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Strategy to Mitigate Recurring Water Deficit in Black Pepper Using Melatonin, *Ascophyllum nodosum*, *Lithothamnium calcareum*

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Abstract

Water deficit is one of the main limiting factors in agricultural production, affecting the growth and photosynthetic performance of plants. The aim of this study was to evaluate the effects of foliar applications of melatonin, *A. nodosum* and *L. calcareum* on black pepper, 'Bragantina', subjected to three cycles of water deficit, on physiological performance, carbohydrate allocation and vegetative growth. The results showed that the applied solutions contributed to the recovery of damage caused by water stress. The first cycle of water deficit affected the electron transfer quantum yield, resulting in the reduction of φP_0 and PI_{ABS}. However, the damage to the photosynthetic apparatus was recovered in subsequent cycles. Water stress reduced chlorophyll indices, possibly as a strategy to minimize photo-oxidative damage. Plants treated with melatonin and *L. calcareum* maintained a stable leaf expansion rate and showed greater water recovery, standing out in the second and third cycles of water deficit.

Keywords: Biostimulants; chlorophyll *a* fluorescence; drought cycles; growth regulator; *Piper nigum* L.; seaweed

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1. Introduction

Black pepper (*Piper nigrum* L.) is the most popular condiment in the world [1]. In 2022, the world produced approximately 812,673.57 tons, with Brazil being the second largest producer, behind only Vietnam (FAO 2025). However, the crop faces production declines due to reduced rainfall [3]. In Brazil, water deficit is one of the main challenges for the expansion of the crop [4].

Little is known about the effect of recurring water deficit on black pepper; however, Ferreira *et al.* [5] reported that water deficit reduced stomatal conductance, photosynthetic rate, transpiration, water potential, leaf area, and number of leaves. However, the cultivar 'Bragantina' exhibited an adaptive response, progressively extending its tolerance to water stress with each new water deficit cycle. Despite these adaptations, the plants were unable to resume vegetative growth after rehydration [5].

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A promising alternative is the use of biofertilizers, these products have emerged as effective tools to improve plant nutrition and increase the plants' ability to tolerate environmental stresses [6]. Biostimulants based on seaweed extract such as Ascophyllum nodosum contain a wide variety of substances, including phenolic compounds, alginic acid, mannitol, laminarin, betaines, proteins, lipids and several mineral nutrients in different concentrations [7]. Among red seaweeds, the genus *Lithothamnium* also has biostimulant potential, as its organic fraction is rich in humic acids with auxinic activity [8]. In addition to seaweed, melatonin (N-acetyl-5-methoxytryptamine) is a biological regulator that modulates physiological and biochemical mechanisms, stimulating plant growth [9]. Biodegradable and non-toxic, it is recommended for sustainable agriculture [10]. Naturally present in plants, its exogenous application induces changes that strengthen resistance to abiotic stresses [9].

It is known that under water deficit, *A. nodosum* and melatonin helped to regulate the osmotic potential in tomato, grapevine, wheat and potato [11, 12, 13]. In wheat, melatonin increased maximum quantum efficiency and root growth [11], while *A. nodosum* increased chlorophyll contents [14]. Previous studies indicated that melatonin at 100 μM L^{-1} applied via foliar and root significantly alleviated the adverse effects of recurrent water stress in black pepper [15]. In sugarcane, *Lithothamnium* with vinasse increased sucrose levels [16], and in grapevines, there were improvements in photosynthetic parameters [17].

Although the efficacy of seaweed and melatonin has been proven in several production systems, there are gaps regarding their effects on black pepper cultivation under recurring water deficit. Thus, the objective of this study was to evaluate the effects of foliar applications of Melatonin, *A. nodosum* and *L. calcareum* on 'Bragantina' black pepper plants subjected to recurring cycles of water deficit, focusing on physiological performance, carbohydrate allocation and vegetative growth.

2. Results

2.1. Water Potential

The results obtained for the Ψ_{Leaf} of the plants showed significant differences between the treatments and the cycles of water deficit and recovery (Figure 1). In DRY1, DRY2 and DRY3, the treatments subjected to water deficit presented higher values of Ψ_{Leaf} compared to the control (Figure 1). However, in the recovery REC1, WD *A. nodosum* presented higher values of Ψ_{Leaf} than the other treatments, while in REC2 and REC3 no statistically significant differences were observed between the treatments (Figure 1).

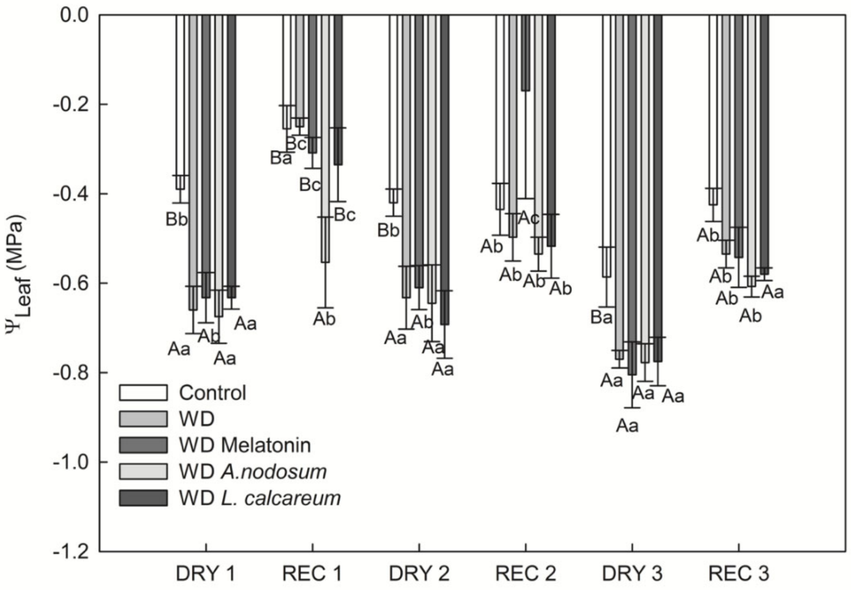

Figure 1. Effects of Control, WD, WD Melatonin, WD *A. nodosum* and WD *L. calcareum* treatments on leaf water potential (Ψ Leaf) of 'Bragantina'. Plants were subjected to three cycles of water deficit (DRY1, DRY2 and DRY3) and recovery (REC1, REC2 and REC3). During the cycles, the WD, WD Melatonin, WD *A. nodosum* and WD *L. calcareum* treatments were subjected to water deficit, while the Control was kept irrigated. The bar corresponds to the standard error of four replicates containing one plant per plot. Means followed by the same letter do not differ from each other by the Scott-Knott test ($p < 0.05$). Capital letters compare treatments within the same water regime, while lowercase letters compare water regimes among themselves.

In the WD treatment, the difference between the values of Ψ_{Leaf} during the cycles of water deficit and recovery was greater in the first cycle. DRY1 had a value of –0.66 MPa, which was recovered to –0.25 MPa in REC1, representing a recovery of 62.12% (Figure 1). In DRY2 and DRY3, the recoveries were lower: 22.22% and 31.17%, respectively. In the WD melatonin treatment, DRY1 had a value of –0.63 MPa, which was recovered to –0.31 MPa in REC1, representing a recovery of 50.79%. In the DRY2 and DRY3 cycles, the recoveries were 27.87% and 32.50%, respectively (Figure 1).

In the WD *A. nodosum* treatment, DRY1 had a value of –0.67 MPa, which was recovered to –0.55 MPa in REC1, resulting in a recovery of 17.91%. In DRY2 and DRY3, the recoveries were 17.19% and 22.08%, respectively (Figure 1). In the *L. calcareum* treatment, DRY1 had a value of –0.63 MPa, recovered to –0.33 MPa in REC1, representing a recovery of 47.62%. In DRY2, the recovery was 26.03%, with no recovery in the last cycle (Figure 1).

2.2. Chlorophyll a Fluorescence

For the variables related to Chlorophyll *a* fluorescence, no significant effects of the interaction between treatments and water deficit and rehydration cycles were observed. Thus, the analysis was performed independently. In the maximum photochemical quantum yield (φP_0), there was a significant difference between the water conditions. The values of the first water deficit and recovery cycle were lower than those of the subsequent cycles (Figure 2A). For the energy absorption flux per reaction center (ABS/RC), an increase was observed in the first water deficit and recovery cycle compared to the two subsequent cycles (Figure 2B).

In the energy captured flux per reaction center (TR_0/RC), DRY1 presented a higher value than the other water conditions (Figure 2C). A higher dissipated energy flux per reaction center (DI_0/RC) was observed in the first drought and rehydration cycle, while the two subsequent cycles presented significantly lower values, with no significant differences between them. The number of active PSII reaction centers per cross section (RC/CS_0) was higher in REC2. Among the treatments, WD *A. nodosum* presented higher averages (Figure 2*E*).

The electron transfer quantum yield of Q_A – for the electron transport chain beyond Q_A– (φE_0) and the performance index for energy conservation of photons absorbed by PSII for the reduction of intersystemic electron acceptors PI_{ABS} followed the behavior of φP_0 (Figure 2A,F,G). Both presented lower values in the first water deficit and recovery cycle compared to subsequent cycles (Figure 2F,G). Among the treatments, WD melatonin and WD *L. calcareum* showed φE_0 values similar to the control (Figure 2F), while PI_{ABS} was lower only for WD *A. nodosum* (Figure 2G).

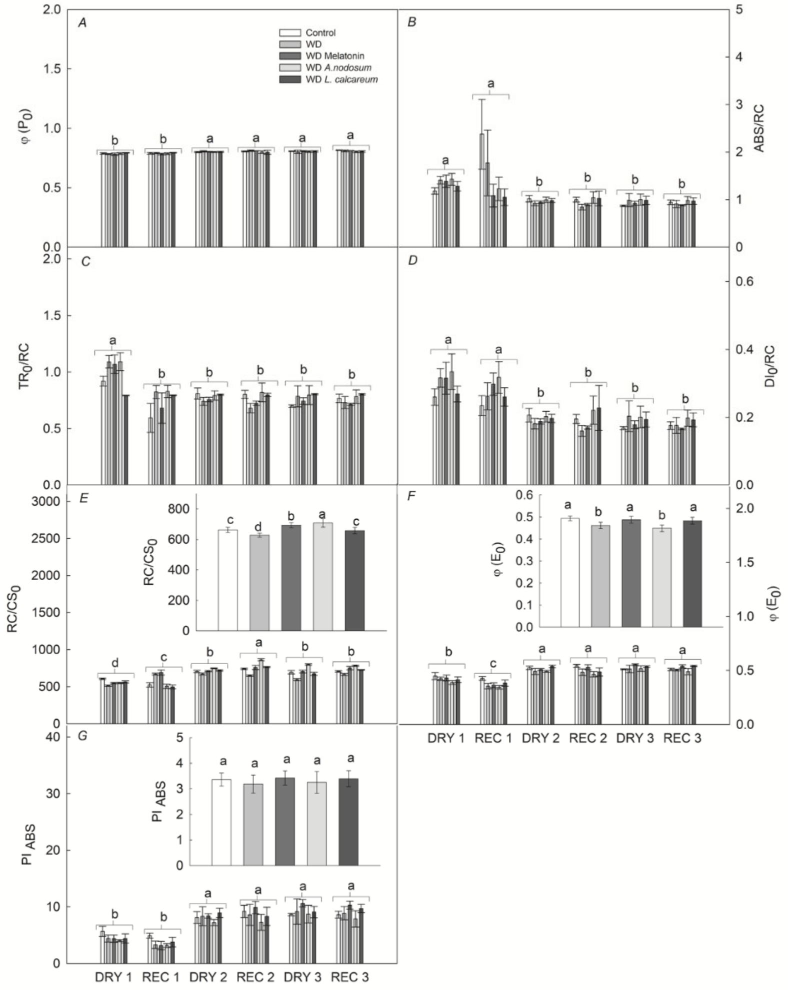

Figure 2. Effect of the treatments Control, WD, WD Melatonin, WD *A. nodosum* and WD *L. calcareum* on the photochemical parameters derived from the analysis of the JIP test of 'Bragantina' subjected to three cycles of water deficit (DRY1, 2 and 3) and recovery (REC1, 2 and 3). During the cycles, the treatments WD, WD Melatonin, WD *A. nodosum* and WD *L. calcareum* were subjected to water deficit, while the Control was kept irrigated. The bar corresponds to the standard error of four replicates containing the mean of two plants per plot. Means followed by the same letter do not differ from each other by the Scott-knott test ($p<0.05$). *A* – Maximum photochemical quantum yield (φP_0), *B* – energy absorption flux per reaction center (ABS/RC), *C* – captured energy flux per reaction center (TR_0/RC), *D* – dissipated energy flux per reaction center (DI_0/RC), *E* – number of active PSII reaction centers per cross sections (RC/CS_0), *F* – electron transfer quantum yield from Q_A- to the electron transport chain beyond Q_A- (φE_0), *G* – performance index for energy conservation of photons absorbed by PSII for the reduction of PI_{ABS} intersystem electron acceptors.

2.3. Photosynthetic Pigments

For the variables related to photosynthetic pigments, in which no significant effects were observed in the interaction between treatments and water deficit and rehydration cycles, the analysis was performed independently. In the chlorophyll a index (Chl *a*), significant differences were observed between water deficit and rehydration cycles, with REC3 presenting higher values compared to the other water conditions (Figure 3 A). Among the treatments, WD *L. calcareum* was the only one that maintained a value similar to the control (Figure 3*A*).

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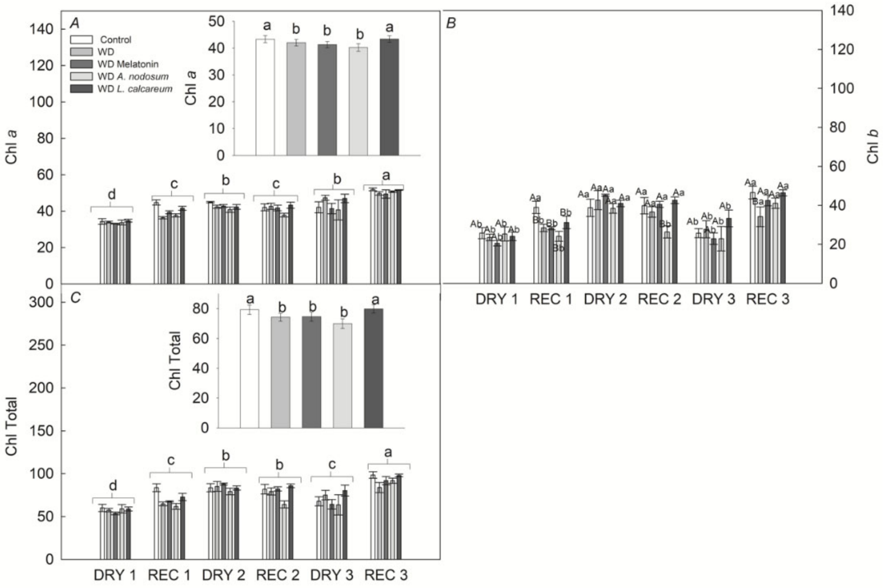

Figure 3. Control, WD, WD Melatonin, WD *A. nodosum*, WD *L. calcareum* and their effects on the photosynthetic pigment content of 'Bragantina'. The plants were subjected to three cycles of water deficit (DRY1, DRY2 and DRY3) and recovery (REC1, REC2 and REC3). During the cycles, the treatments WD, WD Melatonin, WD *A. nodosum* and WD *L. calcareum* were subjected to water deficit, while the Control was kept irrigated. Graphs A and C show the isolated effect of the treatments and the water deficit and recovery cycles. Graph B shows the significant interaction between the water deficit and recovery cycles. Capital letters compare the treatments within the same water regime, while lowercase letters compare the water regimes with each other. The bar corresponds to the standard error of four replicates containing the mean of two plants per plot. Means followed by the same letter do not differ from each other according to the Scott-Knott test ($p<0.05$). *A* – chlorophyll *a* (Chl *a*), *B* – chlorophyll *b* (Chl *b*), *C* – chlorophylls total (Chl Total).

As for the chlorophyll *b* index (Chl *b*), there was a significant interaction between treatments and water deficit and recovery cycles (Figure 3*B*). In the drought periods DRY1, DRY2 and DRY3, all treatments presented statistically similar values (Figure 3*B*). In the recovery periods, treatments subjected to water deficit reduced Chl *b* values compared to the control (Figure 3*B*). In REC2, there was only a reduction in the WD *A. nodosum* treatment, while in REC3, the WD treatment was the only one to show a reduction, when compared to the other treatments, which did not differ statistically from each other.

The Control reduced the Chl *b* value in DRY1 and DRY3, maintaining higher values in the other water conditions (Figure 3*B*). The WD, WD Melatoni and WD *L. calcareum* treatments also reduced the Chl *b* value in the first cycle of water deficit and rehydration, in addition to DRY3, while in the other water conditions they presented higher values, with no statistical differences between them (Figure 3*B*). The WD *A. nodosum* treatment followed a similar pattern, with a reduction in Chl *b* in the first cycle and in DRY3, however, in REC2, it also presented a reduction in the Chl *b* value (Figure 3B).

In the Chlorophyll Total Index (Chl Total), significant differences were observed between the water deficit and rehydration cycles, with REC3 presenting higher values compared to the other water conditions (Figure 3C). Among the treatments, WD *L. calcareum* was the only one that maintained a value similar to the control (Figure 3 C).

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2.4. Carbohydrate Allocation

In the levels of reducing sugars (RS) and total soluble sugars (TSS), there were no significant effects between treatments and vegetative organs, so the analyses were performed independently. However, regardless of the treatments, there was a greater accumulation of RS and TSS in the leaves, followed by the stem and root (Figure 4*A*,*B*).

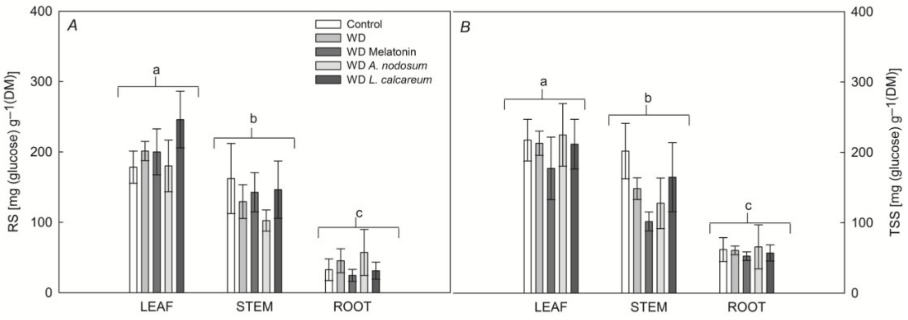

Figure 4. Control, WD, WD Melatonin, WD *A. nodosum*, WD *L. calcareum* and their effects on carbohydrate allocation of 'Bragantina'. Plants were subjected to three cycles of water deficit (DRY1, DRY2 and DRY3) and recovery (REC1, REC2 and REC3). During the cycles, the treatments WD, WD Melatonin, WD *A. nodosum* and WD *L. calcareum* were subjected to water deficit, while the Control was kept irrigated. The graph shows only the isolated effect of the organs. The bar corresponds to the standard error of four replicates containing the mean of six plants per plot. Means followed by the same letter do not differ from each other by the Scott-Knott test (p<0.05). *A* – reducing sugar (RS), *B* – total soluble sugar (TSS).

2.5. Vegetative Growth

There was a significant interaction between treatments and water deficit cycles and recovery for leaf expansion rate (Figure 5*A*). During the cycles, the treatments presented statistically similar values, except in the REC1 water condition, in which the treatments subjected to drought reduced the leaf expansion rate when compared to the control. In REC1, only the *L. calcareum* treatment remained statistically similar to the control (Figure 5*A*).

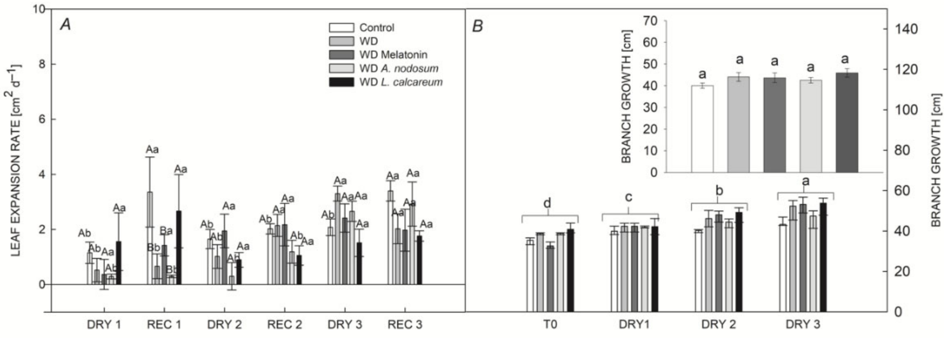

Figure 5. Control, WD, WD Melatonin, WD *A. nodosum*, WD *L. calcareum* and their effects on vegetative growth of 'Bragantina'. Plants were subjected to three cycles of water deficit (DRY1, DRY2 and DRY3) and recovery (REC1, REC2 and REC3). During the cycles, the treatments WD, WD Melatonin, WD *A. nodosum* and WD *L. calcareum* were subjected to water deficit, while the Control was kept irrigated. (T0) before the imposition of the cycles. Capital letters compare the treatments within the same water regime, while lowercase letters compare the water regimes with each other. The bar represents the standard error of four replicates, with one plant per

plot for leaf expansion rate, and six plants per plot for shoot growth. Averages followed by the same letter do not differ from each other according to the Scott-Knott test ($p<0.05$). *A* – Leaf expansion rate, *B* – Branch growth.

The control presented higher averages of the leaf expansion rate in REC1 and REC3 compared to the other water conditions (Figure 5*A*). The WD treatment had lower values in the first cycle and in DRY2, but was higher in REC2 and in the last cycle (Figure 5*A*). The WD *A. nodosum* treatment had higher values only in the third cycle. The WD melatonin and WD *L. calcareum* treatments did not present statistical differences between the water conditions (Figure 5*A*).

No significant effects of the interaction between treatments and water deficit cycles on branch growth were observed, allowing independent analysis of the factors (Figure 5*B*). A progressive increase in branch growth was observed throughout the water deficit cycles, with statistical differences between the values. The lowest growth was recorded in T0, followed by DRY1, DRY2 and, finally, the highest growth occurred in DRY3 (Figure 5*B*). Among the treatments, only WD *A. nodosum* showed lower branch growth compared to the control, while the other treatments maintained similar values (Figure 5*B*).

3. Discussion

In the present study, we evaluated the morphophysiological response of black pepper, ‘Bragantina’, subjected to three cycles of water deficit and rehydration, considering the effects of foliar applications of *A. nodosum*, *L. calcareum*, and melatonin. Water deficit is one of the main factors that affects photosynthesis and, consequently, plant growth, compromising essential processes for plant development [5]. Although *A. nodosum* is widely studied for its potential to alleviate water stress, there is little information on its effects on black pepper, ‘Bragantina’. There is also a lack of information on the role of *L. calcareum* under these conditions.

Melatonin has been investigated as a plant growth regulator, with evidence of its role in mitigating stress [30]. Although studies on the use of melatonin with black pepper, ‘Bragantina’, are scarce, Alagupalamuthirsolai *et al.* [15] investigated the application of the hormone melatonin to alleviate water stress in the cultivar. However, the study considered only one drought cycle. Thus, this study seeks to understand how these foliar applications of melatonin, *A. nododum* and *L. calcareum*, influence the resistance of ‘Bragantina’ to drought and its ability to recover throughout water deficit cycles.

It was observed that the Ψ_{Leaf} of the Control treatment increased significantly in REC1 and DRY3 (Figure 1). Although these plants were kept irrigated and the greenhouse had fans and a cooling system, the climate data from these periods indicate that they were under thermal stress, probably due to high temperatures and relative humidity. On May 31, the date of REC1 collection, the temperature varied between 20°C and 35°C, with relative humidity between 66% and 83%. On June 25, during DRY3 collection, the temperature ranged from 20°C to 33°C, while humidity ranged from 69% to 83%, which may have influenced this physiological response (Figure 1*A*,*C*). Furthermore, a statistically significant increase in Ψ_{Leaf} was observed in treatments subjected to water deficit WD, WD melatonin, WD *A. nodosum* and WD *L. calcareum* under DRY1, DRY2 and DRY3 conditions, compared to the control (Figure 1). Although there was no significant interaction between treatments and organs in TSS contents, leaves showed greater TSS accumulation (Figure 4*B*), since accumulating osmotically active solutes helps in the osmotic potential, contributing to the maintenance of osmotic pressure and to the reduction of ΨLeaf, a recognized strategy to deal with water stress [12, 14].

TSS availability has been used as a physiological indicator of tolerance to water stress, since carbohydrates provide energy and solutes essential for osmoprotective accumulation [14]. In this context, leaf carbohydrates play a central role in regulating osmotic potential, contributing to the preservation of relative water content and cellular functionality [12]. The greater accumulation of TSS levels in leaves suggests an adaptive mechanism that helps maintain water content.

WD melatonin showed a higher percentage of Ψ_{Leaf} recovery in the first cycle of water deficit. However, this percentage of recovery decreased throughout the subsequent cycles (Figure 1).

Nevertheless, in the second and third cycles, this treatment maintained the highest percentage of recovery compared to the other treatments subjected to drought. According to El-Yazied *et al.* [13], melatonin stimulates the production of osmotic solutes, such as sugars, which help minimize water loss and protect cell membranes, in addition to acting as scavengers of reactive oxygen species, through the synthesis of oxidizing enzymes. Soluble sugars play a fundamental role in the stabilization of macromolecules and cell membranes, sugars have the ability to prevent protein denaturation through interactions with membranes and proteins via hydrogen bonds [31].

The WD A. nodosum treatment showed a better percentage of Ψ_{Leaf} recovery in the last cycle. This result may be associated with the chemical composition of *A. nodosum*-based products, which contain a wide range of inorganic components, such as K, Ca, Mg and Zn [32]. These nutrients favor defense responses and osmotic adjustment [33]. The marked presence of K in the commercial product used (Baltiko®) (Table A1) can be explained by the production method via alkaline hydrolysis, which favors the release of this nutrient [7].

In addition to being an essential nutrient, K plays a fundamental role in osmotic adjustment, regulating stomatal opening and closing to improve water use efficiency under water deficit conditions [33, 34]. Previous studies with grasses have shown that the application of *A. nodosum* extracts conferred greater resistance to heat, an effect attributed to increased K absorption by plants [35, 36]. This mechanism reinforces the importance of K in stomatal regulation, also highlighted by Van Oosten *et al.* [37], who emphasized its accumulation as a crucial step in protection against osmotic stress.

Additionally, Spann and Little [38] reported that orange plants (*Citrus sinensis* L.) subjected to water stress and treated with commercial extracts of A. nodosum showed improved water status and greater water use efficiency. These findings corroborate the improvement in Ψ_{Leaf} recovery observed in the present study, suggesting that biostimulants based on *A. nodosum* may contribute to the resilience of ‘Aliança’ papaya under water deficit conditions, favoring the maintenance of water potential and cellular homeostasis.

In addition to the inorganic components, the Baltiko® label indicates an organic carbon content of 176.40 g/L. This high content may be associated with the presence of a diversity of active substances from A. nodosum extract, such as fucoidan, laminarin, alginic acid, mannitol, betaines, oligosaccharides and sterols [7, 33, 39]. These compounds are known to stimulate defense responses in plants and are widely described in the literature as responsible for the biostimulant effects of these extracts [7, 33].

In the soil, alginate reacts with metal ions, forming high molecular weight complexes that improve the structure and moisture retention, increasing water availability for the roots [40]. Inside the plants, compounds such as glycine betaine and sterols act as osmolytes, helping to maintain the osmotic potential and preserve cell turgor, which prevents structural damage under conditions of low water availability [7].

The WD *L. calcareum* treatment showed a better percentage of Ψ_{Leaf} recovery in the second cycle. The biostimulant effects of *Lithothamnion* sp. on tolerance to abiotic stress are still little explored [21]. However, *L. calcareum* can contribute to osmotic regulation due to its high concentration of Ca and Mg, essential cations for maintaining the stability of cell membranes and activating crucial metabolic enzymes, promoting resistance to water deficit and ensuring cellular homeostasis [41, 42]. In addition, the commercial product based on *L. calcareum* used in the present study contains proline. Proline under water deficit conditions helps to reduce cellular osmotic potential and mitigate oxidative damage by protecting plant metabolism against the effects of reactive oxygen species (ROS) [38]. The presence of humic acid in the product also enhances the activation of antioxidant enzymes, improving the efficiency in scavenging ROS and contributing to plant resilience during water stress [38].

The φP_0 reflects the photochemical efficiency of PSII [43]. The reduction in φP_0 values in DRY1 and REC1 (Figure 2*A*) indicates the occurrence of intense damage in the Q_A reduction capacity, due to the impairment in the excitation energy transfer capacity of PSII [44]. Consequently, φE_0 was compromised (Figure 2*F*), the reduction in φE_0 is a result of the failure of the electron transport yield [45]. Despite having had increases in the ABS/RC absorption components for DRY1 and REC1 and an increase in the TR_0/RC energy capture component observed in DRY1 (Figure 2*B*, *C*), they did not result in an increase in φP_0 efficiency and φE_0 yield [46]. The reduction in φE_0 in DRY1 and REC1 resulted in increased DI_0/RC energy dissipation (Figure 2*D*). Plants under stress increase the inactivation rates of reaction centers [46]. Under water deficit, the formation of ROS is common, causing damage to the structures of the photosystems, including the reaction centers [47]. These damages, such as the degradation of the D1 protein, compromise the ability of the reaction centers to capture and process energy efficiently [47], which may have contributed to the reduction in RC/CS_0 in the first cycle of water deficit and recovery (Figure 2*E*).

A reduction in RC/CS_0 was observed during DRY2, DRY3 and REC3 (Figure 2*E*). However, this reduction did not affect φP_0, φE_0 and PI_{ABS} (Figure 2*A*,*F*,*G*). Among the treatments, the decrease in RC/CS_0 was evident in Control, WD, WD melatonin and WD *L. calcareum*, but without impact on PI_{ABS} (Figure 2*G*). The reduction in RC/CS_0 may be an adaptive response of plants in the search to optimize the reaction centers that remained active to reduce Q_A, so that the received excitation energy was able to be transported through the transport chain providing good photosynthetic performance [48, 49].

The photosynthetic performance index PI_{ABS} is multiparametric as it involves other parameters such as the absorption component ABS, the capture component TR and the electron transport component ET [24]. PI_{ABS} is considered the sensitive index for drought stress [24, 50, 51]. The reduction of PI_{ABS} in DRY1 and REC1 (Fig 3*G*) suggests a decrease in overall photosynthetic performance associated with reduced electron transport capacity [51].

Chlorophyll, an essential pigment in photosynthesis, plays a crucial role in light absorption by plants. Water stress accelerates the decomposition of chlorophyll, making its content one of the main indicators of drought stress severity [52]. In the present study, the Chl *a* index in DRY1 decreased dramatically compared to the other water conditions (Figure 3*A*). Maintaining a reduced chlorophyll content under water stress may help plants minimize photo-oxidative damage, which occurs when photosynthesis is inhibited [52]. Therefore, the excessive absorption of excitation energy absorbed by photosynthetic pigments when photosynthesis is inhibited can trigger the accumulation of ROS and, consequently, oxidative stress [52]. Cerri Neto *et al.* [18], when subjecting *Piper aduncum* and *Piper tuberculatum* to three cycles of water deficit and recovery, also observed a reduction in the levels of chlorophyll *a*, *b* and total.

Chl *b* has the function of expanding the light absorption range and transferring energy to Chl *a* [53]. The decrease in the Chl *b* index in the WD melatonin, WD *A. nodosum* and WD *L. calcareum* treatments in DRY1, REC1 and DRY3, may indicate a photosynthetic adjustment of the plants, as a protection mechanism against excess absorbed light, which can lead to photo-oxidative damage, caused by the accumulation of unused light energy [52]. During recovery, if the plant has not yet fully reestablished its physiological processes, a lower amount of Chl *b* can prevent overloading of the photosynthetic system, reducing ROS production and preventing oxidative damage to cells [52].

This adjustment becomes even more relevant when considering that, in the present study, an increase in ABS/RC and DI_0/RC was observed, accompanied by a reduction in φP_0 in REC1 (Figure 2*A*,*B*,*D*). This behavior indicates that the reduction of Chl *b* in DRY1 and REC1 was beneficial, because, if their levels had remained high, the absorbed light would not be efficiently used in the photosynthetic process, which could result in excessive accumulation of energy and greater oxidative stress for the plant.

Over time, Chl *b* values increased in DRY2, REC2, and REC3, except for the *A. nodosum* treatment in REC2 (Figure 3*B*). This increase may have been beneficial in REC2 and REC3, especially because, in these same periods, Chl *a* levels were reduced (Figure 3*A*). Since Chl *b* has the function of expanding the light absorption range and transferring energy to Chl *a* [53], its increase may have compensated for the lower Chl *a* index, ensuring the continuity of the photosynthetic process.

Furthermore, it was observed that, in the last two cycles, the ABS/RC and DI_0/RC values decreased, while φP_0 and φE_0 increased (Figure 2A,B,D), which suggests greater efficiency in the use

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of absorbed light energy [46]. This pattern reinforces the hypothesis that the elevation of Chl *b* at recovery times was a positive adjustment of the plant to optimize its photosynthetic capacity during and after water stress.

The first cycle of water deficit impacted the leaf expansion rate, as indicated by the reductions in the leaf expansion rate values in the WD, WD melatonin and WD *A. nodosum* treatments in REC 1 (Figure 5*A*). Indicating that the plants were unable to present vegetative growth equal to or greater than the control, which can be attributed to the inability of the cultivar to invest carbohydrates in growth when the plants were rehydrated [54].

The WD melatonin and WD *L. calcareum* treatments maintained a stable leaf expansion rate throughout the cycles, unlike the WD treatment. *A nodosum*, which showed an increase in leaf growth in the third cycle (Figure 5*A*). Under water deficit conditions, the plant can reduce the leaf expansion rate to decrease transpiration, or invest in physiological and morphological mechanisms to maximize survival and growth [18].

The stable growth in WD melatonin may indicate a homestatic regulatory effect, helping plants maintain a more balanced water balance and avoiding large fluctuations in leaf expansion. The late increase in leaf expansion rate in the WD *A. nodosum* treatment suggests a resource allocation strategy, in which the plant mobilizes stored carbohydrates for leaf growth, promoting recovery and increased growth after water stress [54].

Although drought cycles cause loss of turgor pressure and tissue dehydration [52], a progressive increase in shoot growth was observed throughout water deficit cycles, with the exception of WD *A. nodosum* (Figure 5*B*). According to [52], plant growth is an important metric of drought adaptation capacity.

4. Materials and Methods

4.1. Plant Material and Cultivation Conditions

The experiment was conducted between May and July 2024, at the Linhares Experimental Farm, of the Capixaba Institute for Research, Technical Assistance and Rural Extension, located at 19° 25' 0.1” S and 40° 4' 35.3” W, in the municipality of Linhares, northern region of the state of Espírito Santo, Brazil. The seedlings of the black pepper plant ‘Bragantina’ were purchased from the commercial nursery Farm Quatro Irmãos, located 19.08392° S, 40.12381° W, in the municipality of Sooretama, northern region of Espírito Santo, Brazil.

The seedlings were transplanted into plastic pots with a capacity of 7 L. The pots were filled with Tropstrato HT vegetable substrate and fertilized with 17 g of Basacote® Mini 3M 16-8-12 (+2). After transplanting, the seedlings were transferred to a greenhouse with polyethylene coverage and 30% Aluminet screen, where they remained for 30 days for acclimatization, being irrigated regularly to maintain field capacity.

4.2. Stress Imposition and Experimental Design

The recurrent water deficit experiment comprised three cycles, each including a dehydration phase (irrigation suspension for ten days) followed by a rehydration phase (irrigation resumption for five days), called DRY1, DRY2, DRY3, and REC1, REC2, and REC3, respectively [18]. With the exception of the control, which was not subjected to water deficit. Before each dehydration phase, the solutions corresponding to the treatments were applied: melatonin (100 µM), *A. nodosum* (3 mL L^{-1}), *L. calcareum* (3 mL L^{-1}), and distilled water (Control). The doses were selected based on studies by Silva *et al*. [19], Ye et al. [20], Alagupalamuthirsolai *et al.* [15], Ramos *et al.* [21], Ferreira *et al*. [22], and Ramos *et al.* [23], who demonstrated positive effects of these biostimulants in mitigating stress in plants under adverse conditions. These applications were made using a 5 L manual sprayer, model 5Lts from Starfer. The control treatment was maintained at field capacity throughout the experiment. And the negative control (WD) was subjected to water deficit.

The melatonin solution was prepared as described by Ye *et al.* [20], by dissolving 0.69 g of melatonin (Sigma Aldrich) in 15 mL of ethyl alcohol as a stock solution. Subsequently, aliquots (2.52 mL) were removed from the stock solution to obtain the concentration of interest (100 µM) and volumeted to 5 L with distilled water.

The 3 mL L^{-1} solution of *A. nodosum* was prepared by dissolving 15 mL of the commercial product Baltiko® from Litho Plant in 5 L of distilled water. According to the manufacturer, Baltiko® is composed of *A. nodosum* extract, amino acids, humic substances and water. The label does not specify the percentage of seaweed extract, humic substance or which amino acids are present in the product. The product guarantees 5% water-soluble potassium (63.0 kg m^{-3}); 2% water-soluble nitrogen (25.20 kg m^{-3}); 14% total organic carbon (176.40 kg m^{-3}); 100 kg m^{-3} solubility in water at 20ºC; 1,260 kg m^{-3} density; 23.50% saline index; pH 7.57; highest solute/solvent ratio recommended by the manufacturer 100 g/L and is of a fluid and suspended physical nature.

To complement the knowledge about the nutritional effects of the product, a sample of the product was sent to the laboratory of Agronomic, Environmental Analysis and Preparation of Chemical Solutions for analysis of macro and micronutrients, results expressed in Table A1.

The 3 mL L^{-1} solution of *L. calcareum* was prepared by dissolving 15 g of the commercial product Litho Micron 3000® from the company Algadermis in 5 L of water. According to the manufacturer, the product is composed of 80% of the seaweed *Lithothamnium* and 20% of calcium and magnesium silicate. The guaranteed levels of the product are 27% calcium, 1.8% magnesium, 2% silicon and 3% moisture. Its physical nature is powder. The chemical composition of nutrients and humic substances of *L. calcareum* used in this study is detailed in Table A2, adapted from the work of Ramos *et al.* [21].

In addition, the study by Ramos *et al.* [21] indicates that the product based on L. calcareum has a concentration of 1,400 mg kg^{-1} of free amino acids (0.15%). Glycine and tryptophan stood out, both with 400 mg kg^{-1}, in addition to aspartic acid (200 mg kg^{-1}), alanine (200 mg kg^{-1}), proline (100 mg kg^{-1}), valine (100 mg kg^{-1}) and glutamic acid (0.01 mg kg^{-1}).

The experiment was conducted in a randomized block design, with four blocks, each containing six plants per plot. During the experiment, the temperature and relative humidity inside the greenhouse were measured using a digital thermometer-hygrometer model Incoterm -7666.02.0.00. Readings were taken twice, at 8:00 and 17:00 (Figure A1).

4.3. Water Potential

Leaf water potential (ΨLeaf) assessments were performed at the end of water deficit (DRY1, 2 and 3) and recovery (REC1, 2 and 3) cycles, with one plant per plot evaluated. Measurements were made at 5:00 a.m. using a Scholander pressure chamber (Model 1000, PMS Instrument Co., Albany, OR, USA) on healthy, fully expanded leaves collected from the middle third of the plants.

4.4. Chlorophyll *a* Fluorescence

Chlorophyll *a* fluorescence assessments were performed at the end of the water deficit (DRY1, 2 and 3) and recovery (REC1, 2 and 3) cycles, in two plants per plot, using a portable Pocket-PEA fluorometer (Hansatech, United Kingdom), as recommended by Strasser *et al.* [24]. For each plant, two leaves were adapted to the dark for 30 min, using specific clips, allowing complete oxidation of the photosystem. Then, a saturating light flash of 3,000 [µmol (photons) m^{-2} s^{-1}] was emitted, lasting 1 s. From the transient fluorescence OJIP, the parameters defined by the JIP test were calculated. The normalization and interpretation of the measured and calculated parameters followed the criteria of Strasser and Strasser [25].

4.5. Photosynthetic Pigments

The evaluations of chlorophyll *a*, *b* and total levels were performed at the end of the water deficit (DRY1, 2 and 3) and recovery (REC1, 2 and 3) cycles, in two plants per plot, using the electronic chlorophyll meter clorofiLOG, model CFL1030, from Falker. For each plant, two readings were performed.

4.6. Carbohydrate Allocation

Carbohydrate allocation was assessed by quantifying reducing sugars (RS) and total soluble sugars (TSS) in the leaves, stems, and roots of six plants per plot. Although the plants underwent three cycles of water deficit (DRY1, 2, and 3) and rehydration (REC1, 2, and 3), carbohydrate quantification was performed only at the end of the third cycle, after the rehydration phase. The dried plant organs were ground in a STAR FT-50 mill and stored in a freezer at –18°C.

Sugar extraction followed the method of Zanandrea *et al.* [26]. 0.2 g of the dried sample was homogenized in 5 mL of 0.1 M potassium phosphate buffer (pH 7.0), incubated in a water bath at 40 °C for 30 min, and centrifuged using a NI 1811-A model at 5,000 rpm for 20 min. The supernatant was collected, and the precipitate was resuspended twice in 5 mL of the same potassium phosphate buffer. The combined supernatants were frozen for RS and TSS quantification, and the precipitate was frozen for starch extraction. The protocol used for RS quantification was the dinitrosalicylic acid method of Miller [27], while TSS quantification followed a modified anthrone method of Yemm and Willis [28], using 2 mL of 0.1% anthrone solution in 93.33% sulfuric acid, plus 1 mL of the plant extract, placed in a water bath at 100°C for 3 min.

4.7. Vegetative Growth

The rate of leaf expansion was determined throughout the water deficit and recovery cycles, which comprised periods of ten days of drought followed by five days of recovery. To do this, the leaf area of each leaf was estimated daily with the aid of a graduated ruler, by measuring the length and width of the leaf. The leaf area was obtained by multiplying these two values, providing an estimate of leaf growth throughout the experiment.

With the leaf area determined for each day, the daily leaf expansion rate was calculated as the difference between the leaf area of the current day and that of the previous day, divided by the time interval between measurements, which was one day. Branch growth was quantified in three periods: before the water deficit cycles (T0) and at the end of the first, second and third dry periods (DRY1; DRY2 and DRY3). Branch length was measured using a tape measure from the stem to the apex.

4.8. Statistical Analysis

For variables such as water potential, chlorophyll a fluorescence, photosynthetic pigments, carbohydrate allocation and vegetative growth, analysis of variance (ANOVA) was performed. The means were compared by Tukey's test at 5% probability ($p<0.05$), using the SISVAR software version 5.8 [29].

5. Conclusions

Water deficit reduced the water potential of black pepper, ‘Bragantina’, suggesting a mechanism for maintaining turgor. However, at the end of the recovery periods, the treatments subjected to water stress showed an increase in water potential, with the WD melatonin treatment showing the highest percentage of water recovery in the second and third cycles.

The first cycle of water deficit affected the quantum yield of electron transfer from Q_A^- to the electron transport chain (φE_0), resulting in a reduction in φP_0 and PI_{ABS}. However, the damage to the photosynthetic apparatus was recovered in the subsequent cycles.

In addition, water stress reduced chlorophyll indices, possibly as a strategy to minimize photo-oxidative damage. Despite these challenges, plants treated with melatonin and *L. calcareum* maintained a relatively stable leaf expansion rate throughout the cycles, in addition to presenting progressive branch growth, with the exception of the WD *A. nodosum* treatment. Plant growth is an essential indicator of the ability to adapt to water deficit.

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Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

ABS/RC	energy absorption flux per reaction center
Chl *a*	chlorophyll a
Chl *b*	chlorophyll b
Chl Total	total chlorophyll
DI_0/RC	energy flux dissipated per reaction center
DRY1	cycles 1 of water deficit
DRY2	cycles 2 of water deficit
DRY3	cycles 3 of water deficit
Hum Max 8h	maximum humidity 8 hours
Hum Max 17h	maximum humidity 17 hours
Hum Min 8h	minimum humidity 8 hours
Hum Min 17h	minimum humidity 17 hours
PI_{ABS}	performance index for energy conservation of photons absorbed by PSII for the reduction of intersystem electron acceptors
RC/CS_0	number of active PSII reaction centers per cross section
REC1	cycles 1 of recovery
REC2	cycles 2 of recovery
REC3	cycles 3 of recovery
ROS	reactive oxygen species
RS	reducing sugar
Tmax 8h	maximum temperature 8 hours
Tmax 17h	maximum temperature 17 hours
Tmin 8h	minimum temperature 8 hours
Tmin 17h	minimum temperature 17 hours
TR_0/RC	energy flux captured per reaction center
TSS	total soluble sugars
WD	water deficit
φE_0	electron transfer quantum yield from
Q_A	to the electron transport chain beyond Q_A
φP_0	maximum photochemical quantum yield
Ψ_{Leaf}	leaf water potential

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Appendix A

Table A1. Analysis of macro and micronutrients of the commercial product Baltiko® based on *Ascophyllum nodosum*, including the following elements: Nitrogen, Phosphorus, Potassium, Calcium, Magnesium, Sulfur, Iron, Zinc, Copper, Manganese and Boron.

Product/parameters	Baltiko®		
	Low	Medium	High
Total nitrogen [%m/m]			2.2
Total phosphorus [%m/m]	0.3		
Total potassium [%m/m]			5.7
Total calcium [%m/m]	0.1		
Total magnesium [%m/m]	0.03		
Sulfur [%m/m]			1.9
Iron [%m/m]	0.04		
Zinc [ppm]	0.27		
Copper [ppm]	0.07		
Manganese [ppm]	0.47		
Boron [ppm]	0.42		

Table A2. Result of the chemical analysis of nutrients and humic substances of the product LT Supra®, from the former company Supramar (currently called Algadermis). The product LT Supra® also changed its name and is currently marketed as Litho Micron 3000®, maintaining the same formulation.

Parameter*	Unit	LT Supra®
Nitrogen	[%]	0.06
Phosphor		0.09
Potassium		0.06
Calcium		31.19
Magnesium		2.06
Sulfur		0.29
Boron	[mg kg $^{-1}$]	48.06
Copper		0.97
Iron		14,765.56
Manganese		481.89
Zinc		10.5
Sodium		8,084.48
Fulvic acid	[%]	9.31
Humic acid		0.93

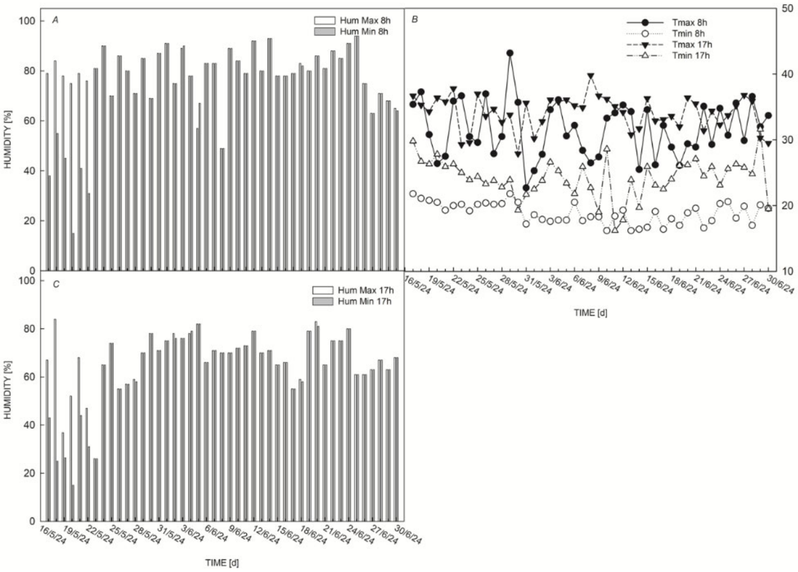

Figure A1. Climatic data. A – Maximum humidity at 8am (Hum Max 8h) [%], minimum humidity at 8am (Hum Min 8h) [%], B – Maximum temperature at 8am (Tmax 8h) [ºC], minimum temperature at 8am (Tmin 8h) [ºC], maximum temperature at 5pm (Tmax 17H) [ºC], and minimum temperature at 5pm (Tmin 17h) [ºC], C – maximum humidity at 5pm (Hum Max 17h) [%], minimum humidity at 5pm (Hum Max 17h) [%].

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