, slowed the development of red colouration of tomatoes, evidenced by a 66% reduction in lycopene values compared to the control fruit (100 kPa). Similar results were obtained in this study at 600 and 800 kPa pressures; these treatments reduced the lycopene content of tomatoes up to 47% after 3 days and 6 days of hyperbaric pressure, even when kept at the optimal temperature for lycopene synthesis (16°C to 26°C).

Thus, our results show that pressures of 600 and 800 kPa slowed the tomato maturation, leading to more resistance to pathogens in the postharvest period. In contrast, tomatoes treated with the lowest hyperbaric pressures (100, 200, 400 kPa) ripened faster as evidenced by the increased lycopene accumulation in the fruit, creating a favourable environment for infection and the development of *B. cinerea*. This is proven by the higher incidence and severity of grey mould, both immediately after treatment (*hp*) and after subsequent storage at ambient pressure (*hp* + d).

Phenolic compounds can be synthesised by plants in response to stress generated by biotic (microorganisms and insects) and abiotic (light, temperature and ionising radiation) factors (Gonzalez-Aguilar et al. 2010). Such stresses activate PAL, the main enzyme involved in the first step of phenylpropanoid synthesis, resulting in compounds such as phytoalexins and lignins, which confer greater resistance of plant cell walls to pathogens (Gayoso et al. 2010). The main phenolic compounds of tomatoes are flavonoids and phenolic acids (Luthria et al. 2006). The accumulation of specific flavonoids, such as myricetin derivatives, can reduce the susceptibility of tomatoes to *B. cinerea* infections (Zhang et al. 2015). The study by Charles et al. (2008) showed that tomatoes treated with UV-C radiation (3.7 kJ m^{-2}) increased resistance to *B. cinerea*. This effect was attributed to the increase in phenolic compounds and the formation of lignin and suberin. This produced a fungistatic effect and a physical barrier that prevented fungal infection of fruits.

We investigated the activity of POD in tomatoes after treatment because an increased activity of POD is associated with activation of the defence system in response to a wide diversity of stressors (attacks of pathogens, insects and abiotic stresses); POD functions as an effective protector of reactive intermediate forms of radicals O_2 and peroxyl, in addition to contributing to the formation of suberin lignin, oxidation of phenolic compounds and signalling for the formation of phytoalexins (Nascimento and Barrigossi 2014).

In a study of strawberries, hypobaric treatments of 50 kPa for 4 h induced the activity of defence-related enzymes such as PAL and POD, promoting reduction of grey mould for 5 days at 20°C (Hashmi et al. 2013). Similarly, our results showed that hyperbaric treatments of 600 and 800 kPa induced greater POD enzyme activity, both after 3 and 6 days of *hp*. In contrast, POD only increased in control fruit (100 kPa) after the storage period of 4 days at ambient pressure (3 *hp* + 4d). This indicates that hyperbaric pressures of 600 and 800 kPa may have induced POD synthesis immediately in the 3 *hp* treatment, activating defence mechanisms that remained until the end of the storage period (3 *hp* + 4d). This was confirmed by the reduction by 68% of the lesion diameter of tomatoes exposed to such pressures compared to the control, indicating the preventive action of the POD to infection.

Although the greatest POD activity was recorded in the control fruit after a 4-day storage period (3 *hp* + 4d), this could be related to oxidative deterioration of the fruit found in the advanced stage of ripening (Chitarra and Chitarra 2005), as demonstrated by the higher lycopene content of these fruit compared to those exposed to higher pressures.

PAL is activated in response to abiotic stresses and pathogen infection, enabling its utilisation as an indicator of stress resistance (Vogt 2010). It is involved in the synthesis of phenylpropanoids, in the regulation of lignin accumulation leading to greater resistance to plant cell walls to pathogens (Gayoso et al. 2010) and in phenol synthesis (Gonzalez-Aguilar et al. 2010).

In our study, hyperbaric pressure significantly affected PAL activity, which increased in tomatoes treated at 800 kPa for 3 days with 4 days storage. This may have activated the biosynthesis of specific phenolic compounds, as these also increased with this treatment, enhancing the fruit's defence mechanisms. This result is supported by the increase in resistance of these fruit to

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infection by *B. cinerea*, with lower disease incidence (25%) and reduced disease severity (68%) compared to control (100 kPa).

In cherry tomatoes, the phenylpropanoid pathway was induced by treatment with hot air (38°C, 12 h), and the increase in PAL activity and the accumulation of phenolic compounds directly interfered with fruit resistance against infection by *B. cinerea* and *A. alternata* (Wei et al. 2016). Similarly, we demonstrated that hyperbaric pressure, as a physical method comparable to hot air treatment, could activate defence-related enzymes and enhance resistance to *B. cinerea*.

Glucanases (GLU) are induced during infection by pathogens and/or other stresses and belong to the group of proteins related to plant defence against pathogenesis, catalysing the hydrolysis of cell wall polymers of fungi; thus, they contribute to increased resistance of plants to pathogens (Pavoncello et al. 2001). Tomatoes in the red-ripe stage were more susceptible to *A. alternata* than in the ripe green stage in three cultivars due to changes in GLU enzymatic activity in response to fungal infection (Cota et al. 2007).

We found that fruit subjected to pressures of 400 to 800 kPa for 3 days showed an increase in GLU activity. This significant effect occurred in fruit treated at 800 kPa for 3 days followed by 4 days at ambient (3 *hp* + 4d), and the increase in activity may have contributed to the strengthening of the plant cell wall, enabling an increase in fruit resistance to *B. cinerea* infection. This was demonstrated by the lower incidence (25%) and reduced severity (68%) of grey mould in these fruits compared to control (100 kPa). Furthermore, the induction in activity showed a prolonged effect until the fourth day of storage at ambient conditions.

CAT plays a crucial role as a scavenger of hydrogen peroxide (H_2O_2) in plant cells, through the dismutation of H_2O_2 generated in peroxisomes by oxidases involved in fatty acid β-oxidation, photorespiration and purine catabolism (Gill and Tuteja 2010; Igamberdiev and Lea 2002). Studies indicate that postharvest physical treatments of strawberries, such as high temperatures (38°C) and UV-C (2.0 kJ m^{-2}) increased SOD and CAT activities in relation to control, enhancing fruit resistance to *B. cinerea* disease up to 12 days at 5°C (Jin et al. 2017). Therefore, the ability of fruit to maintain high CAT activity is a crucial defence mechanism against stress factors (Boonkorn 2016).

Our study results demonstrated that hyperbaric storage at 800 kPa for 3 days increased CAT activity in tomatoes, which remained high after ambient storage conditions for 4 days (3 *hp* + 4d). This increase in CAT activity was correlated with a lower disease incidence (25%) and disease severity (68%) in these fruit compared to the control. Similarly, Inestroza-Lizardo et al. (2021) showed a 54% increase in CAT activity in tomatoes subjected to pressures at 600 and 800 kPa combined with refrigeration (12°C), when compared to tomatoes refrigerated at ambient pressure (100 kPa and 12°C).

In this study, we obtained promising results for the use of hyperbaric pressures as an alternative method for postharvest control of *B. cinerea* in tomatoes. Exposure to high pressures (600 and 800 kPa) for 3 days controlled mycelial growth and conidial germination of the pathogen in the experiments in vitro, as well as reducing disease incidence and severity in tomatoes. The higher hyperbaric pressures combined with shorter treatment periods decreased fungal growth and induced resistance mechanisms in tomatoes, such as the increased enzymatic activity of GLU, increased activity of enzymes associated with oxidative metabolism and biosynthesis of bioactive compounds.

The use of hyperbaric pressures for postharvest disease control is a novel field and this study brings for the first time their use for *B. cinerea* control, increasing shelf life and reducing postharvest losses. We encourage future studies to detail the bioactive compounds synthesised under this treatment and the molecular mechanisms involved in resistance activation.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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