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Systematic Investigation of the Effects of a Novel Protein Hydrolysate on the Growth, Physiological Parameters, Fruit Development and Yield of Grapevine (*Vitis Vinifera* L., cv Sauvignon Blanc) under Water Stress Conditions

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Abstract: In the last decade climate change has impacted viticulture and water deficit has become a major concern in fruit production. Many studies have been carried out to determine the grapevine response to environmental changes and to identify key genetic traits to be used in grapevine breeding. However, in order to better manage climate-related risks, novel viticultural practices are urgently needed. A promising solution for a more sustainable model of viticulture involves the use of biostimulants. In this study, the effectiveness of a novel biostimulant (APR[®]) belonging to the group of protein thermal hydrolysates was tested on grapevine plants subjected to progressive water deficit conditions. Our results showed that this compound applied to roots before imposing water deprivation mitigates the consequences of stress by sustaining the growth of the younger vegetative organs and limiting the extent of cell dehydration; this positive impact on the plant's physiological state persisted during the recovery phase. Furthermore, at the end of the growing season, plants treated with the biostimulant, both in optimal water conditions and under water stress, exhibited a greater accumulation of biomass in the aerial part (6.8% and 21.3 %, respectively) and a higher berry diameter (3.4 % and 9.5 %, respectively). Additional work through field trials will be necessary to further substantiate these results and to translate this knowledge into specific practices that grape growers can easily adopt.

Keywords: plant biostimulants; roots; growth; water deficit; *Vitis vinifera* L.

1. Introduction

The wide geographical distribution and economic importance of grapevine cultivation have placed grape industry worldwide under a major threat from climate change [1]. Indeed, climatic change has been responsible for the most common abiotic stresses, which are defined as environmental factors with potentially unfavorable effects on an organism. These abiotic stresses, such as drought, salinity, and extreme temperatures [2,3], have impaired plant growth and reduced productivity worldwide by more than 50% [4]. There is therefore an urgent need to mitigate the negative effects of such stresses on crop yields.

In the Mediterranean region, the predicted higher frequency of summer heat waves combined with a longer dry season may lead to extended and severe drought events that will affect grapevine growth and physiology, fruit development, and crop yields [5,6]. As traditionally rainfed wine regions

are experiencing more frequent and intense drought spells, in-depth knowledge of possible adaptive strategies to manage water scarcity is critically needed. Many studies have been published on the grapevine's response to these environmental changes (comprehensively reviewed by Geròs et al. [7]). However, there is still insufficient knowledge on effective strategies that need to be adopted [8]. It is imperative that optimal irrigation programs are identified [9], better adapted rootstocks are selected, biotechnological improvements of plants are made, best soil management practices are adopted, and new generations of environmentally friendly agrochemicals are developed and used in grape production regions to enable vines to adapt to changing conditions [10].

In recent years, the use of naturally derived biostimulants to mitigate the detrimental effects of abiotic stresses has become an appealing sustainable method to attain more stable yields in response to the changing environment [11,12]. According to the new Regulation (EU) 2019/1009 of the European Parliament and Council (EC) plant biostimulants are defined as “EU fertilising product, the function of which is to stimulate plant nutrition processes independently of the product's nutrient content with the sole aim of improving one or more of the following characteristics of the plant or the plant rhizosphere: (i) nutrient use efficiency, (ii) tolerance to abiotic stress, (iii) quality traits, or (iv) availability of confined nutrients in the soil or rhizosphere” [13]. Plant biostimulants thus include several different types of natural substances and chemical derivative of natural or synthetic compounds as well as beneficial microorganisms [14,15]. These comprise: (i) humic substances [16]; (ii) vegetal- or animal-based protein hydrolysates [17]; (iii) macro- and micro-algal extracts [18]; (iv) silicon [19]; (v) arbuscular mycorrhizal fungi (AMF); and (vi) plant growth promoting rhizobacteria (PGPR) belonging to the genus *Azotobacter*, *Azospirillum*, and *Rizhobium* spp. [20,21].

Protein hydrolysates (PHs) are “mixtures of polypeptides, oligopeptides, and amino acids that are manufactured from protein sources using partial hydrolysis” [22]. In particular, the use of PHs coming from by-products of vegetables and food manufacturing has sparked a rising interest among commercial enterprises and the scientific community as a sustainable, profitable, and green solution to the problem of waste streams disposal. Globally, the biostimulants market is projected to be valued at USD 2.9 billion in 2021 and was dominated by Europe, in terms of value in 2018, due to the adoption of modern agricultural technology such as precision farming and plant biotechnology [23]. As far as the market of PHs biostimulants is concerned it mainly results from the acid hydrolysis of animal-derived proteins, while the remaining part arises from the enzymatic hydrolysis of plant-derived proteins [14,15].

Several studies on the effects of applying PHs to crops have been published (reviewed by Colla et al., [24] and du Jardin [25]) and provided evidence about their ability to improve plant growth and productivity [26] thus supporting their protective role in grapevine's response to abiotic stress [27]. PHs were shown to stimulate plant primary metabolism and improve productivity [28]. This action seems to be even more pronounced when plants are under stress conditions, as demonstrated for tomato and spinach treated with seaweed extracts and exposed to drought [29,30].

Despite the large number of studies dealing with plant biostimulants [31], most of these studies have been conducted predominantly on vegetable crops, and there are currently very limited data available on the use of biostimulant compounds on fruit crops [12]. For example, two spray applications of PHs have been shown to be effective in enhancing water stress tolerance in grapevine (*Vitis vinifera* L., cv. Corvina) [32].

In recent studies, a novel collagen-derived protein thermal hydrolysate has been demonstrated to affect the transcription of a thousand genes when applied to maize roots grown in a solid medium [33], and its efficacy was subsequently also demonstrated in the same species grown hydroponically [34]. These findings [33] indicate that this PH could be involved in regulating the expression of genes involved in the transport of nutrients and in the signaling and metabolism of reactive oxygen species (ROS), leading to the hypothesis that it acts as an enhancer of plant stress tolerance, which was further supported [34] in experiments investigating its effects on seedlings subjected to different types of abiotic stresses. The results provide strong evidence in support of the idea that this PH might act as

a root growth stimulant, positively affecting nutrient use efficiency (NUE) and the control of ROS metabolism, thus leading to improved resistance to abiotic stresses.

Chemical characterization [35] has revealed a total carbon content and a total nitrogen content of 400 g/kg and 136 g/kg, respectively, the presence of different micronutrients and of various amino acids, such as lysine, phenylalanine, glycine, aspartate, and isoleucine.

The present study aimed to investigate the physiological effects of the plant biostimulant on grapevine plants (cv. Sauvignon blanc) grown in pots in the greenhouse under optimal water conditions and under water stress. After application of the biostimulant to the soil, several biometric parameters together with additional physiological indicators were monitored upon the induction of a gradual water deficit, and after a week of recovery under optimal water conditions.

2. Materials and Methods

2.1. Plant Materials and Treatments

The experimental materials consisted of grapevine, *Vitis vinifera* L., cultivar Sauvignon blanc (clone 108) grafted onto Kober 5 BB (K5BB) (*V. berlandieri* × *V. riparia*) rootstock. These vines were grown in pots, which mimicked growing conditions that similar to those found in the field, in semi-controlled conditions in a tunnel at the “L. Toniolo” Experimental Farm of the University of Padua in Legnaro, northeastern Italy. K5BB was chosen in this experiment, as it is one of the most widely used rootstock in European viticulture and is known to be susceptible to water stress.

The experiments were carried out in 2018. Four-year-old plants were grown in 10 L pots filled with a sand–pumice–peat mixture (2:2:6 in volume). A total of 36 plants were randomized into four groups, according to homogeneous developmental characteristics (i.e., length of cane and number of buds). The plants were pruned before bud burst with three or four dormant buds per plant retained. At the 5-separated-leaves stage (stage 15 according to the extended BBCH scale [36]), the plants were thinned to two shoots and trained vertically, and then placed under the following four experimental conditions: (i) control vines maintained under well-watered conditions throughout the experimental period (WW); (ii) plants treated with APR[®] and maintained under WW conditions throughout the experimental period (WW + PH); (iii) water-stressed plants subjected to progressive water deficit (WS); (iv) plants treated with APR[®] then subjected to progressive water stress (WS + PH).

For the APR[®] treatments (WW + PH and WS + PH), one liter of the novel collagen-derived protein thermal hydrolysate, APR[®] (ILSA S.p.A., Arzignano, Italy) at a concentration of 0.5 g L^{−1} (corresponding to a 50 µM concentration of N) according to Trevisan et al. [34], who identified this concentration as the most effective, was added to the soil of each pot as a soil drench at the BBCH phenological stage 57 (“flowers separating”) according to Lorenz et al. [36], on 15 May (day of the year [DOY] 135). The pots of the untreated plants were given the same amount of water. Water stress (treatments WS and WS + PH) was imposed on July 2 (DOY 183)—48 days after biostimulant application—for an experimental period of 18 days and was controlled by managing the soil water content. Sample pots were selected during soil preparation and were weighed before and after saturating the soil with water at 100% field capacity (Fc 1.0) to calculate the soil water content (g of water/g of dry soil in the pot) at Fc 1.0. The water supply was progressively reduced until 30% of Fc 1.0, and water stress conditions were assessed when the average reading of the stress-exposed pots reached the required soil water content. In order to mitigate the fluctuations in soil water content, all pots were weighed at 6 pm daily and the amount of water needed to maintain the desired soil field capacity was then added.

2.2. Vine Growth and Fruit Parameter

Following bud-break, the internode elongation rate and leaf area increase were measured on four primary shoots per treatment on a weekly basis throughout the entire growing season. Internode length was measured from the lower side of one node to the lower side of the node above using a

ruler. Leaf length (L) was measured along the midrib from the tip of the lamina to the point where the lamina intersected with the petiole, while leaf width (W) was measured across the widest part of the leaf lamina perpendicular to the lamina mid-rib.

The leaf expansion rate was determined as the daily increase in leaf area (LA) calculated from the daily increase in leaf length (L) and maximum leaf width (W). The values of L and W were rounded to the nearest 0.1 cm. At the end of the season, L and W measurements were taken from a sample of 420 leaves and LA was measured using an area meter (LI-3100; LICOR, Lincoln, NE, USA) calibrated to 0.01 cm². The relationship between the biometric measurements and leaf area was evaluated by fitting regression models with the linear regression procedure. The internal validity of the model was tested by coefficient of determination (R²) and root mean square error (RMSE) [37,38]. LA was the dependent variable and the independent variable was the product $L \times W \times a$; the fitting procedure allowed the minimum sum of squares to be achieved by optimizing the “a” variable, the result being 0.944048 with an R² of 0.96 and an RMSE of 4.52 cm².

Internode extension and leaf area increments in grapevine shoots have been described as being sigmoidal over time and can be summarized in four stages [39,40]: Stage I, during which elongation is exponential; Stage II, during which the extension rate increases rapidly; Stage III, during which the extension rate is essentially constant and internode length increases linearly; and Stage IV, during which the extension rate decreases as the internode approaches its final length and the leaf its final area. Internodes were grouped into 5 classes from the basal to the apical: 1–10 (class I), 11–20 (class II), 21–30 (class III), 31–40 (class IV), and 41–50 (class V) [38].

At harvest, all the clusters on all the vines under each treatment were counted and the total fresh cluster weight per vine was recorded. The clusters were immediately brought to the laboratory and bunch fresh weight, berry diameter (50 berries), soluble solids content (°Brix), total acidity (g L⁻¹), and pH were measured in three replicates per treatment. For the biomass measurements, four plants of each treatment were harvested and divided into five vine types: primary stems, lateral stems, leaves (including the petioles that had developed on the primary and lateral stems), roots, and woody truck. All biomass measurements were expressed in dry weight after drying the samples in an oven at 80 °C until a constant weight was reached. Shoot weight was calculated as the sum of the weight of the leaves and the primary and lateral stems. Total dry weight did not include the residual woody parts. Data were expressed as means ± standard errors.

2.3. Leaf Physiological Parameters

The stem water potential (SWP) was measured at noon using a Scholander-type pressure chamber (model PMS-600; PMS Instruments, Corvallis, OR, USA) fitted with a compressed air cylinder. Six randomly chosen, sun-exposed, and fully expanded leaves per treatment, which had been enclosed in an opaque plastic bag for more than 1 h to prevent transpiration and allow them to reach equilibrium with the water potential in the stems [41], were measured from solar noon until the early afternoon. Each leaf was excised from the shoot with a scalpel blade and placed in the pressure chamber with the petiole protruding from the chamber lid. The chamber was pressurized using an air pressure tank, and SWP was recorded as soon as the xylem sap was observed emerging from the cut end of the petiole; pressurization was begun no later than 10 sec after leaf cut.

Chlorophyll content was measured with a SPAD meter (Minolta SPAD-502 chlorophyll meter). On each measurement day three readings per leaf were acquired from leaves randomly chosen from the basal, median and apical part of the shoot of plants of each treatment.

2.4. Statistical Analysis

All data analyses were carried out using the R software package (version 3.5.2; R Core Team, Vienna, Austria). An analysis of variance (ANOVA) of the data was conducted after checking for homogeneity of error variances (Bartlett test), which was verified for each variable studied. When a repeated measure ANOVA was performed, the statistical decision rule at $\alpha = 0.05$, $\alpha = 0.01$ or $\alpha = 0.005$

was deemed significant after applying the Bonferroni correction for multiple comparisons each time a set of pairwise contrasts was used. When treatments and interaction effects were significant, differences between the treatment means were compared using Tukey's test.

3. Results

3.1. Evapotranspiration and Stem Water Potential Trend

Air temperature and photosynthetic photon flux density (PPFD) measured by the weather station located inside the experimental tunnel indicated stable, sunny weather conditions throughout the experimental period, with the exception of 16 and 17 July (DOY 197–198), allowing progressive water deficit to be established through the reduction in the water supply (Figure S1). The extent of evapotranspiration, which gives an indirect estimate of the vine's water status [42], was measured throughout the experimental period by weighing the sample pots each day in the late afternoon before irrigation (Figure 1). No significant differences were observed in either control or stressed plants in response to biostimulant provision. However, as expected, significantly lower levels of evapotranspiration were observed in plants subjected to water deficit on all the dates it was measured, during both the periods of stress and recovery, regardless of whether or not PH was applied (Figure 1).

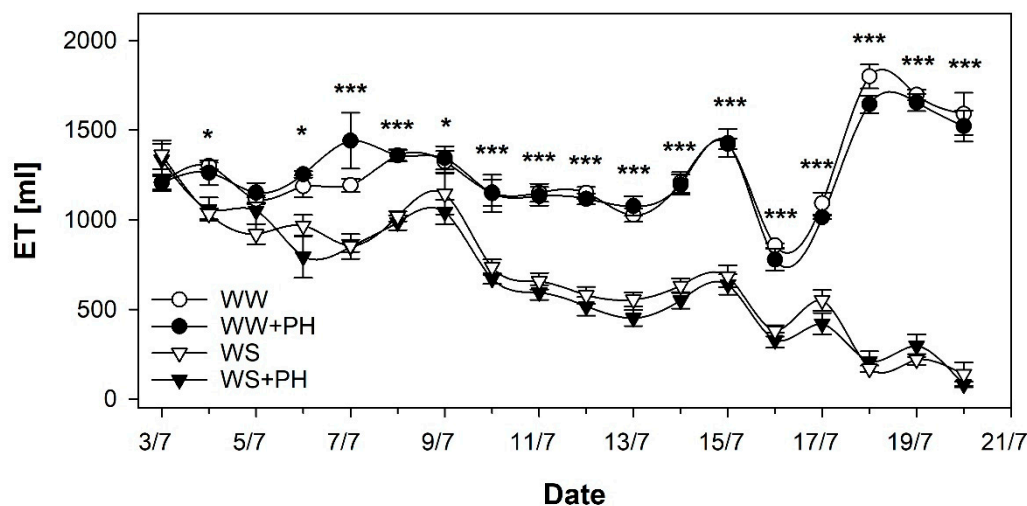


Figure 1. Effects of water stress (WS) compared with well-watered conditions (WW) and PH application, on soil water content. The experimental pots were weighed daily before soil water adjustment in order to measure the daily water loss by evapotranspiration (ET). Values are means $\pm$ SE ($n = 8$). Asterisks indicate significant differences among treatments according to repeated measurements ANOVA ($p < 0.0083$, $p < 0.002$ and $p < 0.0008$ after sequential Bonferroni adjustment for $\alpha = 0.05$ (*) and $\alpha = 0.005$ (**), respectively).

Water stress was induced by reducing the irrigation in the WS and WS + PH treatments the day before the first day of measurements (DOY 186). Figure 2 shows the evolution of SWP in the Sauvignon blanc vines over the duration of the experiment. At days 1 (DOY 187) and 3 (DOY 190) after stress induction, there were no significant differences in the SWP values between treatments. From day 7 (DOY 193) of stress induction onwards, the SWP values of WW and WW + PH were significantly greater than those of WS and WS + PH. The SWP values of the latter (WS + PH), however, were greater than those of the relevant control (WS) and were not significantly different from those of WW and WW + PH 9 days after stress induction (DOY 195). SWP values decreased with progressive water restrictions (DOY 201) and reached minimum values of -1.6 MPa for WS + PH and -1.7 MPa for WS on the last day of stress (DOY 201). No significant differences in SWP values between treatments were found upon recovery to well-watered conditions (DOY 207).

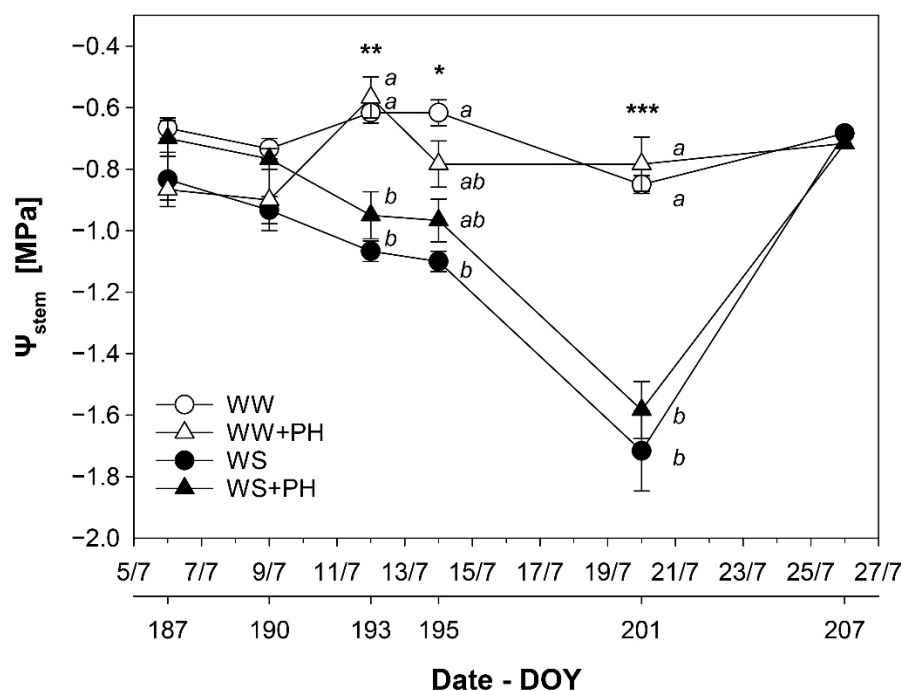


Figure 2. Effects of water stress (WS) compared with well-watered conditions (WW) and PH application, on stem water potential (Ψ_{stem}) (MPa). Values are means $\pm$ SE ($n = 6$), different letters indicate significant differences according to Tukey's test ($p < 0.0083$, $p < 0.002$ and $p < 0.0008$ after sequential Bonferroni adjustment for $\alpha = 0.05$ (*), $\alpha = 0.01$ (**), and $\alpha = 0.005$ (***), respectively).

3.2. Biometric Measurements

To evaluate the effects of water deficit and the biostimulant on grapevine growth, individual leaf area expansion and stem internode elongation were measured starting from the day the biostimulant was applied (Figures 3 and 4). Overall shoot length did not vary significantly as a result of internode extension kinetics in the different treatments (Figure 3A). To understand the shoot growth dynamics in greater detail, the growth of individual internodes was measured throughout the season (Figure 3B–F). To this end, internodes were grouped into 5 classes from the basal to the apical: 1–10 (class I), 11–20 (class II), 21–30 (class III), 31–40 (class IV), and 41–50 (class V), and their elongation dynamics were averaged separately. While in the basal internode classes the same trend as in the total shoot analysis was observed, application of PH resulted in significantly longer internodes in the apical class V, comprising internodes 41–50 that were actively growing at the time water stress was imposed. For this class of internodes, water stress strongly inhibited internode growth, but the application of the biostimulant prevented this effect giving rise to phenotypes with no significant differences from those of the plants under the well-watered conditions (Figure 3F). Leaf area growth dynamics exhibited the same behavior (Figure 4).

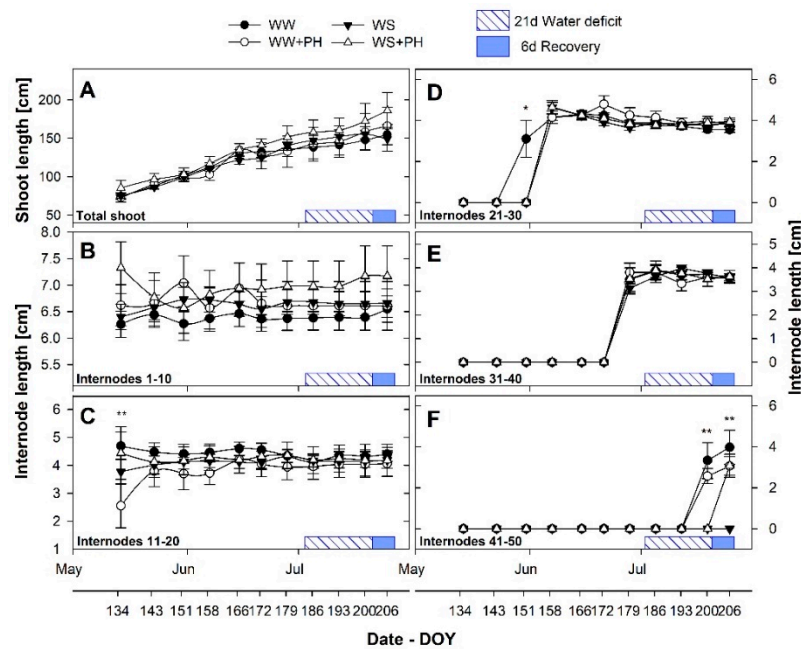


Figure 3. (A) Effects of water stress (WS) compared with well-watered conditions (WW) and PH provision, on shoot length growth dynamics throughout the season. Elongation dynamics of entire shoots (cumulative for all leaves and nodes) (A) and of mean internode classes (B–F): 1–10 (class I) (B), 11–20 (class II) (C), 21–30 (class III) (D), 31–40 (class IV) (E), and 41–50 (class V) (F). Values are means $\pm$ SE ($n = 6$), asterisks indicate significant differences among treatments according to repeated measurements ANOVA ($p < 0.0083$, $p < 0.002$ and $p < 0.0008$ after sequential Bonferroni adjustment for $\alpha = 0.05$ (*) and $\alpha = 0.01$ (**), respectively).

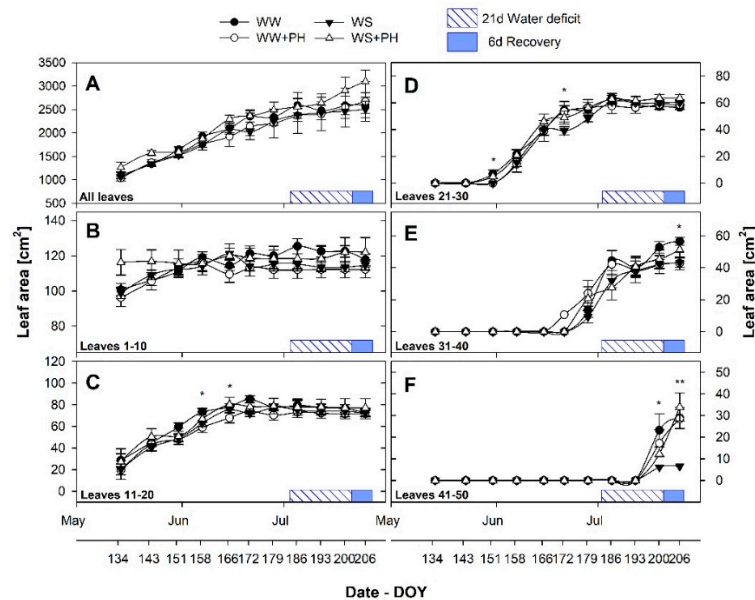


Figure 4. (A) Effects of water stress (WS) compared with well-watered conditions (WW) and PH application, on primary shoot leaf area growth dynamics throughout the season. Dynamics of growth in cumulative leaf area of shoots (A) and in mean leaf area classes (B–F): 1–10 (class I) (B), 11–20 (class II) (C), 21–30 (class III) (D), 31–40 (class IV) (E), and 41–50 (class V) (F). Values are means $\pm$ SE ($n = 6$), asterisks indicate significant differences among treatments according to repeated measurements ANOVA ($p < 0.0083$, $p < 0.002$ and $p < 0.0008$ after sequential Bonferroni adjustment for $\alpha = 0.05$ (*) and $\alpha = 0.01$ (**), respectively).

Overall, no significant differences in leaf area were observed for almost all the foliar stages examined in relation to PH application, except for the youngest leaves of class V (leaves 41–50), on which the PH induced a highly significant effect in water stressed plants (Figure 4F). As the figure shows, the application of the biostimulant triggered a sustained, significant internode and leaf area increment in the youngest organs as they developed, even under severe water stress conditions. This effect continued into the recovery phase.

3.3. SPAD Measurements

SPAD readings were taken on basal, median, and apical leaves along the shoots of the vines to assess the effect of both water stress and PH application on chlorophyll content, which has been shown to be a reliable indicator of the N status of crops as it is highly correlated with leaf N content (Figure 5) [43,44].

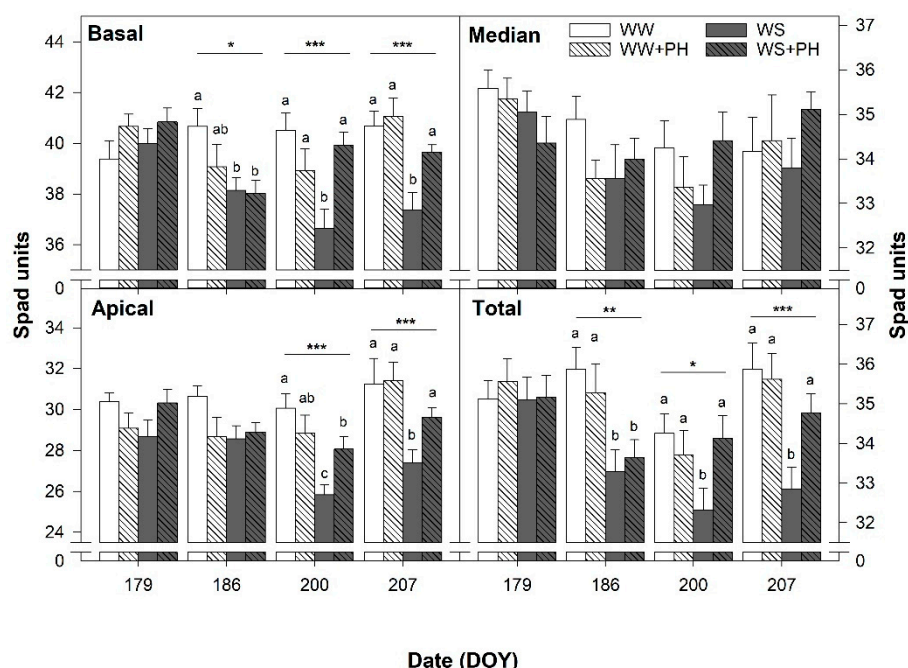


Figure 5. Effects of water stress (WS) compared with well-watered conditions (WW) and PH application, on the leaf chlorophyll content of basal, median, apical, and all (total) leaves measured as SPAD units before the drought trial (DOY 179), under light (DOY 186) and severe water stress (DOY 200), and upon recovery to well-watered conditions (DOY 207). Means $\pm$ SE values indicated with the same letters are not significantly different according to Tukey's test ($p < 0.0083$, $p < 0.002$ and $p < 0.0008$ after sequential Bonferroni adjustment for $\alpha = 0.05$ (*), $\alpha = 0.01$ (**) and $\alpha = 0.005$ (**), respectively).

In general, basal and apical leaves, the oldest and youngest respectively, showed a significant decrease in SPAD values as stress progressed, while the median leaves exhibited no significant effects. This specific trend was consequently clearly visible in the total SPAD values, obtained by averaging the basal, median, and apical leaves together, which were significantly lower in plants subjected to water deficit conditions after light water stress had already been imposed (DOY 186) (Figure 5).

However, the biostimulant markedly increased the SPAD values for both basal and apical leaves during severe stress conditions (DOY 200) and during the subsequent recovery phase (DOY 207), giving rise to phenotypes with no significant differences from those of unstressed plants (Figure 5).

3.4. Root and Shoot Development and Berry Diameter

To assess the overall effect of the PH treatment on grapevine plant development, the total root and shoot biomass were evaluated at the end of the vegetative season (Figure 6).

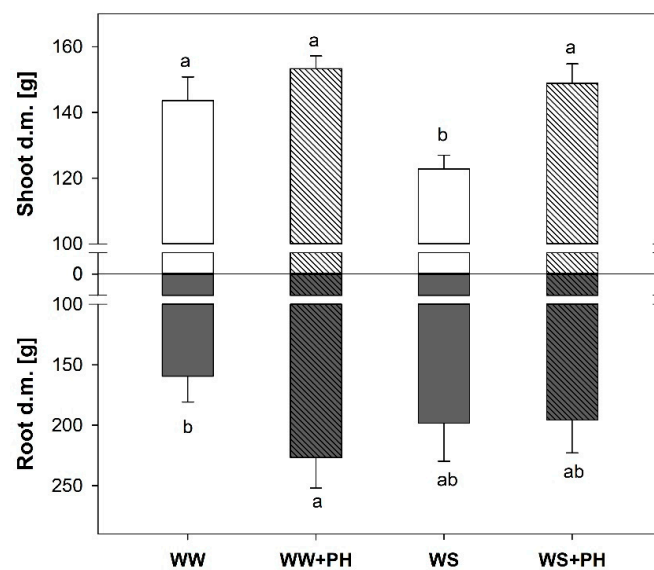


Figure 6. Effects of water stress (WS) compared with well-watered conditions (WW) and PH application, on dry matter partitioning among shoots (comprising the sum of the weight of the leaves and the primary and lateral stems, but not including the residual woody cutting), and roots. Values are means $\pm$ SE ($n = 4$), different letters indicate significant differences according to Tukey's test ($p < 0.05$).

As far as the effect of stress was concerned, a notable decrease in shoot growth and a slighter increase in root development were observed. The negative impact of water stress on shoot development was entirely reversed in stressed plants treated with PH, which led to full restoration of shoot growth, possibly by stimulating the development of the shoot tip, comprising the apical internodes and youngest leaves, as shown above (Figures 3 and 4). However, no significant effects on the root biomass of stressed plants treated with PH were observed, yet there was significant root biomass accumulation in non-stressed plants in response to the biostimulant, suggesting that PH acts differently on root and shoot growth depending on the stress level (Figure 6).

The effectiveness of the PH was also evaluated in terms of its effects on fruit development, quantified by measuring the berry diameter, which increased significantly in response to the biostimulant in both control and stress conditions (Figure 7). No significant differences were observed in the number of clusters per vine and their weight, nor in the soluble solids content ($^{\circ}$ Brix), total acidity, and pH of the berries of either control or water-stressed plants, regardless of the absence or presence of PH treatment (Table 1).

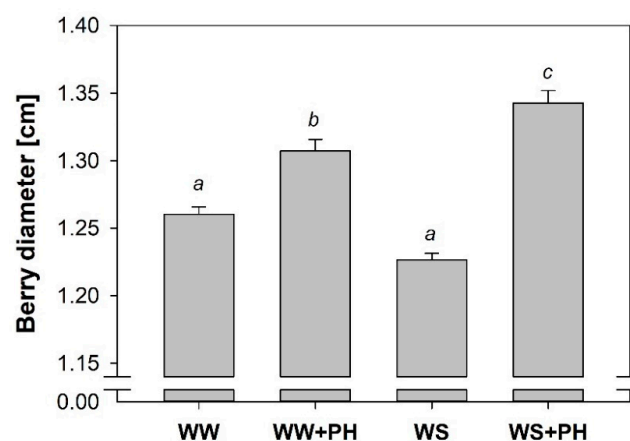


Figure 7. Effects of water stress (WS) compared with well-watered conditions (WW) and PH application, on berry diameter at harvest. Values are means $\pm$ SE of 50 berries in three replicates per treatment. Different letters indicate significant differences according to Tukey's test ($p < 0.05$).

Table 1. Effects of water stress (WS) and PH application on cluster numbers, cluster weight, and grape composition at harvest compared with well-watered conditions (WW). Values are means $\pm$ SE of 50 berries in three replicates per treatment. *ns* = no significant differences according to Tukey's test ($p \leq 0.05$).

	Mean Cluster Number	Mean Cluster Weight	Soluble Solids	Total Acidity	pH
	<i>n</i>	<i>g</i>	°Brix	<i>g L⁻¹</i>	
WW	1.42	100.94	22.44	1.51	3.08
WW + PH	1.75	112.29	21.73	1.06	3.06
WS	1.92	97.09	24.00	1.23	3.09
WS + PH	2.00	112.71	23.30	1.22	3.13
<i>p</i> -value	0.186	0.707	0.073	0.165	0.483
	<i>ns</i>	<i>ns</i>	<i>ns</i>	<i>ns</i>	<i>ns</i>

4. Discussion

Increases in aridity and shifts in the amounts, seasonality, and distribution of precipitation are predicted to occur in the Mediterranean region more regularly over the coming decades [45], with severe consequences for viticulture [46].

In order to better manage climate-related risks, viticultural practices should be reevaluated [47], and in this context the use of biostimulants represents an innovative and sustainable tool for mitigating the negative effects of abiotic stresses, especially water stress [48,49].

This study has shown the effectiveness of a protein hydrolysate (PH) in improving the tolerance of grapevine (cv Sauvignon blanc) to water deficit.

Previous papers have shown that application of this same compound affected the expression of a thousand genes in maize roots, most of which were involved in regulating oxidative stress signaling and response [33]. When the protein hydrolysate was supplied in a hydroponic solution to maize seedlings which were then subjected to various abiotic stresses, the plants' tolerance was increased, likely as a result of modulation of the transcription of a set of genes involved in ROS detoxification and nutrient acquisition [34]. Ebinezer et al. [50] have also hypothesized that this compound, and PHs in general, could exert this effect by regulating the control of ROS homeostasis and the response to oxidative stress.

To our knowledge only a few experiments have been conducted to test the effectiveness of biostimulants on perennial fruit crops, and especially grapevine. Moreover, in the few available studies, the products were applied exclusively as foliar treatments [12,32,51–53].

In the present work, a PH was applied directly to the soil (roots) 48 days before stress imposition, and its effects on plant growth performances were assessed throughout the period of stress and afterwards during the recovery phase (re-watering). It is important to point out that the most striking differences between the treated and untreated plants were observed in the presence of stress conditions, consistent with the view that a common feature of PHs' effectiveness as enhancers of stress tolerance [24,25]. Through a detailed survey of biometric parameters, we have provided evidence that APR[®], applied preventively to the roots, counteracts or alleviates the detrimental effects of water stress by stimulating plant development in water deficit conditions.

As expected, the stem water potential of the grapevine plants was highly affected in response to water shortage, after just a few days of stress and throughout the entire duration of the water deprivation period, as also shown by Choné et al. [41] and Meggio et al. [54] for the same species.

Stem water potential values are reliable indicators of plant hydric status, hence allowing the actual extent of stress to be assessed [6,55,56]. The application of the biostimulant significantly alleviated the negative effects of water stress, even though the SWP values were lower than those of unstressed plants. Nevertheless, this result suggests that this compound could mitigate the severity of the effects of water deficit and could be already helping cells to counter cell dehydration soon after stress imposition.

To assess the long-term effects of the treatment, biometric measurements were taken throughout the entire duration of the experiment. The effects of APR[®] were evident from observation of both the length of the internodes and the area of the leaves on the most apical part of the shoots. In fact, the growth of the internodes and leaves in the 41–50 range (class V), i.e., those actively growing during the period of water deprivation, significantly increased in plants subjected to water stress and supplied with the PH compared with the untreated plants. Indeed, applying the biostimulant to the roots prior to water stress enabled the youngest tissues—those that were in active growth during stress imposition—to maintain an adequate growth rate, albeit lower than that of the control plants. Nevertheless, this slight advantage with respect to the stressed plants that had not been treated with the biostimulant, later in the recovery phase turned into a clear benefit in terms of growth for the treated plants. These results are consistent with the previously discussed findings regarding leaf water potential and indicate less dehydration in PH-treated plants, suggesting also improved metabolism. Trevisan et al. [34] previously showed the same compound to have a positive effect on the growth of maize plants subjected to abiotic stresses, suggesting that it has a general aptitude for stimulating growth by counteracting the growth limitations imposed by stress.

Similarly, Oancea et al. [57] showed that applying a micro-algae-based biostimulant to water-stressed tomato plants increased plant height, root length, and leaf number and area.

The effect of the biostimulant application on biomass accumulation was evident also at the end of the vegetative season. The significant decrease in shoot growth in response to water stress was completely reversed in PH treated plants, which exhibited a similar aerial biomass to that of the unstressed plants, likely as a consequence of the above described sustainment of shoot tip growth. However, application of the compound to the roots of water-deprived plants had no significant influence on the biomass of this organ, suggesting that in stress conditions its bioactivity is totally concentrated in the aerial organs, unlike in the control conditions. Our results suggest that the biostimulant has two different action mechanisms for roots and shoots depending on the presence or absence of stress conditions.

SPAD measurements, likely reflecting the physiological state of the leaves in terms of chlorophyll content and N status [43,44,58,59], were also highly influenced by water deprivation. Plants supplied with the biostimulant had values that were essentially equivalent to those of unstressed plants, an effect that was evident in the basal (older) and apical (younger) leaves.

The SPAD results are consistent with those for leaf water potential and suggest that the preventive application of the biostimulant to roots may support primary cellular and physiological activity, thus limiting the extent of the stress-induced senescence that was otherwise observed in untreated plants.

Leaf yellowing due to chlorophyll degradation is a reliable indicator of drought-derived metabolic disorders and senescence in plants [60].

An increase in chlorophyll content in response to *A. nodosum* [30] and Megafol [61] has previously been reported in tomato plants subjected to water stress after PH application.

Regardless of the presence of a stress, many authors have reported an increase in chlorophyll content in response to various biostimulants (for a review see Bulgari et al. [62]).

PH application also positively influenced the berry diameter in both control and stressed conditions. In line with this result, Ullah et al. [63] showed that moringa leaf extracts stimulated fruit weight, volume, and firmness, and enhanced titratable acidity in tomato plants. Our data did not reveal any differences in titratable acidity among treatments, nor in soluble solids content or pH at harvest/berry maturity. It is worth noting that pre-treatment of grapevine plants with the PH importantly ensured maintenance of berry growth throughout the period of active berry growth, while maintaining the quality of the berry juice, evidenced by the uniform size of the berries and by the soluble solids and titratable acid contents.

Overall, the results suggest that applying the biostimulant preventively to roots before the imposition of water stress could protect the plants by enhancing their stress tolerance, confirming Trevisan et al.'s previous suggestion [33,34], and supporting the role of biostimulants as stress tolerance enhancers [24,25].

It is widely recognized that protein hydrolysates can improve nutrient uptake through different mechanisms, including the modification of the root architecture, the complexation of nutrients by peptides and amino acids, or the stimulation of microbial activity [26]. Indeed, the enhanced growth of the aerial organs reported here could also be an indirect consequence of the above mentioned features. The effects of the biostimulant on the root biomass of water-stressed plants appeared to be negligible, even though positive significant effects on the root biomass of control unstressed plants were observed. We may therefore hypothesize that the positive effects indirectly exerted by the compound on the upper organs may be due to an overall improvement in root physiology (e.g., nutrient uptake).

In general, amino acids have been demonstrated to improve the response to several abiotic stresses in plants [64–67]. Moreover, amino acids can form relatively stable complexes with metals i.e., zinc (Zn) via carboxylic groups [68].

The aminogram of this compound allowed to identify lysin as the most represented amino acid and the chemical analyses revealed a high Zn content [35]. Previous studies showed the effectiveness of micronutrient and amino acid complexes for enhancing plant growth and yield and showed that Zn complexed with lysine, methionine, and threonine could be the most effective combinations [69]. In addition, lysine per se, besides being an important essential amino acid, is also a precursor of glutamate [70] which is widely recognized as a crucial molecule for the plants stress signaling and response [71,72].

Based on the present results and on the chemical features of this compound [35], it may be hypothesized that the increased tolerance to water deficit induced after this treatment might at least in part be an indirect effect of an improved micronutrient uptake and of an increased level of glutamate. Previous studies [33,34,50] have already conjectured that this PH could act as a cue in priming the plant's defenses against environmental stresses through molecular regulation of the signaling pathways underlying the response to oxidative stress. It has also been proposed that the effectiveness of chitosan-based products as plant protectors is based on their ability to interfere with the ROS scavenging machinery in many species [73–76]. These findings lead us to hypothesize that various biostimulants, independently of their origin, share common molecular action mechanisms, mainly based on strengthening the ROS detoxification pathways. This hypothesis opens new promising scenarios and new opportunities for the early screening, phenotyping and development of new putative biostimulants.

This study provides further knowledge on the activity of these types of molecule in semi-controlled conditions and on a fruit crop, strengthening their promising potential as biostimulants for application in the field. Furthermore, these preliminary results highlight the importance of roots as the proper target for protein hydrolysate application and demonstrate the existence of long-term effects deriving from a single root application before exposure to stress.

Further studies should be carried out to gain a better understanding of the mode of action of these compounds and to further assess the reliability of their application in the open field with the aim of allowing precise protocols to be established for their effective utilization in vineyards.

Supplementary Materials: The following is available online at <http://www.mdpi.com/2073-4395/10/11/1785/s1>: Figure S1: Meteorological trend.

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