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Exploring *Opuntia ficus-indica* as a Strategy to Mitigate High Temperatures Effects in Vineyards: Insights into Physiological and Proteomic Responses

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Abstract: High temperatures significantly impact grapevine growth and development and lead to severe losses in grape quality and production. To minimize the impact of those environmental constraints, the application of biostimulants (BSts) has emerged as one of the most interesting strategies. BSts application derived from cactus species has been described as a successful approach to enhance tolerance to biotic and abiotic stresses. In this study, an aqueous extract prepared from the cladodes of *Opuntia ficus-indica* was applied through foliar spraying to grapevine plants (*Vitis vinifera* L.) 'Aragonez' already under heat stress. The effect of the extract application on protecting grapevine plants against heat stress was assessed in an experiment running during 15 days after extract application by determining several physiological parameters and detecting the changes in the whole proteome profile by comparing non-treated and extract-treated samples. Results show that physiological parameters directly related to photosynthesis showed a positive effect of the extract in mitigating heat stress in grapevines. Proteomic analysis indicated that the extract significantly upregulated proteins associated with photosynthesis and stress responses. This study provides new insights about the effect of *O. ficus-indica* extract in grapevines, offering a valuable strategy for future applications under field conditions.

Keywords: *Vitis vinifera* L.; *Opuntia ficus-indica*; biostimulants; abiotic stress; physiological response; proteomic response

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1. Introduction

Grapevine (*Vitis vinifera* L.) is one of the four most widely cultivated fruit crops globally, with its yield and fruit quality significantly influenced by various environmental stresses, including biotic and abiotic factors. Among the abiotic stress factors,

extreme temperatures stand out as one of the critical factors, with elevated temperatures being particularly important in Mediterranean regions by adversely impacting plant growth and morphology, seed germination, metabolic processes, fruit quality, and overall production [1–3]. Additionally, under high-temperature conditions, stomatal conductance and photosynthesis rate also decrease significantly as the leaf temperature rises [4]. Water scarcity, combined with high temperatures and radiation, is a reality of the Mediterranean agricultural ecosystems, impacting the crop production with negative effects well known in the grapevine sector. These conditions are the primary cause of a symptomatology known as sunburn [5].

Sunburn in vineyards can have several detrimental effects that impact both grape quality and overall yield. Depending on the severity of the damage, sunburn symptoms range from the appearance of brown or necrotic spots on the epidermis of grapes to the complete desiccation of the berries. Consequently, the effects of sunburn on grapes alter their chemical composition, leading to higher sugar levels and lower acidity, which negatively impacts the final quality of the wine [5]. To mitigate the effects of sunburn in grapes, farmers may adopt various strategies, such as canopy management and protective sprays, though these methods can be challenging and expensive to implement. Because grapevines are one of the oldest and most economically significant fruit crops in the world, largely due to the wine-making industry, which substantially contributes to cultural and economic activities in many regions, new and more environmentally friendly strategies have been developed to protect this crop against the adverse effects of environmental constraints [6]. The application of biostimulants (BSts), first defined by Kauffman and colleagues [7] as 'substances, distinct from fertilizers, that enhance plant growth when used in small amounts', could be a potential approach in the current crop production scenario.

Although the definition of BSts may vary slightly depending on the regulatory context, BSts are currently defined as a compound, a microorganism, or an amalgamation of both that, when applied to seeds, plants, or soil, enhances nutrient uptake, plant growth, and tolerance under different environmental stresses—without being classified as pesticides, plant growth regulators, or fertilizers [8]. BSts can trigger several mechanisms, including physiological, biochemical, and molecular alterations that mitigate the negative impacts of stress on plants. Different reports highlight the role of BSts in facing biotic or abiotic stresses by increasing nutrient uptake, stimulating beneficial microbial activity in the rhizosphere, increasing plant immune responses by developing cellular hypersensitivity, increasing callose deposition and lignin synthesis, and enhancing the antioxidative defenses to scavenge excessive reactive oxygen species (ROS) production [6,9–11].

According to their origin, BSts can be classified into two groups: non–microbial BSts (chitosan, humic and fulvic acids, protein hydrolysates, phosphites, seaweed extracts, and silicon) and microbial BSts (arbuscular mycorrhizal fungi, plant growth–promoting rhizobacteria, and *Trichoderma* spp.) [12]. Depending on the type of biostimulant extract, foliar fertilization, fertigation, or direct soil application are the primary methods for applying BSts [13].

The most frequently used biostimulants in the vineyard were grouped according to the stress they mitigate (biotic, abiotic, or both) and those that act as elicitors. Some of these biostimulants are plant extracts, namely nettle, Japanese Knotweed, and seaweed extracts, but also other products, such as yeast extracts, urea, and kaolin. The application of these biostimulants is predominantly carried out through foliar application, which ensures direct and efficient absorption of the active compounds by the vine leaves [14].

Previous studies have found that the application of biostimulants to grapevines resulted in greater tolerance to abiotic stresses, such as drought and soil salinity, stimulating various physiological, biochemical, and molecular responses [6,15]. In an extensive review, Cataldo and colleagues [16] highlight the effects of different BSts applied to grapevines against biotic and abiotic stresses. They cover various BSts categories, including seaweed extracts, humic and fulvic acids, inorganic compounds such as silicon and phosphites, protein hydrolysates, chitosan, and microbial biostimulants, such as *Trichoderma* spp., Plant-Growth-Promoting Rhizobacteria (PGPR), and Arbuscular Mycorrhizal Fungi (AMF). Grapevines treated with BSts showed increased tolerance to drought by improving stomatal regulation and photosynthetic activity, increasing the concentration of soluble proteins, enhancing the accumulation of abscisic acid (ABA), proline, and total phenols, and boosting the activity of the antioxidant enzymes guaiacol peroxidase (GPX) and catalase (CAT) [16,17]. In addition, BSts help mitigate the oxidative stress caused by higher temperatures, increasing the production of antioxidants and other protective compounds [16,18,19]. Other studies have shown that grapevines treated with BSts exhibited higher levels of bioactive compounds in grapes, enhancing grape quality and confirming the foliar absorption of biostimulants when applied through foliar spraying [20].

The application of BSts derived from desert plants, which are characteristic of arid climates, to agricultural crops has been described as an effective technique to enhance tolerance to biotic and abiotic stress. In an extensive review, Zhang and White [21] highlighted studies on the effects of desert endophytes in combating fungal pathogens. For example, the endophytic *Piriformospora indica* from the Thar Desert enhanced barley resistance to root pathogens, while *Bacillus* and *Enterobacter* isolated from *Thymus vulgaris* in Egyptian deserts increased tomato resistance to *Fusarium oxysporum*. However, none of these studies involve biostimulants derived from cactus species. Cactus species are characterized by their rich content of vitamins and bioactive compounds, which exhibit biological activities such as anti-inflammatory and antimicrobial properties [22]. They also have high nutritional importance, containing significant amounts of pectin, flavonoids, phenolic acids, carotenoids, vitamin C, minerals, and free amino acids [23]. In addition to their physical adaptations that enable them to thrive in dry, nutrient-deficient soils, desert plants also benefit from their microbial endophytes. In the current context of climate change, where extreme events increasingly simulate desert-like conditions, endophytes from desert plants may become more relevant for agriculture. In this study, we investigated the protective potential of an extract from the cladodes of a prickly pear cactus (*O. ficus-indica*) in grapevine plants (*V. vinifera* L. 'Aragonez') exposed to high temperatures. Plant responses were evaluated through analyzing different physiological parameters and identifying the main changes that occur at the proteome level.

2. Materials and Methods

2.1. Experimental Place

The experimental work was conducted under controlled environmental conditions at the facilities of the University of Évora (Évora, Portugal), which include a complex of greenhouses and plant growth chambers.

2.2. Opuntia ficus-indica Extract

An aqueous extract from wild prickly pear (*O. ficus-indica*) cladodes was prepared to explore its effectiveness in protecting grapevines from heat stress. Cladodes were crushed, and the viscous material was then filtered to separate two phases. The phase correspondent to plant material was discarded, and the supernatant correspondent to the viscous material extract, containing phenolic compounds, was diluted at a ratio of 300 g of extract to 2 L of

sterile distilled water. The final extract was applied to six plants by foliar spraying at a rate of 100 mL per plant, with a total of 15 g of *O. ficus-indica* extract applied per plant [24].

2.3. Plant Material and Experimental Design

Twelve P1103 field grown bench-grafted grapevines of *V. vinifera* L. 'Aragonez' (provided by PLANSEL Lda. Montemor-o-Novo, Portugal) were potted in twelve 20 × 31 cm containers with an appropriate commercial substrate (*Pot Plant Bordnamona*) on February 2024. P1103 refers to the 1103 Paulsen rootstock, which is widely used in viticulture due to its high tolerance to drought and resistance to calcareous soils [25]. To promote efficient budbreak, the pots were kept in a greenhouse. After two months, 12 plants were transferred to a growth chamber set at a temperature of 30 °C and 75% relative humidity, where they were kept for 15 days. During this period, a gradual increase in temperature occurs, reaching 42 °C end. After the 15 day period, of the 12 plants transferred to the growth chamber, six plants were treated with the extract from *O. ficus-indica* cladodes by foliar spraying (treatment group), while the remaining six were sprayed with water (control group). Each plant corresponded to a biological replicate. Both groups remained in the growth chamber, where the temperature was adjusted in a day/night cycle, with a minimum of 10 °C at night and a maximum of 42 °C during the day under a 14 h photoperiod for fifteen days. The plants were irrigated daily with only 100 mL of water to mimic the natural stress conditions found in Mediterranean vineyards during summer. The effect of the extract application on the grapevines plants was evaluated by measuring various physiological parameters at 2 days, 7 days, and 15 days after the extract application. At the end of the experiment, leaves from both groups were collected for whole proteome profile analysis through a two-dimensional electrophoresis (2-DE) technique.

2.4. Measurements of Selected Physiological Parameters

The effect of extract application on the grapevine plants was evaluated by measuring different physiological parameters, namely stomatal conductance (g*s*), leaf water potential (ψ), chlorophyll content, and relative water content (RWC), at 2, 7, and 15 days after the extract application. The mentioned physiological parameters were analyzed in all plants from both groups. For stomatal conductance and chlorophyll content, measurements were taken on three different leaves per plant.

2.4.1. Stomatal Conductance and Leaf Water Potential

Stomatal conductance was measured with an AP4 porometer (Delta-T Devices, Burwell, Cambridge, UK), with three measurements taken on three different leaves per plant. Leaf water potential was measured with a Scholander pressure chamber (PMS 1000, PMS Instruments, Albany, OR, USA) at predawn growth chamber control conditions on mature leaves. Both parameters were evaluated on the same days. Results were expressed in mmol $m^{-2}s^{-1}$ for stomatal conductance and in Mpa for leaf water potential, respectively.

2.4.2. Chlorophyll Content and Relative Water Content

To determine total chlorophyll content, the MINOLTA chlorophyll meter SPAD-502 (Minolta Co., Ltd., Osaka, Japan) was used, with three measurements taken on three different leaves per plant. Results were expressed in SPAD units. To determine relative water content (RWC), one leaf was collected from each plant, and fresh weight (FW) was immediately measured. Leaves were then immersed in water for 24 h to obtain the turgid weight (TW) and subsequently dried at 70 °C for 72 h to measure the dry weight (DW). RWC was calculated using the formula $RWC(\%) = [(Fw - DW) \div (TW - DW)] \times 100$.

2.5. Proteome Profile Analysis Through Two-Dimensional Electrophoresis (2-DE)

Fifteen days after the extract application (at the end of the experiment), from each grapevine plant with and without prickly pear extract application, a pool of three leaves per plant was collected for protein profile analysis by two-dimensional electrophoresis. Six biological replicates were considered per condition (each biological replicate corresponded to an individual plant). After collection, leaves were immediately frozen in liquid nitrogen and stored at −80 °C prior to protein extraction.

2.5.1. Protein Extraction and Quantification

The protocol for total protein extraction was adapted from Wessel et al. [26], with some modifications. Briefly, samples were homogenized with liquid nitrogen, crushed into a fine powder, and 100 mg was suspended in 1 mL of cold methanol. After being vigorously mixed for 30 s, chloroform and ultra-pure water were added to the samples, with the mixture being vigorously mixed for 30 s between the addition of each component. The mixture was centrifuged (15,000× *g* at 4 °C for 5 min), and the supernatant was discarded. 1 mL of methanol was added to the resulting precipitate, followed by gentle agitation. After centrifugation under the previously mentioned conditions, the protein pellet was washed with 90% acetone. Finally, the purified pellets were air-dried in a fume hood for 30 min and then reconstituted in 1 mL of solubilization buffer (7 M urea, 2 M thiourea, 4% CHAPS, 100 mM tris-HCl, pH 7.5). Total protein quantification was carried out using the Bradford method [27]. Results were expressed as µg/mL.

2.5.2. 2-DE and Gel Image Analysis

The volume corresponding to 80 µg of protein was mixed with 2% of IPG *buffer* (GE Healthcare) and applied by passive rehydration into immobilized nonlinear pH gradient 3–10 Immobiline Dry strips (7 cm) for 16 h at room temperature in the Multiphor II system (GE Healthcare). Each strip was covered with 2 mL of mineral oil to prevent evaporation. Isoelectric focusing (IEF) was performed using an IPGphor system (GE Healthcare, Chicago, IL, USA) at 12 °C by increasing the voltage in the following steps: 200 v a 1 vh, 200–3500 V until reaching 2800 vh; 3500 V until reaching 10,000 vh; 3500 V until reaching 5200 vh. After IEF, strips were equilibrated for 15 min in equilibration buffer [75 mM Tris-HCl, pH 8.8; 6 M urea; 29.3% (*v*/*v*) glycerol; 2% (*w*/*v*) sodium dodecyl sulfate (SDS); and 0.002% bromophenol blue] with 1% (*w*/*v*) dithiothreitol (DTT), then replacing DTT by 2.5% iodoacetamide (*w*/*v*), followed by separation of proteins based on molecular masses using a 14% polyacrylamide sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) mini-gel (Protean xi, Bio-Rad, Hercules, CA, USA), using the Laemmli buffer system [28]. Each sample was run in triplicate, and three biological replicates of each group were considered. Electrophoresis was run at constant voltage (130 V) until the dye front reached the end of the gel. Gels were fixed for 1 h in 40% methanol/10% acetic acid, followed by staining for 2 h with Coomassie Brilliant Blue (CBB) G-250. Images of the 2-DE gels were acquired using a scanning molecular dynamics densitometer with internal calibration and LabScan software v.6.0 (GE Healthcare). Gel analysis was performed using the software SameSpots v.1.0 (TotalLab programme) with automatic spot detection, followed by manual editing for spot splitting and noise removal.

2.5.3. Protein Identification by Mass Spectrometry Analysis (MS)

To maximize the likelihood of successful protein identification, the six most abundant differentially expressed spots were selected and excised from the reference gel.

Excised spots were trypsin digested, and the tryptic peptides were desalted and concentrated using POROS C18 (Empore, 3M) and eluted directly onto the Matrix-Assisted Laser Desorption/Ionization (MALDI, Framingham, MA, USA) plate using 1 µL of 5 mg/mL CHCA (alpha-cyano-4-hydroxycinnamic acid, Sigma, Kawasaki, Japan) in 50% (*v*/*v*) acetonitrile and 5% (*v*/*v*) formic acid. The data was acquired in positive reflector MS and MS/MS modes using a 5800 Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF/TOF) (AB Sciex, Framingham, MA, USA) mass spectrometer and using TOF/TOF Series Explorer Software v.4.1.0 (Applied Biosystems, Foster City, CA, USA). External calibration was performed using CalMix5 (Protea, Morgantown, WV, USA).

The raw MS and MS/MS data were analyzed using Protein Pilot Software v. 4.5 (ABSciex, Framingham, MA, USA) with the Mascot search engine (MOWSE algorithm). Monoisotopic peptide mass values, a maximum precursor mass tolerance (MS) of 50 ppm, and a maximum fragment mass tolerance (MS/MS) of 0.3 Da were considered. The search was performed against protein database UniProt with taxonomy restriction to *V. vinifera* or SwissProt without taxonomy restriction when the first search had a score lower than 70 (just protein scores greater than 70 are significant). Cysteine carbamidomethylation (C) was set as a fixed modification; methionine oxidation (M), asparagine and glutamine deamidation (NQ), and the transformation of N-terminal glutamine to pyroglutamic acid (N-term Q) were set as variable modifications. Protein identification was only accepted when significant protein homology scores were obtained and at least one peptide was fragmented with a significant individual ion score ($p < 0.05$).

2.6. Statistical Analysis

Statistical analyses were performed by SPSS version 28.0. Normality and homoscedasticity were evaluated using the Shapiro-Wilk [29] and Levene [30] tests, respectively. For physiological parameters and volume of the protein spots, mean comparisons between non-treated and extract-treated plants, and comparisons among the different time points were performed by Student *t*-test and one-way ANOVA (followed by a Tukey HSD test), respectively, or Mann-Whitney and Kruskal-Wallis nonparametric tests when the data did not meet the assumptions for parametric tests. The Pearson or Spearman correlation coefficient was used to verify the correlations among the physiological parameters. Statistical significance was considered at $p < 0.05$.

3. Results

3.1. Physiological Parameters

Before spraying the vine leaves with prickly pear extract, predawn leaf water potential (ψ_{PD}) (Figure 1a) was measured in all vine plants. Both groups of grapevines showed similar ψ_{PD} values, highlighting the homogeneity among plants. However, 48 h after extract application, a significant decrease in ψ_{PD} was observed in the extract- treated group when compared to control plants (sprayed with water). A similar result was observed 7 days after the extract application. Nevertheless, at the end of the experiment, no significant differences were observed between extract-treated grapevine plants and the control plants. In extract-treated plants, the ψ_{PD} reached lower values at 2 and 7 days, significantly different when compared with values obtained previously to spraying (T0). In control plants, the ψ_{PD} reached lower values at 7 and 15 days, significantly different when compared with values obtained previously to spraying (T0).

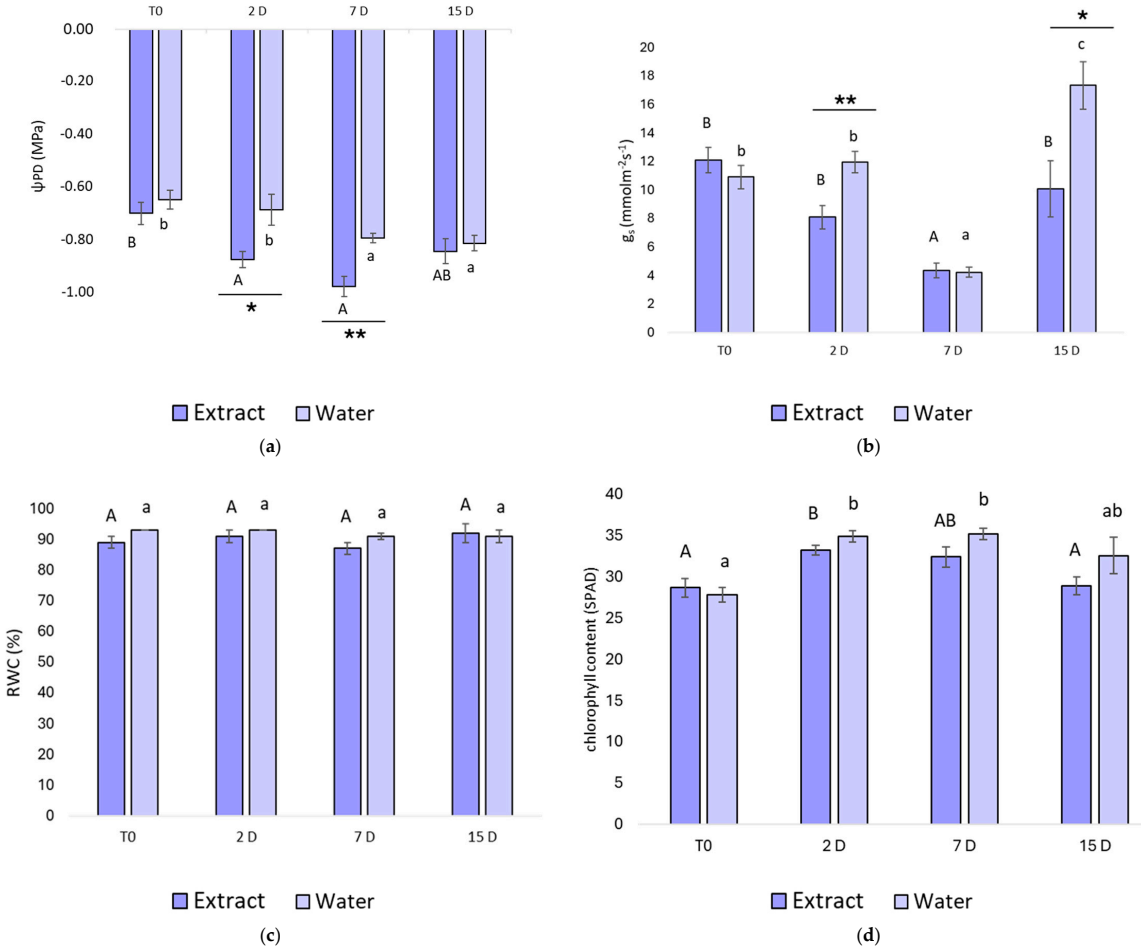

Figure 1. (**a**) Leaf predawn water potential (ψ_{PD}), (**b**) stomatal conductance (gs), (**c**) relative water content (RWC), and (**d**) chlorophyll content in plants of *V. vinifera* 'Aragonez' sprayed with *O. ficus-indica* extract (dark bars) or water (light bars). The measurements were taken at four time points: T0 (before application of the extract or water), 2, 7, and 15 days after spraying. In both groups, data are the mean value of measurements taken from six plants ± SE. Statistical significance was considered for $p < 0.05$. Different capital letters indicate significant differences among time points for plants sprayed with *O. ficus-indica* extract, and different small letters indicate significant differences among time points for plants sprayed with water. Significant differences between plants sprayed with extract and water are indicated with * for $p < 0.05$ and ** for $p < 0.01$.

The effect of foliar application of prickly pear extract on stomatal conductance (gs) was assessed at different time points, as shown in Figure 1b. Prior to the application of extract or water (T0), both groups exhibited similar gs values, indicating no initial differences between the groups, which is in accordance with ψPD values exhibited at T0 for both groups. A significant decrease in gs was observed in plants treated with the extract at timepoints 2 and 15 days after spraying. In both groups, gs reached significantly lower values 7 days after spraying. In the control group, gs increased after that moment, reaching significantly higher gs values at the end of the experiment.

No significant differences were observed in relative water content (RWC) (Figure 1c) and chlorophyll content (Figure 1d) between treatments. Over time, chlorophyll levels in both groups significantly increased 48 h after foliar spraying, then gradually declined to near T0 levels by the end of the experiment.

Figure 2 shows the significant correlations among the physiological parameters under study. A moderate positive correlation is seen in plants sprayed with *O. ficus-indica* extract when ψ_{PD} is correlated with stomatal conductance (Figure 2a). On the other hand, in the

group of plants sprayed with water, a higher positive correlation is observed between ψ_{PD} and relative water content (Figure 2b). No significant correlations were observed among the remaining parameters.

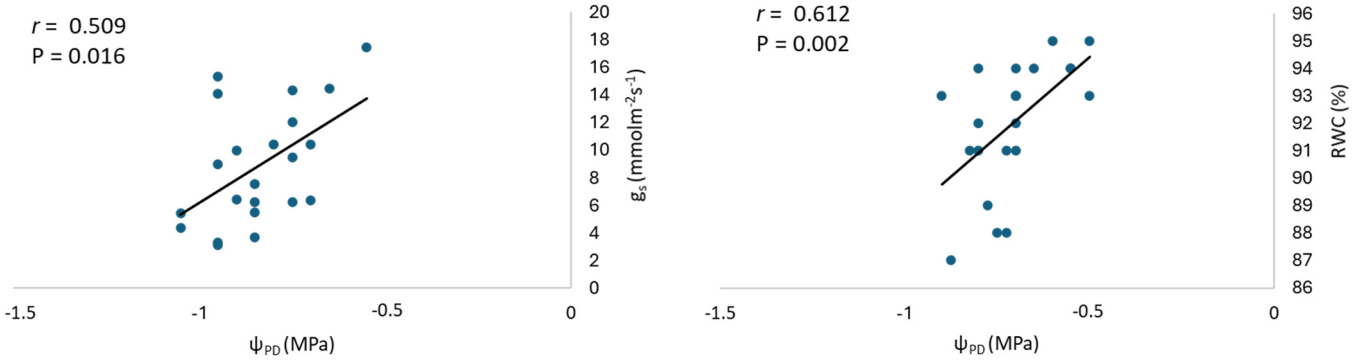

Figure 2. Correlation between (**a**) predawn leaf water potential (ψ_{PD}) and stomatal conductance (gs) in plants sprayed with *O. ficus-indica* extract and between (**b**) predawn leaf water potential (ψ_{PD}) and relative water content (RWC) in plants sprayed with water. r represents the Pearson correlation coefficient in (**a**) and the Spearman correlation coefficient in (**b**). *p* represents the significance level of the correlation. The correlation is significant for $p < 0.05$.

3.2. Two-Dimensional Protein Profile

After the analysis of the two-dimensional gels, 144 protein spots were considered for statistical comparison. Significant differences between grapevine plants of both extract-treated and non-treated (control) were observed for 12 protein spots (the position of the spots on the gel is shown in Figure 3): 5 spots [spot 77 (p = 0.040), 114 (p = 0.042), 183 (p = 0.007), 187 (p = 0.035), and 214 (p = 0.002)] with significantly higher expression in plants sprayed with *O. ficus-indica* extract (Figure 4a) and 7 spots [spots 15 (p = 0.029), 24 (p = 0.021), 32 (p = 0.021), 101 (p = 0.043), 166 (p = 0.046), 168 (p = 0.033), and 198 (p = 0.046)] with significantly higher expression in the control group (Figure 4b).

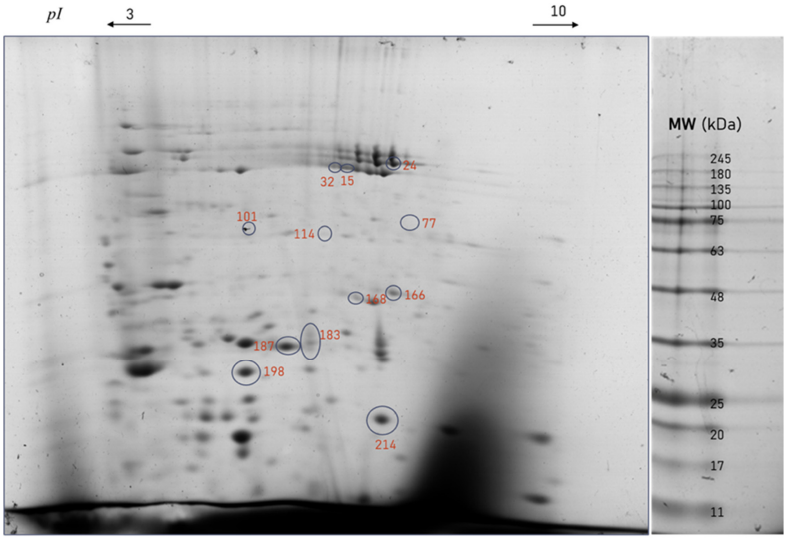

Figure 3. Representative 2-DE profile of proteome extracted from *V. vinifera* L. 'Aragonez' leaves. Circles represent the spots differentially expressed among plants sprayed with *O. ficus-indica* extract and sprayed with water. MW—molecular weight (kDa); *pI*—isoelectric point.

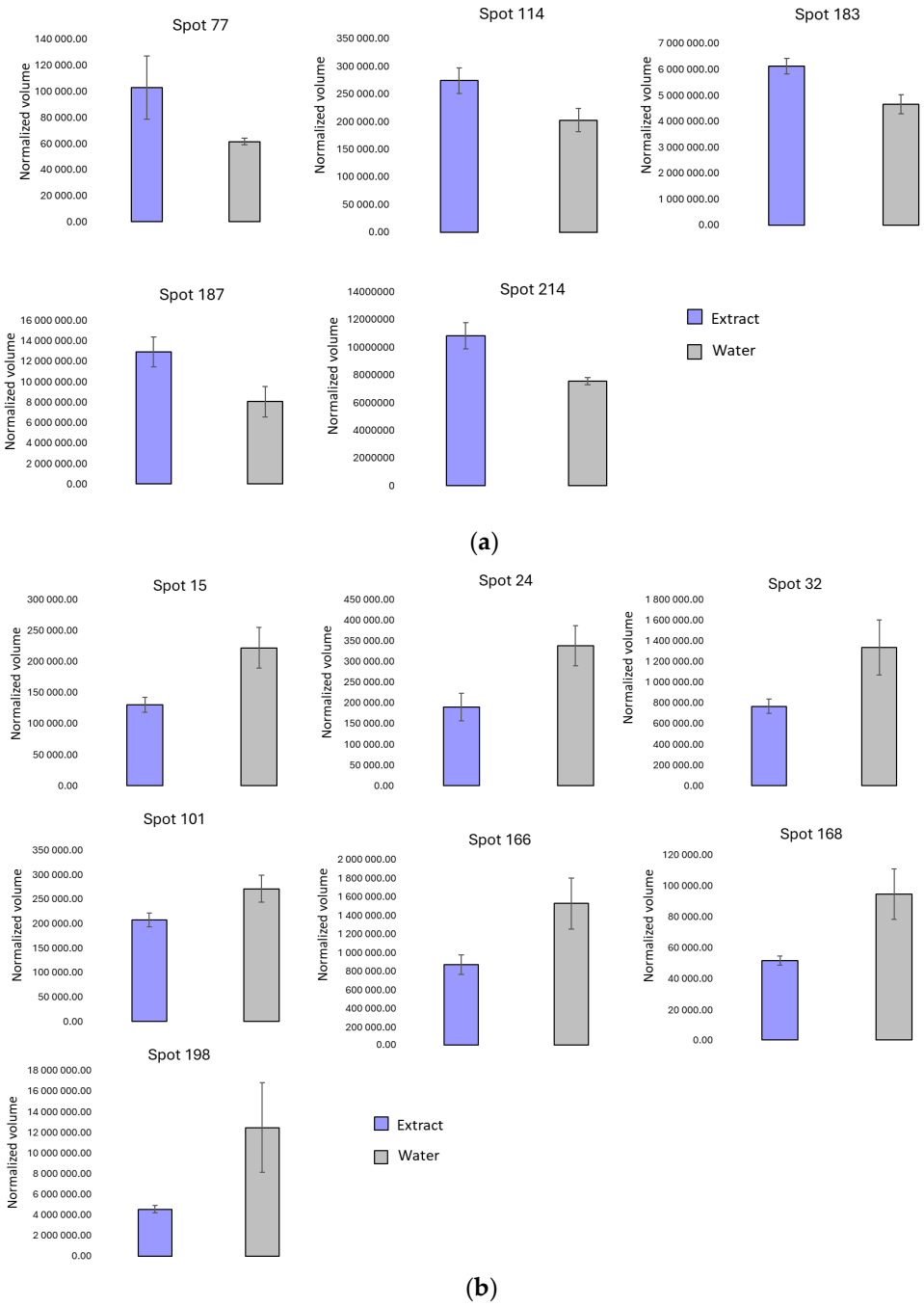

Figure 4. Expression level of protein spots identified in 2-DE gels (normalized volume) differing between grapevine plants sprayed with *O. ficus-indica* extract and sprayed with water (control). (**a**)—spots with significantly higher expression in extract- sprayedplants; (**b**)—spots with significantly higher expression in the water sprayedplants. Data are the mean value of volume from three biological replicates ± SE. Statistical significance was considered for $p < 0.05$.

Of the 12 differentially expressed spots detected when comparing extract-treated and non-treated grapevine plants,, those with high-quality resolution on 2-DE gels were further identified by mass spectrometry (MS). Results of the MS identification are presented in Table 1. Except for one spot, the search was performed against protein database UniProt with taxonomy restriction to *V. vinifera* L.

Table 1. Proteins differentially expressed among plants sprayed with *O. ficus-indica* extract and water identified by MS. The arrow indicates the group of plants in which the protein has significantly higher expression. Protein scores greater than 70 are significant ($p < 0.05$).

Spot	Protein	Swissprot/Uniprot Entry Reference	Theoretical MW (kDa)	Theoretical pI	*Score* ID	Seq. C (%)	N° Peptide Matched	*O. ficus-indica* Extract Pulverization	Water Pulverization
24	α-Amy 1A	P0DUB6	58.4	9.25	221	38	27		↑
166	RBC (large chain)	A0A0K1CWJ6	22.3	8.33	201	50	17		↑
168	RBC small subunit, chloroplastic	A0A438JZ20	20.7	9.11	60	21	6		↑
187	RBC (large chain)	A0A0D4BPD8	29.5	9.18	111	31	13	↑	
198	RBC (large chain)	A0A6G8J0R0	21.2	6.23	78	15	12		↑
214	HSP18.1	F6HNP7	18.1	6.78	264	47	11	↑	

MW—molecular weight (kDa); *pI*—isoelectric point; Seq. C (%)—Sequence coverage α-Amy—Alpha-amylase; RBC—ribulose bisphosphate carboxylase (RuBisCO); HSP—Heat Shock Protein.

Of the five spots with significantly higher expression in plants sprayed with *O. ficus-indica* extract, only two were identified by MS, namely spot 187, identified as ribulose bisphosphate carboxylase (RuBisCO), with an estimated molecular mass of 29.5 kDa, and spot 214, identified as heat shock protein (HSP), with an estimated molecular mass of 18.1 kDa.

Proteins from the ribulose bisphosphate carboxylase (RuBisCO) family were identified in spots 166, 168, 187, and 198. Of the spots identified as RuBisCO, only spot 187 showed significantly higher expression in the plants treated with *O. ficus-indica* extract, while the others were increased in the plants sprayed with water. Spot 168, although identified as RuBisCO, showed a low score in both databases used for identification. The protein spots identified as RuBisCO exhibited a range of molecular masses between 20 and 29 kDa, which may indicate the presence of different RuBisCO isoforms.

Spot 24, due to the low score when searched in the UniProt database with taxonomy restriction to *V. vinifera*, was identified as α-amylase by searching in the database SwissProt without taxonomy restriction. This protein showed significantly higher expression in plants sprayed with water when compared to the extract-sprayed plants.

4. Discussion

To address the negative impacts of high temperature on grapevine plants, viticultural practices should be reconsidered. The use of BSts appears as an innovative and sustainable approach to mitigating the adverse effects of abiotic stress, including high-temperature stress. BSts influence plant growth by inducing molecular changes and modulating physiological, biochemical, and anatomical processes. Their diverse nature arises from the variability in bioactive compound composition, enabling them to operate through multiple modes of action [6].

O. ficus-indica is native to arid and semi-arid regions, where it thrives under extreme heat and sunlight. Its natural adaptations include high levels of antioxidants, mucilage, and phenolic compounds that protect against oxidative stress [23]. To our knowledge, this is the first study to apply cladode extracts from *O. ficus-indica* to grapevine leaves for protection against abiotic stress. In the present work, the extract was applied to grapevine leaves exposed to high temperatures, and the effects were evaluated at physiological and proteomic levels.

Leaf water potential (ψ) and stomatal conductance (gs) are two key physiological parameters for characterizing the plant's water status at a given moment and are used as indicators of the physiological activity of the plants. ψ reflects the overall water status of the plant, indicating the plant's hydration level and its capacity to maintain turgor pressure. In opposition, gs measures the rate at which water vapor exits the leaf through stomata, directly influencing transpiration rates and, consequently, the plant's water loss [31]. These parameters provide significant insights into the plant's capacity to manage water under several environmental conditions and are essential tools in understanding plant responses to abiotic stress [32]. Although the values may depend on the cultivar, under normal conditions ψ_{PD} typically ranges between −0.10 MPa and −0.30 MPa, while gs varies from 150 to 400 mmol m^{-2} s^{-1} [33,34]. In this work, before the foliar spraying of the plants with prickly pear extract, ψ_{PD} and gs displayed lower values compared to those reported under normal physiological conditions, which is a possible indicator of plant stress caused by high temperatures. This result is in accordance with those obtained by Edwards and co-workers [35], who observed similar pre-dawn ψ values (approximately −0.6 MPa) and *gs* values in grapevines ('Cabernet Sauvignon') subjected to high-stress temperatures (45 °C) [35]. The application of biostimulants often improves the plant's water-use efficiency and enhances osmotic adjustment. These effects may result in less negative ψ values, indicating improved water retention and reduced water stress [36]. In this work, as expected, a positive correlation between ψ and g*s* in plants sprayed with *O. ficus-indica* extract was found, indicating that grapevine stomata strongly respond to plant water status [31]. However, the application of *O. ficus indica* extract did not show a positive effect on ψ_{PD} and g*s* values in grapevine plants. A significant decrease in both parameters was observed in the plants sprayed with the extract, compared to those sprayed with water. A possible explanation for this result could be related to the fact that the grapevine may already possess inherent mechanisms to tolerate high temperatures, such as stomatal closure and adjustments in water transport systems [37]. This natural adaptation may have masked or reduced the impact of the *O. ficus-indica* extract.

The composition of the extract is also a key factor that can influence the plant's physiological behavior. The chemical composition varies with the cultivar, the development stage, the cultivation site, the climatic conditions, and the cladode order [38]. The efficacy of the extract largely depends on the presence of specific bioactive compounds, such as antioxidant compounds, polysaccharides, or secondary metabolites (e.g., flavonoids and phenolic acids) [39]. If the extract lacks sufficient concentrations of these active components, its impact on physiological parameters like ψ and g*s* may be limited. On the other hand, specific polysaccharides present in *O. ficus-indica* cladodes, such as pectin and mucilage [40,41], might form a thin film or coating on the leaf surface. This could create a barrier that inadvertently affects the natural stomatal function, thereby limiting gas exchange and transpiration. Future research must be considered, including a chemical characterization of the extract, as well as the time of the exposure to the stress factor and the use of different genotypes.

RWC is recognized as an important measurement of plant water status, as it effectively reflects the metabolic activity within the tissues [42,43], being a valuable parameter in determining the effects of changing temperatures [44]. In this experimental work, no significant differences were observed in RWC between plants sprayed with the extract and plants sprayed with water under high-temperature conditions. Similarly, no differences in this parameter were observed over time in either group. Previous studies have demonstrated that the foliar application of Cycocel™ (artificial plant growth regulators) [45] and strigolactones (carotenoid-derived phytohormones) [46] increases the leaf RWC in grapevines subjected to drought stress. However, no studies have been reported on the effects of bios-

timulants on RWC in grapevines exposed to high temperatures. In our study, high temperatures likely did not affect the RWC, as typical values range between 80% and 95% when plants are not under abiotic stress [33]. Studies indicate that RWC was suppressed only under severe drought stress, with values below 70%, which significantly impairs photosynthesis and plant metabolism [45–48], while in other studies, it was observed that stress caused by high UV-C radiation did not affect the RWC in grapevine leaves [49]. Small changes in leaf RWC could be related to the hydrostable nature of grapevines, a species with the ability to maintain a relatively stable water balance even under water stress conditions [50].

Most studies found in the literature regarding the effects of biostimulants on grapevines for protection against abiotic stresses are primarily focused on water stress, and the results are highly variable, depending on the type of biostimulant applied. In a recent study [51], the application of protein hydrolysate as a biostimulant directly to the soil of grapevine ('Sauvignon blanc') significantly alleviated the negative effects of water stress, even though the stem water potential values were lower than those of unstressed plants. Nevertheless, these findings suggest that this compound may help to mitigate the severity of water deficit effects and could already be assisting cells in counteracting dehydration shortly after stress imposition. On the other hand, Jalil and Sabir [52] verified that glycine betaine grapevine foliar application had no remarkable alleviating effect on leaf water status under deficit irrigation.

Despite the negative effects of high temperatures on grape quality and overall yield, the effects of biostimulants on grapevines under high-temperature stress remain poorly understood. Wu and colleagues [53], in a recent study, analyzed the effect of three different types of biostimulants, whose main components were β-Myrcene, protein, and Aspergone, on grapevine seedlings provided from seeds exposed to naturally high temperatures (maximum temperatures between 42–45 °C) during their development. However, this study primarily focused on the biostimulants' effects on the growth, development, and photosynthesis of grapevine seedlings, without addressing other physiological parameters. The authors found that the three biostimulants either enhanced chlorophyll accumulation or slowed its degradation in grapevine leaves, helping to maintain consistently elevated chlorophyll levels and supporting the growth of grapevine seedlings under high-temperature conditions. Indeed, the results suggest that biostimulants can mitigate or prevent damage to the photosynthetic processes of seedlings exposed to high temperatures, thereby preserving relatively high photosynthetic activity. In our experimental work, no significant differences were observed in total chlorophyll content between plants sprayed with the extract and those sprayed with water. Nevertheless, consistent with the findings of Wu et al. [53], the application of the extract led to a significant increase in total chlorophyll levels, which returned to initial values 15 days after exposure to high temperatures. However, this increase was also observed in plants treated with water, which may indicate a general plant defense strategy against high temperatures, thereby casting doubt on the specific effect of the extract. In a previous study [54], Xiao and colleagues reported that the Chl *a* and Chl *b* contents in grape leaves initially increased and decreased six days after exposure to high temperatures (36, 38, and 40 °C).

Proteomics approaches enable a specific evaluation of plants response to abiotic stresses and provide insights into the mechanisms underlying plant biostimulant activity, identifying molecular and biochemical pathways influenced by these biostimulants. However, to the best of our knowledge, few studies have been focused on proteome changes induced by biostimulants, even fewer on the effect of biostimulants on the protein composition of *V. vinifera* L. In this experimental work, the proteomic profile was accessed by bidimensional electrophoresis, and a total of 12 proteins were differentially expressed. Al-

though these differences were not extensive, other studies have reported similar findings. Foliar application of plant-derived protein hydrolysates to *V. vinifera* L. 'Merlot', under field conditions shows a moderate effect of the treatment on leaf proteome, since only 13 proteins have been modified. Some proteins increased (e.g., ATP synthase, superoxide dismutase, elongation factor Tu), having a positive role in vegetative growth, while proteins related to photosynthesis decreased [55].

Protein spots showing significant differences and reproducible changes between plants sprayed with water and *O. ficus-indica* extract were selected for MS identification. Among the six differentially expressed spots identified by mass spectrometry, four of them correspond to the protein ribulose bisphosphate carboxylase (RuBisCO) (commonly known as RuBisCO), an enzyme with a key role in photosynthesis. RuBisCo is involved in the Calvin cycle, being responsible for fixing carbon dioxide (CO_2) into an organic form that plants can use for growth and energy [56]. Structurally, RuBisCo is an enzymatic complex composed of eight large subunits and eight small subunits arranged in a dodecameric structure [57]. In this research, two spots were identified as the RuBisCO large subunit and one as the RuBisCo small subunit chloroplastic and were up-regulated in plants sprayed with water, whereas only one spot identified as the RuBisCO large subunit showed up-regulation in extract-treated plants. Proteins associated with photosynthesis are commonly affected by abiotic stress conditions. In a comprehensive study about differential proteomic analysis on leaves of 'Cabernet Sauvignon' under heat stress, Liu and colleagues [58] demonstrated that RuBisCO subunits were upregulated during heat stress but were then downregulated after recovery. A similar result was also reported by Król and Weidner [59], where both the small and large subunits of RuBisCO were upregulated in grapevine leaves during prolonged drought stress [59]. On the other hand, Azri et al. demonstrated that changes in RuBisCO expression are dependent on the genotype [60].

Changes in RuBisCO expression were also reported under drought conditions. Bavaresco and co-workers demonstrated that plant-derived protein hydrolysates significantly impacted *V. vinifera* leaf proteomic profile, leading to the upregulation of ribulose biphosphate carboxylase large chain. Similar results were obtained by Ilangumaran and coworkers [61] in soybeans inoculated with Rhizobacteria under salinity stress.

Unfortunately, no studies were found in the literature regarding the effect of biostimulants on the proteome of grapevine leaves under high-temperature stress. To the best of our knowledge, this is the first work to provide insights into the effect of bioactive compounds on grapevines subjected to high temperatures. The differential expression of RuBisCO subunits observed in this study may reflect the complex interplay between the plant's inherent physiological responses to heat stress and the effects of the *O. ficus-indica* extract. The upregulation of multiple RuBisCO subunit spots in grapevine plants sprayed with water and under high temperatures could indicate a stress-induced activation of the photosynthetic machinery. This response could be an adaptive mechanism to maintain photosynthesis and ensure energy production under high-temperature stress. In opposition, the reduced upregulation of RuBisCO subunits in plants treated with *O. ficus-indica* extract may suggest that the extract mitigated the stress perceived by the plant, leading to a more moderated response. This result is consistent with the elevated chlorophyll levels after extract application observed in this work. This could indicate an attempt to increase photosynthetic efficiency to meet the plant's energy needs under adverse conditions.

Another protein spot identified as upregulated in grapevine plants sprayed with water and downregulated in plants sprayed with the extract was the α-amylase. This enzyme plays a key role in starch degradation into simpler sugars, such as maltose; then, under maltase enzyme action, glucose is obtained, which is used by the plant as an energy source [62]. This result may be an adaptation of plants to the high-temperature stress, working to main-

tain essential cellular functions, such as photosynthesis. At the same time, the extract may have a protective effect, minimizing the impact of high temperatures and, consequently, reducing the need to activate mechanisms such as starch mobilization.

Along with other stress-responsive proteins referred to in this study, plants also induce the synthesis of proteins from the heat shock protein (HSP) family in response to different abiotic stresses. These proteins play a crucial role in protecting plants against stress by re-establishing normal protein conformations, helping to maintain other proteins in their proper structure, and preventing aggregation or denaturation of proteins caused by high temperatures [63]. In this study, one HSP was upregulated in grapevine plants sprayed with *O. ficus indica* extract compared to those sprayed with water. The increase in HSPs in plants treated with the extract may suggest a protective effect, activating mechanisms against thermal stress, which could help mitigate the negative effects of high temperatures. Similar studies in the literature indicate that, in grapevine plants under water stress conditions, the application of biostimulants does not cause changes in the expression of HSPs. However, a proteomic study on tea plants exposed to drought conditions revealed a positive effect of fulvic acid, with an increase in the abundance of HSPs [64].

5. Conclusions

In the current context of climate change, high-temperature conditions are exerting a substantial impact on plant growth, development, and crop yield. Heat stress has become one of the major limiting factors in the development of the grape industry. In this context, viticultural practices should focus on developing new solutions to mitigate the effect of abiotic stresses, with the application of biostimulants being increasingly considered. The molecular and physiological responses of grapevine to biostimulant application represent a significant challenge in contemporary plant research, with no information available in the literature about this specific response.

The results of our research make a significant contribution to the current understanding of the mechanisms involved in the grapevine's response to high temperatures following the application of bioactive compounds from *O. ficus-indica* extract. In an initial approach, although the leaf water potential did not yield the expected result, the physiological parameters directly related to photosynthesis indicated a positive effect of the extract application in mitigating the effects of heat stress in grapevine. Furthermore, the proteomic analysis of the leaves indicates a positive effect of the extract, with differentially expressed proteins in the plants treated with the extract likely playing a role in mitigating the effects of heat-induced stress. Based on these findings, we propose that some proteins related to photosynthesis and stress response may play key roles in protecting grapevines from heat stress following the application of *O. ficus-indica* extract.

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Abbreviations

The following abbreviations are used in this manuscript:

BSts	Biostimulants
ROS	Reactive Oxygen Species
AMF	Arbuscular Mycorrhizal Fungi
ABA	Abscisic acid
GPX	Guaiacyl peroxidase
CAT	Catalase
ψ	Leaf water potential
gs	Stomatal conductance
RWC	Relative water content
FW	Fresh weight
TW	Turgid weight
DW	Dry weight
2-DE	Two-dimensional electrophoresis
IEF	Isoelectric focusing
SDS	Sodium dodecyl sulfate
DTT	Dithiothreitol
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
CBB	Coomassie Brilliant Blue
MALDI-TOF	Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry
CHCA	Alpha-cyano-4-hydroxycinnamic acid
MS	Mass spectrometry
MW	Molecular weight
pI	Isoelectric point
RuBisCO	Ribulose bisphosphate carboxylase
HSP	Heat Shock Protein
CO_2	Carbon dioxide

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