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Alleviation of drought stress in tomato by foliar application of seafood waste extract

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To manage the adverse effects of garbage pollution and avoid using chemicals, a natural extract of seafood shells was obtained and explored for its beneficial role. Physical characterization highlighted that its active compounds correspond to chitin and its derivative, chitosan. The ability of the extracted biostimulant to foster tomato tolerance was tested on drought-stressed plants. Along with changes in morphological parameters, the accumulation of chlorophyll and carotenoids was improved. The biostimulant also mediates the accumulation of osmoprotectants and an increased leaf water content. Furthermore, the biostimulant effectively promotes tolerance by increasing drought-stress *SlERF84* Transcription factor and decreasing both *SlARF4* and *SlWRKY81* transcript levels, which in turn, mediates stomatal closure. In addition, the up-regulation of key genes related to NO_3^- uptake (NTR1.1/2) and assimilation (NR) coupled with the downregulation of ammonium transporters' genes (AMT1.1/2), allowed the uptake of NO_3^- over NH_4^+ in the tolerant genotype which is likely to be associated with drought tolerance. Overall, the biostimulant was effective in alleviating water stress and showed similar effects to commercial chitosan. Besides the benefits of a circular economy framework, this biostimulant-based approach is innovative to promote a sustainable eco-agriculture, in the face of persistent water scarcity.

Keywords Seafood waste, Biostimulant, Chitosan, Drought, Tomato

Over the past decades, water deficit has become the most prevalent abiotic stress negatively impacting plant growth, development, and crop yield worldwide[1,2]. Rather, soon, both the frequency and severity of drought constraints are expected to be accentuated in several areas of the world due to the ongoing climate change[3]. FAO statistics predict that by 2050, drought stress together with other abiotic stresses will negatively impair crop yield with up to a 50% decline in average productivity[4]. Water deficit tends to decrease various plant features and has wide-ranging detrimental impacts on plant growth, physiology, and productivity[5]. This is driven by reductions in CO2 intake, stomatal conductance, leaf area, and photosynthetic efficiency[6]. Water scarcity thus poses a significant threat to the agricultural sector leading to a substantial decline in food production while affecting populations and economies. In countries belonging to arid and semi-arid regions such as Tunisia, the negative impact of water stress is driven by the scarcity of water resources and the frequency of droughts due to lack of rainfall[7].

Tomato, currently a globally cultivated crop, is exposed to the negative effects of water deficit, affecting quality and production[8]. Tomato crops, especially commercial cultivars, require a fully sufficient water supply hence drought stress severely limits their biomass accumulation and production[9]. Drought disrupts growth, photosynthesis, and key physiological and metabolic machinery[10,11]. It leads to oxidative stress and impairment of biological membranes and macromolecules[12]. To prevent cellular damage, plants accumulate osmolytes (proline, soluble sugars and soluble proteins) in the cytoplasm. Due to their high solubility, soluble compounds don't interfere with the cellular processes while contributing to maintaining cellular osmoregulation[13].

To withstand limited water resources, plants gradually establish an active and adaptative network that conjugates regulatory stress-responsive genes and a panel of -related transcription factors (TFs)[14]. Ethylene-responsive factors (ERFs) belong to a superfamily of transcription factors identified in plants[15]. *Arabidopsis*

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disposes of 122 ERFs factors while 139 are found in rice[15], 200 in poplar[16], 121 in barley[17] and 85 in tomato although they are mostly still uncharacterized[15,18].

Extensive studies have focused on the role of ERFs through their Knock-out or over-expression in transgenic plant species of interest. These factors belong to the AP2/ERF superfamily which modulates the expression of target genes by binding to elements in the cis-acting promoter region known as the CRT/DRE element, or GCC-box[19]. They also regulate plant developmental cycles and various environmental stresses[20–22]. Indeed, overexpression of either the SlERF5 gene or the GmERF7 improves drought and salt stress tolerance in tomato and tobacco transgenic plants, respectively[23,24]. The abundance of SlERF9/16/80 transcripts allowed tomato tolerance to salt stress[22]. *SlERF84* acts as a drought-responsive transcription factor in tomato[25] and overexpression of the OsERF71 isoform confers drought stress tolerance[25].

ARFs (Auxin response factors) also participate in plant responses to environmental stimuli by mediating the plant response to auxin[26,27]. They are encoded by the ARF gene family in several plants including tomato[28,29]. ARF4, a member of the ARF family, has been shown to alleviate drought stress in tomato[30].

It is noteworthy that the potential WRKY genes identified so far are likely to be involved in modulating plant responses to a plethora of various environmental constraints[31]. Among the WRKY factors, several isoforms are stress-responsive[22,32]. In tomato plants facing abiotic stress, SlWRKY8/31/39 were shown to display distinct expression patterns being either overexpressed or downregulated[21,22]. SlWRKY81 downregulation reduced leaf water loss by decreasing the stomatal aperture, thus constituting a negative regulator of tomato drought tolerance[33,34].

Notably, plants employ a range of strategies to mitigate water stress damages including a rapid and precise stomatal aperture regulation[35]. Stomata play a key role in modulating transpirational water loss as plants cope with drought stress.

Crosstalk between signaling pathways encompasses signaling molecules, among which nitrate (NO) is involved in plant cell functions such as stomatal movement, biomass accumulation, and mitigation of oxidative stress[32,36]. The steps of N-assimilation start with the uptake of inorganic NO_3^-, and culminate in its incorporation into organic compounds or amino acids with the intermediate synthesis of $NH4^+$ via the catalytic activities of NR and NTR-related genes[37]. Tolerance to drought stress is the result of a synergistic expression of NR genes, NO-transporters (NRTs), and NH4 transporters (AMTs)[38,39].

To mitigate the detrimental effects of multiple abiotic threats, chemical fertilizers have been widely used in agricultural practices[40]. Although they can be effective in ensuring an increase in plant growth and crop yield, agrochemicals contaminate soil, water, and air, thereby affecting the balance and viability of ecosystems. To assess the vulnerability and adverse effects of chemicals and to meet the food demands of a growing population, the implementation of an ecologically sustainable and durable agricultural system is an urgent practice. Organic fertilizers, rich in mineral elements and beneficial compounds, promote plant growth and development[41]. Besides, their overuse can disturb the natural balance of nutrients in the soil, leading to reduced soil quality, fertility, and environmental degradation, as well as contaminating water resources and the human food chain[42]. Thus, the natural biostimulant represents an alternative practice to the use of mineral synthetic fertilizers[39]. Accordingly, natural biostimulants constitute an alternative and accurate solution to cope with the frequent outbreaks of drought stress and to better guarantee both the quality and quantity of crops.

Biostimulatory effects are driven by mechanisms that enhance nutrient uptake, phytohormone synthesis, osmotic adjustment processes, and network regulation of candidate gene expression[43,44]. The ability of biostimulants to tremendously promote plant growth coping with stressors is often attributed to their ability to provide an exogenous supply of macro- and micronutrients[45]. Biostimulants correspond to a wide range of natural products such as seaweed extracts, chitin, and chitosan, PGPG bacteria (plant growth promoting growth), biopolymers; humic and fluvic acids[46,47]. For certain criteria such as origin, type, or composition, the assortment of biostimulants into categories or classes has been proposed[48].

Seafood waste from market fisheries and processing industries generated daily and discarded as waste, is a focal point of the blue economy concept[49]. In Tunisia, fisheries markets and industries lack elementary waste management procedures and services. As a result, much of the waste is not collected by the community and is dumped as garbage, including both biodegradable and non-biodegradable materials. Inadequate management of marine waste addresses environmental issues and makes its exploitation as a valuable biomaterial urgent[50]. To solve the problems related to the increase in human population and demand for seafood, with the consequent increase in the amount of waste generated, the adoption of a circular economy approach is key to a long-term resilient ecosystem[51].

Added values from marine wastes are achieved through the widespread use of chitin and its derivative, chitosan. Recently, both have gained importance in ensuring sustainable and eco-friendly agriculture[52]. Their beneficial application in crops, especially in vegetable species, has been largely proven[53,54]. Despite the variability of their natural sources (insects, mollusks, fungi), the main origin of chitosan and chitin corresponds to crustaceans (shrimp, lobster, king crab)[55–57]. Due to its valuable properties such as hydrophilicity, biodegradability, biocompatibility, antibacterial properties, and heavy metals removal, chitosan is used in a broad range of applications, such as biotechnology, biomedicine, food packaging, wastewater treatment, and agriculture[58]. In addition, chitosan has been involved in agricultural practices to protect crops from abiotic stressors[59,60]. Chitosan supplementation alleviates the negative effects of drought through a set of mechanisms including increasing photosynthetic activities[25] and water absorption capacity[61]. Positive effects of chitosan on yield during water deficit have been reported for many crops (cowpea, potato, common bean, wheat, and mung bean)[62–64].

In this context, our current study focuses on the use and valorization of a biostimulant extracted from fishery waste (fish and shrimp shells). A mixture of chitosan and chitin was extracted, physically characterized, and used as a biostimulant. As it is biodegradable, its use avoids the generation of additional waste and prevents

environmental pollution. We further investigated how two contrasting drought stress-tolerant and sensitive tomato genotypes responded to a natural biostimulant compared to commercial chitosan when facing water scarcity. We also identified specific genes involved in the plant's response to drought and suggested that the biostimulant could help improve tomato plants' ability to withstand water stress. Differentially expressed candidate genes can provide a valuable framework for genetic improvement of tomato to water deficit limitation under the efficient contribution of the biostimulant extract. Overall, the seafood waste extract-based biostimulant shows relevant potentialities to mitigate the adverse effects of water stress in both tolerant and sensitive tomato genotypes.

Results

Biostimulant characterization and effect on plant morphology

To extract the natural biostimulant, we adopted a common method currently applied on a commercial scale and corresponds to a three-step chemical extraction[65]. The extracted biostimulant was further characterized for its compound content, together with commercial chitosan.

FTIR spectroscopy

Infrared (IR) spectroscopy of the structural changes of the natural extract compounds was confirmed by Fourier Transform Infrared (FTIR) spectroscopy (Fig. S1A) and compared with the commercial chitosan (Fig. S1B). Indeed, the IR spectrum revealed the presence of both chitin and chitosan. Chitin displayed a panel of bands at 3447, 2910, 1655, 1375, and 1077 cm^{-1} (blue color), whereas chitosan lays out bands at 3375, 2890, 1645, 1420, and 1062 cm^{-1} (red color). The peak with moderate intensity at 3447 cm^{-1} is partly covered by the N-H stretching whose functional group is amine. The significant drop in transmittance in this band region underlines that the chitin attachment at a strong intensity of 2910 cm^{-1} affects the C-H alkane vibration. The strong absorption peak at 1655 cm^{-1} is attributable to ketone C=O, α, and β. The presence of the peak with variable intensity raised at 1375 cm^{-1} is due to the bending vibration of C-H groups. Concomitantly, the presence of the band at 1077 cm^{-1} confirms the presence of C-O stretching of the alcohol.

X-ray powder diffraction (XRD)

The XRD approach allowed to obtain, in the 2θ range of 3−35°, a set of patterns corresponding to the biostimulant composed of the assortment of both chitosan and chitin and to the commercial chitosan, as well (Fig S2). Worth noticing that the major reflections within the natural extract compounds match perfectly with those of the control. Besides, some characteristic peaks of chitin can be also detected. The XRD patterns indicate that the natural biostimulant is composed of compounds corresponding to chitin and chitosan.

Electronic study

The solid-state UV-vis spectra of the biostimulant, recorded at room temperature, displayed similarities between chitosan and chitin (Fig. S3) The two bands, revealed in the UV regions and centered around 250 and 358 nm, can be referred to $\pi - \pi^*$ and $n - \pi$ intraligand transitions of the chromophoric C=O unit.

Plant morphological parameters

Under water stress (WS), the phenotypic appearance of tomato plants was negatively affected and there were genotype-specific differences compared to the controle conditions (C) where plants are well watered. Overall, the Riogrande (T) genotype was well maintained and showed slow growth associated with slight leaf curling, whereas the sensitive (S) genotype showed severe leaf yellowing, curling, and wilting (Fig. S4). Therefore, phenotypic differences were assessed by monitoring morphological parameters of genotypes grown under drought stress regime and foliar spray treatment with either natural biostimulant (NB) or commercial chitosan (CC) (Fig. 1).

As expected, the lack of water affected the height of tomato plants regardless of their genotypes (Fig. 1a), with a more pronounced decrease in the S genotype (48.79%) than the T genotype (29%). Furthermore, the exogenous supply of natural biostimulant and commercial chitosan improved significantly plant growth in both genotypes. The highest improvement was observed within the T genotype after the natural biostimulant application (49.1%), compared to the water stressed state (WS) without any exogenous supply. Comparably, water deficit tended to notably shorten root length in T and S genotypes (12.5% and 21%, respectively). Foliar application of biostimulant and commercial chitosan increased root length significantly in both genotypes (Fig. 1b).

In comparison to the control conditions (C), the S water-stressed genotype showed a significant decrease in the number of leaves and branches (55.5% and 44%, respectively). The T genotype was less affected, with a significant reduction of 44.3% in the number of leaves, while the decrease in the number of branches was not significant (3.9%) (Fig. 1c, d).

Within the T and S genotypes, both the natural biostimulant and commercial chitosan helped to reduce the impact of water stress on the number of leaves (Fig. 1C). However, in the S genotype, only the commercial chitosan led to a significant increase in the number of branches compared to the stressed state (WS).

A similar trend was observed for the weight of the 7th leaf which was more affected in the S (50%) than in the T genotype (26%). Foliar treatment with biostimulant or commercial chitosan allowed the recovery of the leaf weight in both genotypes (Fig. 1e).

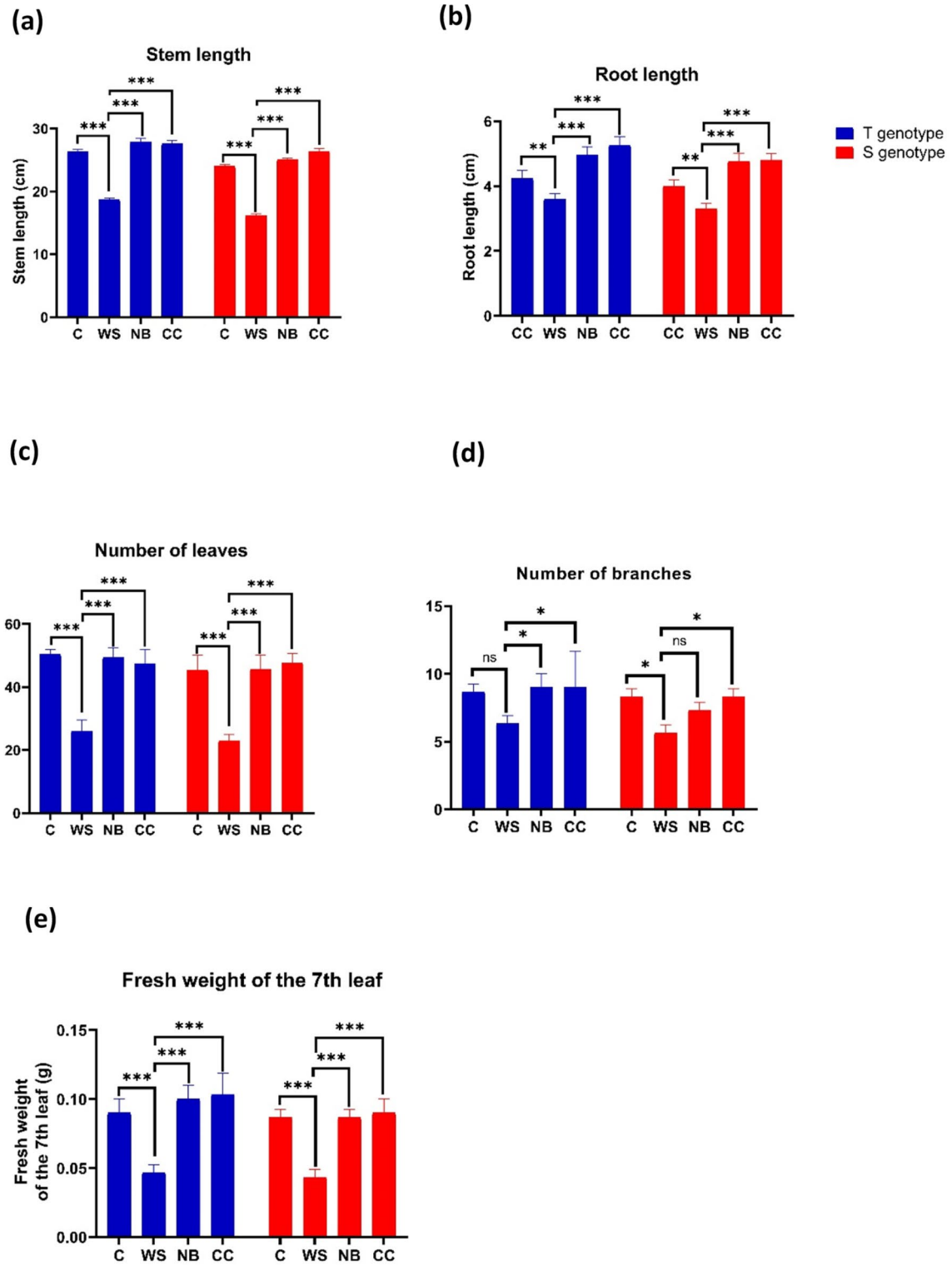

Fig. 1. Monitoring of plant growth parameters within the two contrasting tomato genotypes T and S. Each data represents the average of at least three independent biological replicates. The bars represent the mean ± standard deviation. The number of asterisks indicates the level of significant differences according to Tukey's test. Asterisks *; **; *** and **** indicate that differences by Tukey's HSD test are considered statistically significant at $p < 0.05$; $p < 0.01$; $p < 0.001$ and $p < 0.0001$, respectively.(ns) indicates no significant statistical differences.

Biostimulant effects on plant physiological attributes

Chlorophyll and carotenoid content

Since chlorophyll content is crucial for photosynthesis[66], we assessed it (Fig. 2a) and found a significant decrease in both T and S drought-stressed genotypes (53.5% and 60.17%, respectively). Treatments with natural biostimulant or commercial chitosan allowed the level of chlorophyll content to increase in both genotypes. The highest improvement was significantly observed in the T genotype (148%), under commercial chitosan, with regard to the stressed conditions (WS). Similarly, carotenoid content (Fig. 2b) decreased significantly in both drought-stressed T (35.48%) and S genotypes (35.37%) A significant increase in carotenoid content was observed in both genotypes under natural biostimulant or commercial chitosan foliar treatments compared to the water stress conditions (WS).

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Osmoprotectants content

Drought stress led to an obvious increase in soluble sugars (Fig. 3a) and proline (Fig. 3b) contents in the T (89.66% and 103.65%, respectively) and in the S genotype (92.28% and 125%, respectively). Moreover, osmoprotectants contents were significantly enhanced upon foliar treatment with natural biostimulant, particularly in the T genotype. Commercial chitosan was the best treatment for significantly improving the content of soluble sugars and proline in both T and S genotypes.

NO_3^- and NH_4^+ content

During water deficit (WS), the NO_3^- content decreased significantly in both the T and S genotypes (53.2% and 45% respectively), but it was notably increased by biostimulant and artificial chitosan treatments (Fig. 4a). Compared to the water stress stage, NH_4^+ accumulation was significantly reduced when natural biostimulant or artificial chitosan was applied (Fig. 4b). It's worth noting that NH_4^+ content tended to accumulate more in the S genotype during all stages of the experiment, regardless of the conditions imposed.

Stomatal aperture VS RWC

Our data revealed that foliar application of the biostimulant promoted drought-induced reduction in stomatal aperture within both S and T genotypes (Fig. 5a). Changes in stomatal aperture were screened by determining the ratio of length to width within both genotypes, under control conditions (C), water stress (WS), and natural (NB) or artificial (CC) stimulant treatment (Supplementary T2). Regarding control conditions, the stomatal aperture was relatively larger in genotype S (24,31%). The drought stress regime led to a significant stomatal aperture decline with 21% and 37% for the T and S genotypes, respectively.

In parallel, the relative water content (RWC) of both genotypes T and S showed a drastic decrease (16.85% and 22.49%, respectively) (Fig. 5b). Nevertheless, the stomata of the S genotype remained 10% more open than those of the T genotype. After foliar treatments with natural biostimulant or commercial chitosan, the stomata of both genotypes were more closed, improving significantly RWC only in T genotype.

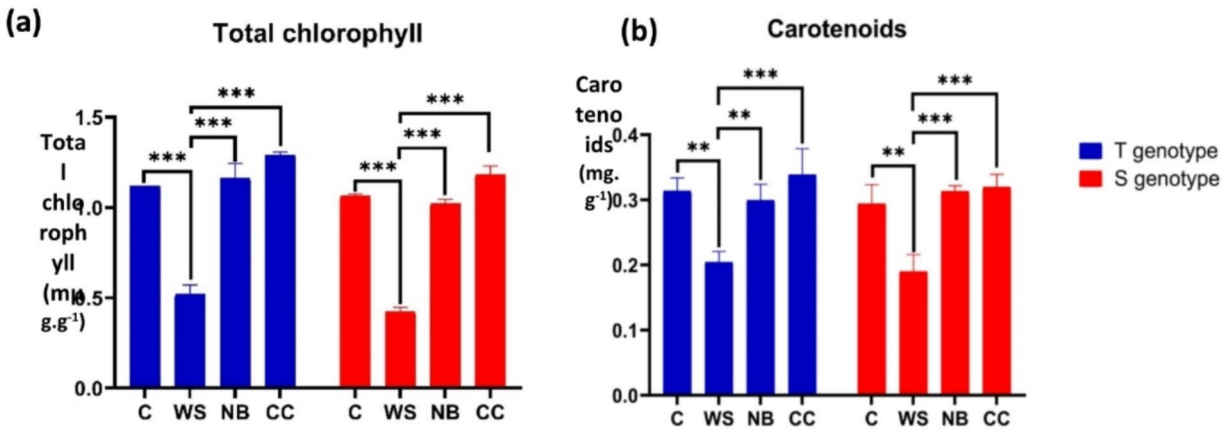

Fig. 2. Contents of photosynthetic pigments (**a**) total chlorophyll and (**b**) carotenoids within the two contrasting T and S tomato genotypes. Each data represents the mean of at least three biologically independent replicates. Bars represent mean ± standard deviation. The number of asterisks indicates the level of significant differences according to Tukey's test. Asterisks *; **; *** and **** indicate that differences by Tukey's HSD test are considered statistically significant at $p < 0.05$; $p < 0.01$; $p < 0.001$ and $p < 0.0001$, respectively.

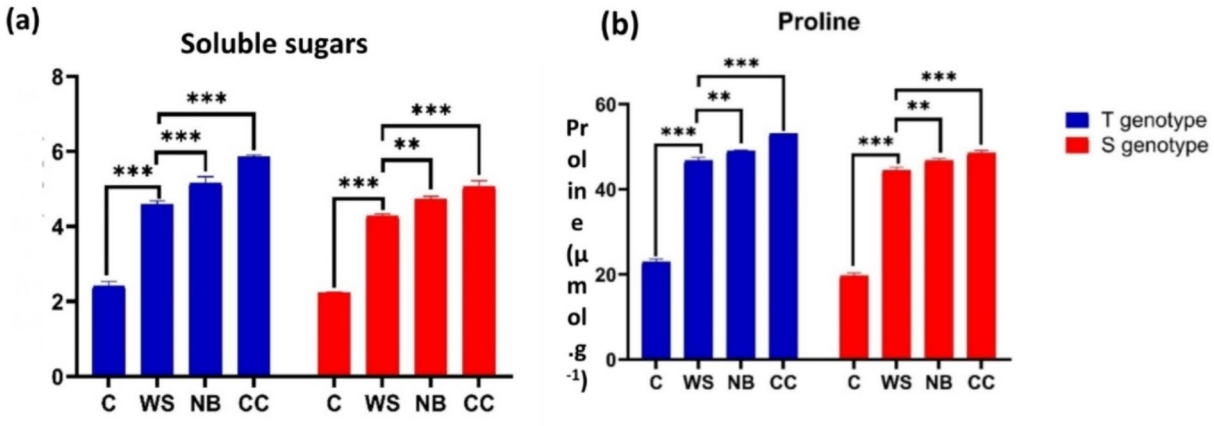

Fig. 3. Osmoprotectants contents of (**a**) soluble sugars and (**b**) proline in the two contrasting T and S tomato genotypes. Each data represents the mean of at least three biologically independent replicates. Bars represent mean ± standard deviation. The number of asterisks indicates the level of significant differences according to Tukey's test. Asterisks *; **; *** and **** indicate that differences by Tukey's HSD test are considered statistically significant at $p < 0.05$; $p < 0.01$; $p < 0.001$ and $p < 0.0001$, respectively.

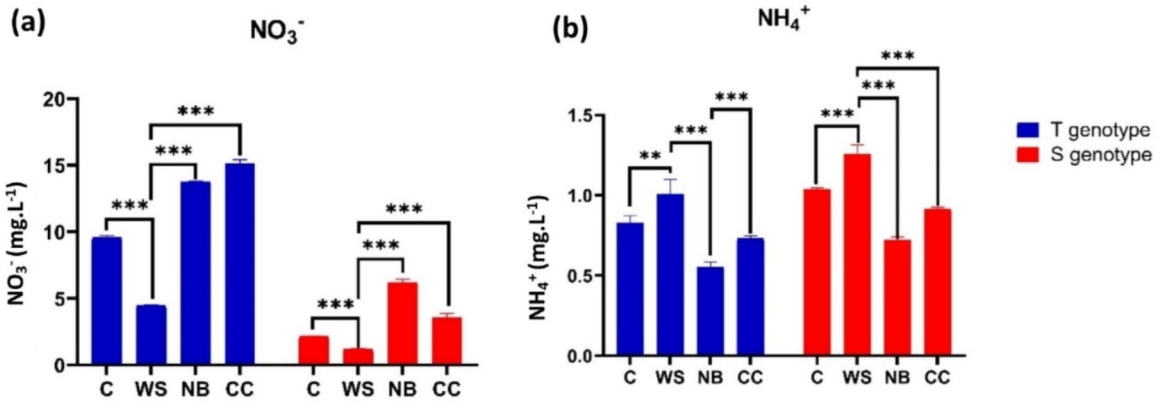

Fig. 4. (**a**) NO_3^- and (**b**) NH_4^+ contents within the two contrasting T and S tomato genotypes. Each data represents the average of at least three biologically independent replicates. Bars represent mean ± standard deviation. The number of asterisks indicates the level of significant differences according to Tukey's test. Asterisks *; **; *** and **** indicate that differences by Tukey's HSD test are considered statistically significant at $p < 0.05$; $p < 0.01$; $p < 0.001$ and $p < 0.0001$, respectively.

Biostimulant effects on the expression of stress-responsive genes

To gain insight into the effect of foliar treatments on plants under water deficit, expression levels of a panel of candidate genes were assessed using qRT-PCR. Heat maps of transcript expression were generated and data were presented as relative expression changes with respect to the control values (Fig. 6).

Data analysis outlined that all genes were found to be differentially expressed under drought and exogenous supply of biostimulant or commercial chitosan. Overall, the expression level allowed the assignments of candidate genes into two clusters of (i) downregulated and (ii) upregulated genes under foliar treatments when compared to the control (well-watered conditions).

Indeed, the *SIWRKY81* gene was actively expressed within either tolerant or sensitive tomato genotypes during drought stress whereas it showed a decreased expression when the plants were treated with natural biostimulant or commercial chitosan. Notably, *SIARF4* was actively expressed in the S genotype whereas it was weakly expressed in the T genotype. However, the accumulation of *SIARF4* gene transcripts was markedly reduced in plants treated with natural biostimulant or artificial chitosan treatments irrespective of the type of the tomato genotype. Similarly, transcript abundance of the AMT family genes showed significant downregulation patterns regardless of whether the genotype was sensitive or not. Meanwhile, the AMT1.2 gene was significantly expressed in the drought-stressed S and T genotypes. Conversely, the second cluster was characterized by the

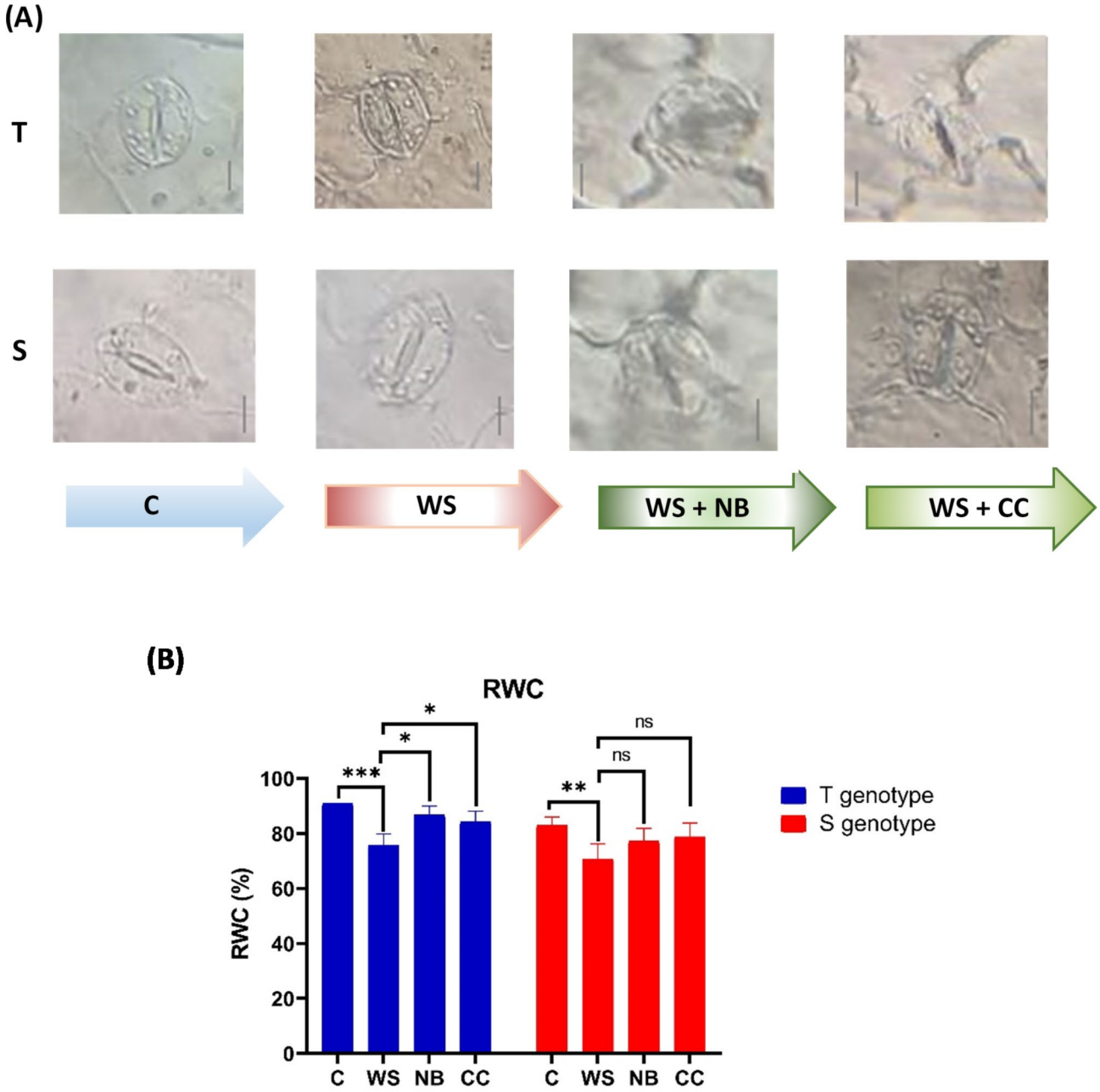

Fig. 5. Effect of natural biostimulant and commercial chitosan on (**A**) stomatal aperture status, bars = 6 µm and (**B**) relative water content (RWC) in the two contrasting T and S tomato genotypes. Each data represents an average of at least three independent replicates. Bars represent means ± Standard deviation. The number of asterisks indicates the level of significant differences according to Tukey's test. Asterisks *; **; *** and **** indicate that differences by Tukey's HSD test are considered statistically significant at $p < 0.05$; $p < 0.01$; $p < 0.001$ and $p < 0.0001$, respectively. (ns) indicates no significant statistical differences.

upregulation of NR and NTR1.1/2 genes under an exogenous supply of biostimulant or commercial chitosan. Comparably, *SlERF84* transcripts were downregulated in both T and S genotypes under drought stress before being markedly induced upon foliar treatment with natural biostimulant.

The Pearson correlation coefficients between gene expression and nutrients were assessed (Fig. S5). In the T genotype, NTR1.2 is positively and highly correlated with NO_3^- content whether the genotype is treated with the natural biostimulant or the commercial chitosan. Following the natural biostimulant supply, NH_4^+ content was strongly correlated with the AMT1.2 gene expression in the T genotype and highly correlated with both AMT1/2 in the S genotype.

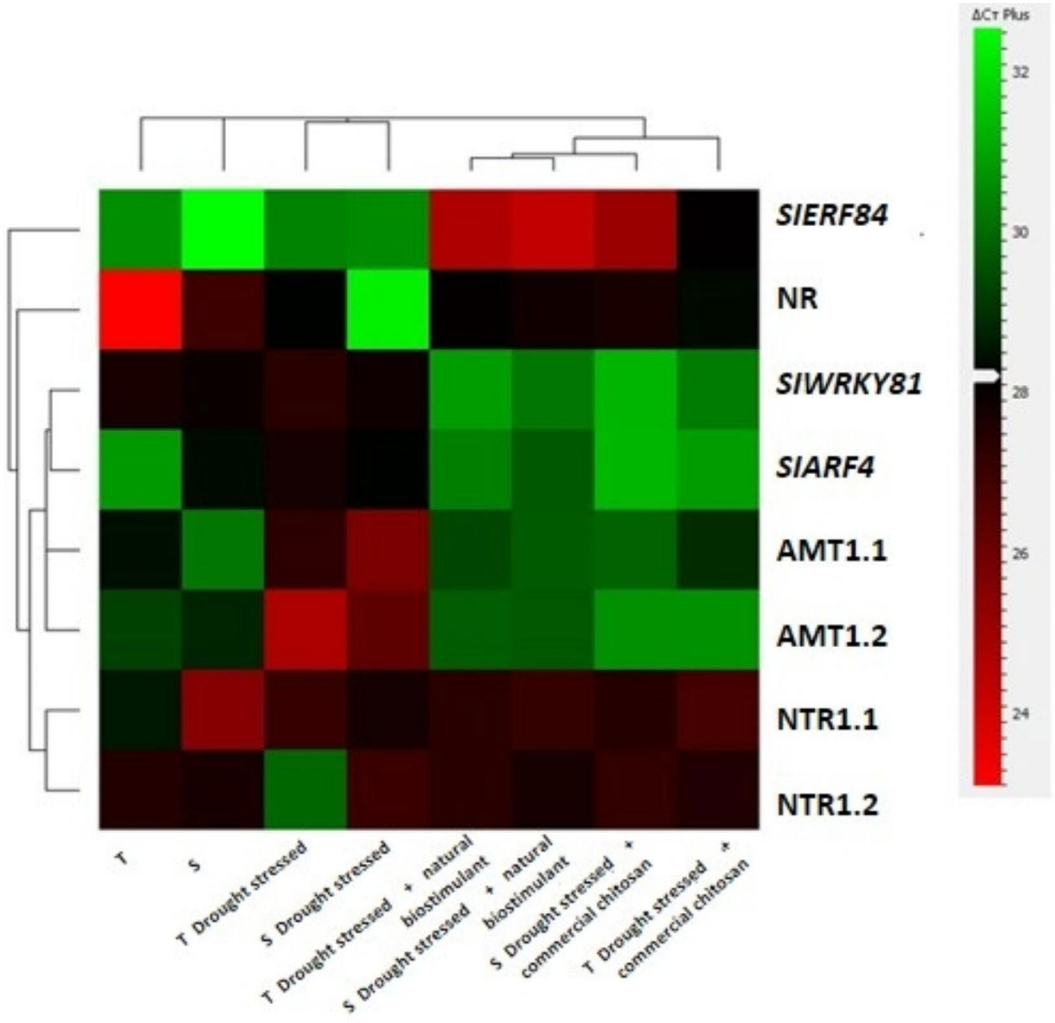

Fig. 6. Heat map representation of the effects of natural biostimulant and commercial chitosan foliar treatments on the relative expression level of target genes in the two contrasting T and S genotypes. Clustering (Average linkage, Euclidean distance) was applied to group genes with similar expression levels. The color intensity indicates expression level with a color scale ranging from red (Low ΔCt, up-regulation) to green (high ΔCt, down-regulation).

Discussion

This work is carried out within the framework of a circular bio-economy practice, which includes the valorization of fishery waste in sustainable tomato agriculture under water scarcity. Implementing a strategy based on innovative bioactive compounds to mitigate drought stress is challenging[67].

In this context, we investigated the effect of a natural extract from fishery waste on tomato plants subjected to a drought stress regime. Interestingly, the extraction process from crustacean and fish shell waste collected from the market resulted in a natural biostimulant consisting of a mixture of chitin and chitosan. Such a result could be expected given that the currently applied extraction methods produce a final product that mixes chitosan with different biomolecules[52]. Due to its properties and large-scale application, chitosan has gained interest driven by the growing interest in green processes for application in agriculture[53].

Therefore, the obtained mixture of chitosan and chitin was considered and used as a natural biostimulant to be applied in a foliar spray manner. Henceforth, it is more likely that the beneficial effects of the extracted biostimulant will be attributed to chitosan. During the subsequent experimentations, the effect of the natural biostimulant will be evaluated and compared with the commercial chitosan. Although this is an ecological practice aimed at promoting sustainable agriculture, this integrative and innovative approach has been used to seek the efficient use of the biostimulant in improving the response of tomato plants to drought stress.

Under drought stress regimes, morphological and physiological attributes pointed to a significant difference between tolerant T and susceptible S tomato genotypes. Nevertheless, the T genotype showed a better potential to recover quickly and efficiently after biostimulant treatment. Drought stress-tolerant genotypes are reported to display a quick discernment and perception towards drought. This potential allows them to quickly enhance

dynamic and functional crosstalk between various processes that trigger downstream signaling molecules and stress-responsive factors to overcome drought[68].

The recordings of morphological parameters indicated that both phenotypes were negatively affected by water deficit, with inhibition of stem length and reduced number of leaves and branches. Roots of tomato-stressed genotypes showed a significant reduced length between the T and S genotypes. Even drought stress has been reported to increase root biomass and length in many crops[69–71], in some crops like maize, moderate stress regime did not affect root development[69,70] whereas water deficit shortened root length of two species of basil[71]. On another side, it has also been found that morphologically, drought stress causes a progressive senescence phenotype in mature leaves of plants[71].

Exogenous foliar application of natural biostimulant or commercial chitosan significantly improved the growth of both genotypes. However, the T genotype exhibited enhanced recovery. This result is consistent with previous reports indicating that foliar application of chitosan improved several growth parameters such as stem height, number of roots and leaves in garlic, okra, or mungbean plants[59,63,72]. An analogous trend has been observed in many other crops such as cowpea, potato, common bean, and wheat[62–64,73]. Chitosan supply has been reported to improve plant growth and development, as it can potentially be used as an additional carbon source, or it can enhance nutrient uptake such as nitrate[74,75]. When chitosan is supplied hydroponically to common bean, both root and shoot morphology and biomass can be improved[76]. Along with chitosan, chitin also showed beneficial effects such as promoting plant growth and nutrition, and improved tolerance to environmental stress[52].

Concomitantly, drought stress induced a significant and drastic decline in physiological parameters such as photosynthetic pigments, and relative water content, along with increased osmoprotectants (sugar and proline) contents, in both drought-stressed genotypes, which was more pronounced in the S genotype. Nevertheless, foliar application of either natural biostimulant or commercial chitosan was effective in improving their physiological characteristics. These results are in agreement with those reporting that foliar application of chitosan enhanced drought-induced responses by improving morphophysiological, and biochemical attributes, in both the tolerant and susceptible mungbean genotypes[77].

Drought-stressed T and S tomato genotypes showed lower water content than the well-watered plants (control). This is consistent with reports showing that the decrease in RWC is typically associated with the drought stress response[78]. Our data also indicated that biostimulant-treated plants were able to maintain a significantly higher RWC under drought compared to water-stressed plants. Subsequently, the biostimulant can mitigate the negative effects of water deficit, probably by affecting the osmotic potential through the net accumulation of osmoprotectants[79,80]. To withstand drought stress, osmoprotectants including proline, and soluble sugars were recorded and shown to increase and accumulate better in the tomato-tolerant T genotype. Furthermore, our findings underlined that the supply of biostimulants enhanced the accumulation of soluble sugars and proline in tomato under drought stress. Hence, it has been reported that plants tend to accumulate osmoprotectants such as proline, soluble sugars, and other soluble proteins to counterbalance oxidative stress and damage to biological membranes and macromolecules[13,81]. Metabolites accumulate in high amounts and assist the plant to face oxidative stress promoting tolerance to water deficit[82,83].

Rather, water content is closely linked to changes in stomatal aperture[84,85]. It appears that response to the water deficit conditions is intimately connected with fluctuations in stomatal conductance, which affects the delivery of carbon dioxide to the chloroplasts, thereby lowering the photosynthetic rate and slowing plant growth and development[86]. Under drought stress, plants gradually close their stomata until they are completely closed under extreme water deficit conditions[87]. Sensitivity to water stress appears to be strongly correlated with differential reponses of contrating genotypes. Indeed, the tomato T genotype showed better stomatal closure after water stress compared to the S genotype. This data is corroborated by previous studies in pea plants showing that the complete closure of their stomata fluctuates according to their tolerance to drought stress[88,89]. In the present study, foliar application of the biostimulant improved stomatal closure in both T and S genotypes. Chitosan has been described as likely to act as an anti-transpirant by acting on the stomatal aperture[74]. The benefits of chitosan application seem to be mainly related to its hydrophilic nature and thus its ability to reduce transpiration rate while improving water uptake[75,90].

Previous reports supported that chitosan application in crops is currently associated with increased photosynthetic activity to overcome drought stress[73]. Our data demonstrated the drastically reduced number of tomato leaves under drought stress. The number, biomass, and area of leaves are rather crucial than those of shoots and roots because photosynthetic activities take place in leaves[91,92]. Under drought stress, the drastic decline in chlorophyll content accounts for oxidative stress and or photo-oxidation of chlorophyll pigments[93]. Conversely, increased photosynthesis has been reported in chitosan-treated leaves of maize, soybean, and tomato[94,95]. Our findings comply with these reports. Accordingly, the content of photosynthetic pigments decreased dramatically in the water-stressed T and S genotypes whereas the biostimulant and chitosan foliar treatments allowed chlorophyll and carotenoids to accumulate advantageously. Both are essential components required for photosynthesis and are strongly affected by drought-stress conditions in plants[96,97]. Therefore, the exogenous addition of the biostimulant was found to be effective in alleviating the negative effects of water stress, allowing for increased photosynthetic pigment content.

In wheat, the drought stress altered the plant's metabolic machinery and resulted in low nutrient uptake[98]. In addition, a foliar supply of chitosan has been reported to improve nitrogen accumulation and transport by improving the activities of key associated enzymes[74,75,79,80]. Compared to the S genotype, the T genotype showed a preferential uptake of NO_3^- over NH_4^+ at all stages of the experiment. Furthermore, NO3- uptake was emphasized under foliar treatment and was positively correlated with higher expression of NTR.1.1/2. Conversely, to nitrate, NH_4^+ content displayed a different pattern and was advantageously assimilated by the S genotype. Meanwhile, NH_4^+ decreased with the exogenous supply of the natural biostimulant and was

significantly correlated with lower AMT1.1/2 transcription levels. The expression of these stress-responsive genes has been explored in contrasting genotypes to highlight their involvement in abiotic stresses such as salinity[99]. Hence, our results pointed to that the ammonium transporters (AMT1.1 et AMT1.2) were notably down-regulated in response to foliar biostimulant supply under drought stress, whereas genes related to nitrate uptake, reduction, and N metabolism were up-regulated in response to the natural biostimulant. On the other side, our result argues that NO_3^- uptake over NH_4^+ is achieved to overcome drought stress in the T genotype. It was established that the water deficit conditions reduce the uptake of NH_4^+ and/or NO_3^-[100,101].

Depending on the Solanaceous plant species and environmental conditions, NH_4^+ and NO_3^- may display different trends in net fluxes[102–106]. In tomato, candidate gene expression analyses using bioinformatic and molecular tools revealed that AMT1 family genes were down-regulated in plants challenged with either drought or salt[107]. NH_4^+ supply alleviates growth damage when the rice is challenged with drought stress (*Oryza sativa*)[108]. Conversely, NO_3^- is crucial for other species undergoing water deficit stress[109,110]. In several abiotic-stressed plant species, the NH_4^+ flux was higher than that of NO_3^- in roots[111,112]. NH_4^+ uptake is privileged because it requires less energy[113]. This stress-adaptive trait was correlated with the up-regulation of 13 AMT genes and the down-regulation of NR genes in root tissues. The highly salt-tolerant *Spartina alterniflora* preferentially uptakes NH_4^+ over NO_3^-. Conversely, when this species is exposed to drought stress, an opposite trend is observed[114]. NRT1 and NRT2 genes have been proposed as NO_3^- signal transducers in Arabidopsis[115], while NRT1.1 modulates stomatal opening and closing under drought stress[116]. NO_3^- acts as a signaling molecule that is actively involved in response to environmental stimuli, especially drought[119,120114,115].

In particular, NO_3^- is an endogenous ABA-dependent signal to prevent water loss via stomatal closure and opening[32,117–119]. Accordingly, NO_3^- is produced via a network of cascade responses involving NR-related genes, whose differential expression alters plant responses to water deficit stress[39,120]. NO-ABA signaling mitigates drought stress by allowing greater total N content and assimilation, and up-regulation of NRT and also NR relative expression[120]. balanced endogenous change in NO and ABA levels, coupled with NO accumulation, has been described to alleviate the effects of drought stress in *Brassicacea*[37].

Besides candidate genes, transcription factors are crucial components in shaping plant responses by interacting with cis-acting elements in the promoter regions of stress-related genes. Ethylene response factors (ERFs) are stress-responsive transcription factors that are differentially expressed in response to abiotic stress[18,22]. Our current study demonstrated that *SlERF84* is induced by foliar biostimulant or commercial chitosan treatments. Previously, *SlERF84* was functionally characterized as a drought-induced isoform in tomato[25]. Additionally, SlERF84 might play a key role in an ABA-dependent signaling pathway in response to abiotic constraints. A correlation between *SlERF84*-overexpression and a slower rate of water loss was found in transgenic plants with reduced stomatal apertures. The tolerance of transgenic plants was attributed to the increased stomatal closure, which in turn prevented water loss[25].

The WRKY transcription factors are known to be involved in plant growth, development, and responses to environmental stresses[21,121]. They act by activation or repressing candidate genes by binding to their promoter regions[122,123]. Expression pattern analysis revealed the upregulation of WRKY81 in the contrasting genotypes challenged with water deficit. Conversely, the tolerance is mediated by the downregulating of *SlWRKY81* expression under biostimulant treatment. Concomitantly, we have shown above that an exogenous supply of the natural biostimulant mitigates the adverse effects of drought by enhancing the nitrate uptake and assimilation genes with the subsequent accumulation of NO_3^-. This result meets previous reports in water-stressed tomato, describing a functional network in which the expression of *SlWRKY81* leads to the repression of NR-related genes, resulting in the reduction of NO_3^-content and photosynthetic capacity, allowing stomatal opening, promoting thus drought sensitivity[33]. Our findings corroborate the consistent link between NR-mediated NO accumulation and *SlWRKY81*-regulated stomatal aperture in ensuring tomato drought tolerance.

ARFs have been reported to play key roles in hormone signaling[124] and plant development. In tomato, 24 ARF genes have been reported to be involved in plant growth and response to environmental stress[27,29,125,126]. Among them, the ARF4 isoform played a role in drought stress response. *SlARF4* factor is likely to take part in the ABA signaling pathways and thereby improve tomato tolerance to drought stress by influencing stomatal movement[30]. In our current work, *SlARF4* was downregulated within tomato genotypes, particularly following the foliar application of a natural biostimulant, promoting drought tolerance. Consistent with our results, genome editing knock-out of *SlARF4* improved tomato plant tolerance to water deficit and enhanced rehydration capacity[30]. Within our biostimulant-treated plants, monitoring of relative water content suggested a reduction in water loss. Accordingly, the loss of *SlARF4* function in mutant plants results in a more developed xylem, enabling these plants to maintain a persistent hydrated state[30].

The extraction and efficient use of the natural biostimulant is an added value to a material that is currently considered waste. Ultimately, the exogenous foliar application of the biostimulant allows tomato plants to progressively implement a dynamic system based on the coordinated and reinforced regulation of functional isoforms like *SlARF4*, *SlWRKY81*, and *SlERF84* that (i) promotes NRT and NR-mediated NO accumulation and signal transduction (ii) further regulates the expression of stomata-related genes rendering them capable of closing (iii) improves the accumulation of photosynthetic pigments and osmoprotectants, thus mitigating the adverse effects of drought.

Conclusion

This work encompasses a circular bioeconomy based on the recycling of seafood waste to produce a natural extract with high added value composed of a mixture of chitin and chitosan. The foliar application of the natural biostimulant improved morphological parameters and was associated with better biomass maintenance under water deficit. Furthermore, it activates an effective network by improving (i) numerous physiological processes (photosynthetic pigments, proline, soluble sugars, and relative water content (ii) stomatal closure (iii)

modulation of stress-responsive gene expression and, consequently, seems tightly related to the alleviation of drought stress. The benefits of the natural biostimulant were comparable to those obtained by supplementing the artificial and commercial chitosan. We propose the use of this natural biostimulant in an effective eco-friendly approach to sustainable agriculture.

Materials and methods

Plant material

Tomato genotypes corresponding to Riogrande and Heinz are commonly used by Tunisian farmers and are currently reported to be drought-tolerant and susceptible, respectively. Seeds were provided by the laboratory of seeds and plant analysis (Ministry of Agriculture, TUNISIA). Tomato seeds were sterilized with a 95% ethanol, 0.1% tween solution and sown into 20 cm diameter pots filled with a mixture of plant compost (40%), peat moss (40%), sand (20%) and enriched with NPK 20–20–20 fertilizer (pH 6.8, Terranum). The experimental site corresponds to an environmentally controlled greenhouse located at the Faculty of Sciences of Tunis (GPS Coordinates 36.806389; 10.181667), Tunisia. Pots were randomly arranged under supervised conditions (40–60% relative humidity; 8/16 h dark/light (100 µmol/m2) with a day/night temperature of 25 °C/18°C.

Biostimulant extraction

An agreement with the Municipality of Megrine (Tunisia) was obtained, allowing us to access, collect, and treat fisheries waste. Extraction of the natural biostimulant,, from waste mainly composed of shrimp and fish shell, was performed following three main steps[65] (i) demineralization (ii) deproteination and (iii) deacetylation. For the demineralization step, 10 g of the waste sample, previously washed and dried, was treated with 2 N HCl (ratio 1:15) for 2 h at room temperature with constant stirring at 150 rpm/min. After demineralization, the sample was repeatedly washed with distilled water until the pH was neutral. A Final washing step was performed with hot distilled water and the sample was dried at 80 °C for overnight. Afterward, a treatment with 2 N NaOH (ratio 1:20) was done for 2 h at 50 °C, with constant stirring, followed by a repeatable process of washing and drying. These two steps resulted in a chitin end product, which in turn, was subjected to the deacetylation step. 1 g of the chitin product was treated with 50% NaOH for 1 h at 121 °C and washed repeatedly until the pH became neutral. The dried samples were stored at room temperature until further use[127]. They constitute a biostimulant that was further characterized to screen physical parameters and to identify associated components. The biostimulant effects of the natural extract (100 $mg.L^{-1}$) were explored in subsequent experiments and further compared with the equivalent concentration of a commercial chitosan (Sigma Aldrich, Iceland).

Biostimulant physical characterization

Fourier Transform Infrared (FTIR) was recorded at room temperature in the wave number range of 400–4000 cm^{-1} by a Nicolet IR 200 FTIR spectrophotometer (Thermo Fisher, USA) with a resolution of 4 cm^{-1}. The X-ray powder Diffraction (XRD) pattern was recorded using a D8 ADVANCE X-ray diffractometer (Bruker, Germany) by a Cu tube ($\lambda_{CuK} = 1.5418$ Å) operating at 40 kV and 40 mA at a rate of 0.02°s^{-1}. The UV—Vis spectra were recorded using a Lambda 19 spectrophotometer (PerkinElmer, US) in the 200–800 nm range. Fluorescence emission spectra were obtained by an LS55 spectrofluorometer (PerkinElmer, USA) supplied with a 450 W xenon lamp as the excitation source for chitosan at room temperature. All experiments were carried out with the compounds in the solid state.

Drought stress imposition, biostimulant application, and experimental design

The two contrasting drought-responsive tomato genotypes were subjected to drought stress imposition with or without exogenous foliar application of natural biostimulant and commercial chitosan. Drought stress was achieved by withholding water up to 50% of field capacity at the 4–5 leaf stage. First, pots were watered to field capacity and left to drain overnight. They were then weighed to obtain the weight corresponding to 100% field capacity. Afterwards, the soil was oven-dried and its weight was recorded. The pot weight at 50% field capacity was kept and maintained by frequent measurements and watering according to the pot weight.

The experiments were carried out according to the following four patterns: For each tolerant or sensitive genotype, we assigned four different sets of plants: (1) untreated plants grown under well-watered conditions (control), (2) untreated plants grown under drought stress conditions; (3) natural biostimulant treated plants grown under drought stress conditions and (4) commercial chitosan treated plants grown under drought stress conditions (Fig. 7).

At the end of the experiments, plant material (leaves) was collected and used for morphometric, physiological, and molecular analyses. Three biological replicates for each tomato genotype were performed for each pattern. Each replicate consisted of a pool of 10 plants. The uppermost fully expanded leaves were collected for physiological and molecular analysis.

Monitoring of morphological parameters

The growth and development of tomato plants were recorded by manually measuring the plant's height (from the base of the plant to the top of the main stem), the number of branches, the size of the secondary roots, and the weight of the 7th upper leaf and the number of leaves. The average of three biological replicates was considered for the final scoring.

Photosynthetic pigments content

Leaves were grounded in liquid nitrogen. 0.1 g of the vegetable powder was then mixed with 5 ml of 80% cold acetone and kept in the dark at 4 °C for 72 h. The content of photosynthetic pigments was determined by measuring the absorbance at three wavelengths (663 nm and 647 nm for chlorophyll a and b) and 470 nm (carotenoids)

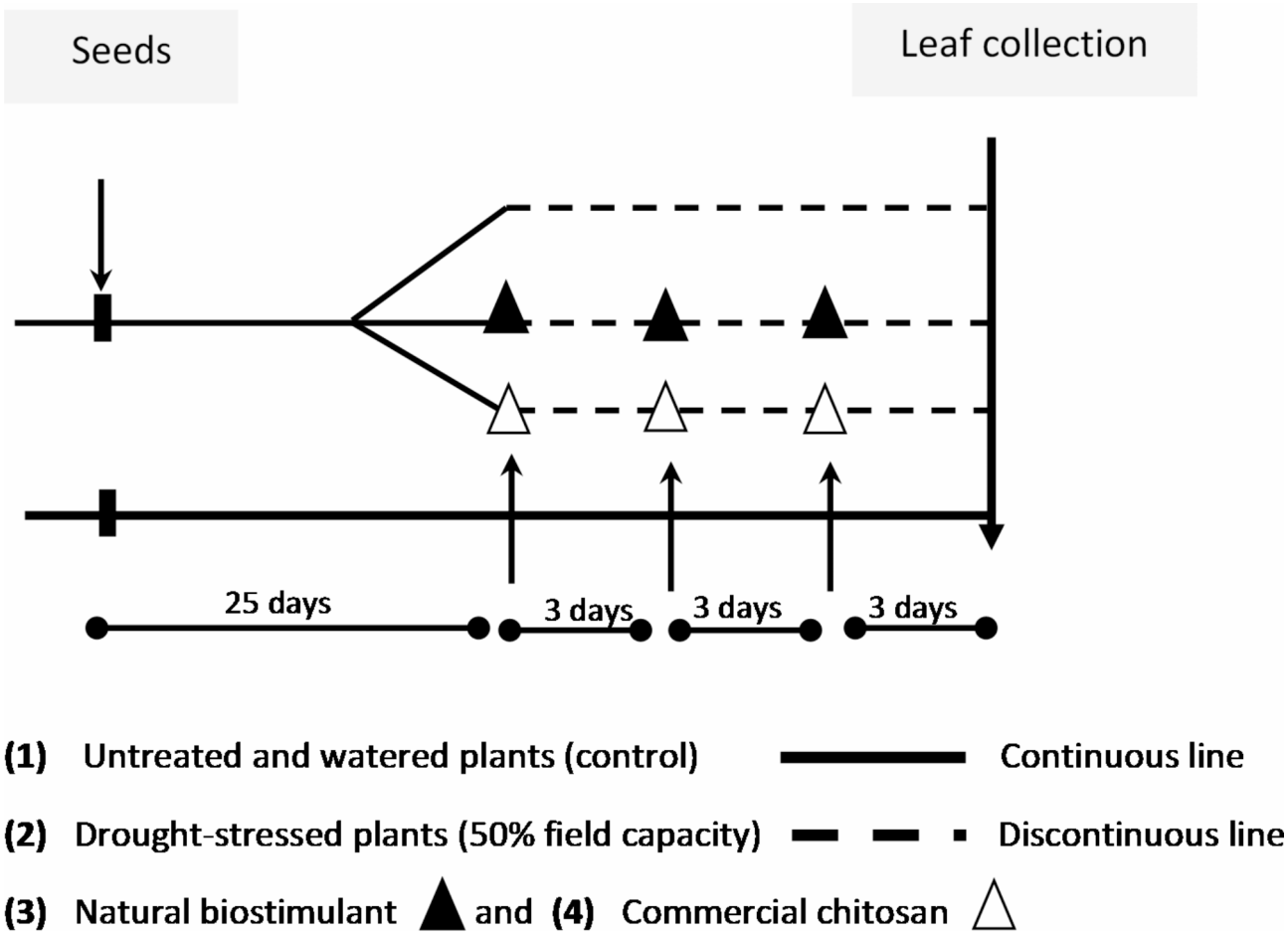

Fig. 7. A schematic pattern of the experimental design. At the beginning and after seedling emergence, all the plants were irrigated with a full water supply. 25-day-old seedlings, plants were divided into four clusters (C) untreated and well-watered plants (WS) untreated and water-stressed plants (50% field capacity), and the remaining clusters of drought-stressed plants were set up according to the compound to be sprayed on the leaves (WS + NB) natural biostimulant (WS + CC) commercial chitosan.

using a UV–Vis spectrophotometer (6850 UV spectrophotometer, JENWAY, USA). The photosynthetic pigments content was calculated according to the the following formula[128].

$$\text{Chlorophyll } (\mu g/mL) = 7.15 \times \text{DO } 663\,\text{nm} + 18.71 \times \text{DO } 647\,\text{nm}$$

$$\text{Carotenoids } (\mu g/mL) = (1000 \times \text{DO}470 - (1.90 \times \text{Chlorophyll a} + 63.14 \times \text{Chlorophyll b})/214$$

Proline content

Proline leaf extraction was carried out[129]. For colorimetric determinations, a solution of proline, ninhydrin acid, and glacial acetic acid (1:1:1) was incubated at 90 °C for 1 h and allowed to be cooled in an iced bath. The chromophore was extracted using 2 ml of toluene and its absorbance at 520 nm was determined (6850 UV/VIS spectrophotometer, JENWAY, USA).

Soluble sugar content

The soluble sugar content was determined[130]. 25 g of a dry sample was ground and homogenized in 80% ethanol. The mixture was kept at 70 °C for 30 min, cooled, and then centrifuged at 3000 rpm for 30 min at room temperature. Reducing sugars were recovered from the supernatant as follows: 0.1 ml of the supernatant was diluted in 0.9 ml of 80% ethanol, mixed with 2 ml of anthrone sulfuric acid, and boiled for 10 min. The soluble sugar content was determined based on a standard range of glucose and measured by spectrophotometry at 620 nm (UV-VIS spectrophotometer, JENWAY USA).

Total NO_3^- and NH_4^+ content

NO_3^- content of leaves was performed[131]. A total of 100 mg of dry tissue was mixed with 2 ml of deionized water and incubated for 1 h at 45 °C, in a water bath. The mixture was centrifuged at 5,000 rpm for 15 min. The supernatant (0.2 ml) was mixed with 0.8 ml of 5% (w/v) salicylic acid in concentrated H_2SO_4 and incubated

for 20 min at room temperature. A total of 19 ml of 2 M NaOH was added to raise the pH above 12 and the absorbance was measured at 410 nm (Shimadzu UV-1800 UV-Vis spectrophotometer, Fisher Scientific, USA). The NO_3^- concentration was determined using a calibration curve with KNO_3.

The NH_4^+ content was measured)[132]. Ammonium was extracted from 100 mg of leaf with 3 ml of 0.3 mM sulphuric acid (pH 3.5). The mixture was centrifuged for 10 min. 100 µl of the supernatant were diluted with 0.3 mM sulphuric acid to a final volume of 4 ml. The colorimetric reaction was assessed by mixing 0.5 ml of solution A (25 mg sodium nitroprusside and 5 g phenol dissolved in 100 ml deionized water) with 0.5 ml of solution B (2.5 g NaOH mixed with 40 ml 5% sodium hypochlorite) with deionized water to a final volume of 100 ml. After incubation in a water bath at 37 °C for 20 min, the NH_4^+ content was estimated using the standard ammonium sulfate. Absorbance was measured at 625 nm (Shimadzu UV-1800 UV-Vis spectrophotometer, Fisher Scientific, USA).

Stomatal aperture

To determine the measurement of stomatal aperture, the abaxial epidermis was delicately peeled and the peels were then floated on a buffer (10 mM MES containing 30 mM KCl, and 0.5 mM Ca^{2+})[31,119]1 For each treatment, 10 stomata from three tomato plants were taken into account. Each data represents an average of at least three independent replicates. Pictures of stomata were analyzed and measures were performed using Image J software (National Institute of Health, USA) http://imagej.nih.gov/ij/.

Relative water content (RWC)

Relative water content was estimated in the 7th upper leaf at the pre-flowering stage)[133]. The fresh leaf weight (FW) was recorded for each sample. Thereafter, leaves were saturated in distilled water at 4 °C, for one day in the dark and the turgid weight (TW) was recorded. Leaf samples were then allowed to dry at 65 °C for 72 h and the dry weights (DW) were determined.

The RWC was calculated using the following formula:

$$\mathrm{RWC}\ (\%) = [(\mathrm{FW}\ -\ \mathrm{DW})\,/\,(\mathrm{TW}\ -\ \mathrm{DW})]\ \times\ 100.$$

Candidate gene expression

Total RNA extraction

Total RNAs were extracted from tomato leaf tissue using TRIZOL Reagent (Trizol RNA stabilization solution, Invitrogen; Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Total RNA was quantified by ND-1000 spectrophotometer (Nanodrop Technologies, USA).

qRT-PCR amplification

First-strand cDNA was synthesized from 2 µg of total RNA with oligo (dT) and M-MLV reverse transcriptase (200 U/µl, Invitrogen, USA) according to the manufacturer's instructions.

An ABI A Prism 7000 sequence detection system (Applied Biosystems, Beverly, USA) was used for quantitative real-time PCR (qPCR). Reactions were performed in a final volume of 25 µl under the following thermal profile: 50 °C for 2 min, 95 °C for 2 min, followed by 39 cycles, each consisting of 95 °C for 15 s and 60 °C for 1 min, followed by melting at a temperature between 65 and 95 °C with 0.5 °C increments for 10 s. The tomato β-actin gene was used as an internal reference gene[134].

Candidate genes and their corresponding primers are listed (Supplementary S2). Reactions were carried out in 96-well optical reaction plates (Applied Biosystems, Beverly, USA). The reaction mixture included 2 µl of 20-fold dilution of cDNA, each primer at 2.5mM concentration, and 12.5 µl of IGreen PCR master Mix-Rox (BIOMATIK, Wilmington, USA). Each qPCR assay was run in three technical replicates and three biological replicates. Relative quantification was performed by applying the comparative $2-\Delta\Delta Ct$ method[135] Data correspond to the fold change in gene expression normalized to the endogenous reference gene (β-actin) and relative to the calibrator (untreated control).

Statistical analysis

Gene expression analyses were performed using DataAssist TM v3.0 software (Applied Biosystems, USA). Data were analyzed using two-way ANOVA with treatments and genotypes as the two predictor variables. Asterisks *; **; *** and **** indicate that differences by Tukey's HSD test are considered statistically significant at $p < 0.05$; $p < 0.01$; $p < 0.001$ and $p < 0.0001$, respectively. For real-time PCR experiments, three independent biological replicates and three technical replicates for each cultivar were analyzed. Analyses were performed using GraphPad Software (version 8.0, CA, USA). The relationship between the expression of genes and nutrient content was evaluated by Pearson's correlation coefficients using R software (R Core Team, Vienna, Austria, 2020).

Permission

The plant collection and use were in accordance with all the relevant guidelines.

Data availability

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

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Author contributions

F.G. and S.W. designed the experiments. I.B.S. performed all the experiments with the help of S.W. S.W. contributed to molecular data analysis. A.H., R.Z. and S.Z. contributed to statistical analysis. A.M. and S.A. performed physical experiments and supervised data analysis. H.H. performed stomatal isolation and analysis. F.G. wrote the paper. H.F. obtained funds to carry out the work and revised the manuscript.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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