

1-1-2019

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SILVA, HEWSLEY; NETO, ANDRÉ AZEVEDO; MENEZES, RENATA; SILVA, PETTERSON; and GHEYI, HANS (2019) "Use of hydrogen peroxide in acclimation of basil (*Ocimum basilicum* L.) to salt stress," *Turkish Journal of Botany*. Vol. 43: No. 2, Article 7. <https://doi.org/10.3906/bot-1807-80>
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Use of hydrogen peroxide in acclimation of basil (*Ocimum basilicum* L.) to salt stress

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Received: 26.07.2018 • Accepted/Published Online: 10.12.2018 • Final Version: 07.03.2019

Abstract: The aim of this study was to verify the effects of application of H₂O₂ on salt tolerance and oil production in basil cv. 'Gennaro de Menta' grown in a hydroponic system. Seedlings were subjected to 3 levels of H₂O₂ (0, 1, and 1000 µM) in a nutrient solution during 2 different exposure periods (24 and 48 h) and 2 levels of NaCl (0 and 80 mM). The plants were harvested at preflowering (at 15 days) and postflowering (at 25 days). At 25 days, the application of 1 µM H₂O₂/48 h and its reapplication at preflowering increased leaf dry mass production compared to the other salt treatments. Relative water content decreased while leaf succulence and sclerophylly index increased with salinity. The increase of succulence was more pronounced with the application of H₂O₂. Salinity increased the chlorophylls content only in plants without the application of H₂O₂. Salinity increased Cl⁻, Na⁺, and organic solutes content, this effect being more pronounced in salt treatments that received H₂O₂. Essential oil content, yield, and productivity were induced by salinity and H₂O₂. This study shows that H₂O₂ application in nutrient solution for basil cultivation in hydroponic systems using brackish water is a viable technique for promoting biomass or essential oil yield.

Key words: Salinity, salt tolerance, essential oil productivity, *Ocimum basilicum* L.

1. Introduction

Salinity is one of the major challenges for crop production, especially in semiarid regions such as the Northeast of Brazil (Cova et al., 2016). In this region, the excessive use of fertilizer, lack of drainage, and low quality of irrigation water are considered the main factors contributing to soil salinization (Niu and Cabrera, 2010).

In this context, studies have been carried out to investigate the use of brackish water in agricultural production, especially in hydroponic systems (Santos et al., 2012; Bione et al., 2014). However, hydroponic cultivation with saline water can reduce crop productivity (Paulus et al., 2010) due to osmotic and ionic effects that alter water and nutrient uptake, membrane integrity, gas exchange, and hormonal balance reducing cell expansion and division (Azevedo Neto et al., 2015).

To maintain osmotic and ionic homeostasis plants can accumulate organic and inorganic solutes, reducing water potential and favoring water uptake. This mechanism is essential for maintaining plant growth in saline environments (Silveira et al., 2009; Azevedo Neto et al., 2015).

Salt stress also induces the accumulation of reactive oxygen species (ROS), such as hydrogen peroxide, superoxide radical, and hydroxyl radical, which are highly reactive and can alter cellular metabolism leading to oxidative damage and programmed cell death (Azevedo Neto et al., 2015). Among the ROS, hydrogen peroxide (H₂O₂) is noteworthy because in high concentrations it causes cellular damage, whereas at low or moderate concentrations it acts as a secondary messenger in intracellular signaling cascades that mediate the responses of plant cells against different types of stress (Azevedo Neto et al., 2005; Christou et al., 2014). Based on this information, several authors reported that the exogenous application of H₂O₂ increased the salt tolerance in maize (Azevedo Neto et al., 2005; Gondim et al., 2011), strawberry (Christou et al., 2014), and rice (Carvalho et al., 2011).

Basil (*Ocimum basilicum* L.) is an aromatic herb of great economic interest. It is cultivated for the *in natura* trade and mainly for the industrial production of essential oils widely used in the pharmaceutical industry (Lorenzi and Matos, 2008). According to Alves et al. (2015), the highest concentrations of essential oil are

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found in leaves, floral calyces, secretory channels, and glandular hairs. In addition, the amount of oil may be affected by environmental stresses such as water deficit and salinity, but this effect depends on factors such as type, intensity, and duration of the stress (Alves et al., 2015).

Considering the importance of basil and the possibility of its cultivation in regions with water and soil salinity problems, the aim of this study was to evaluate the effects of the application of H_2O_2 on tolerance to salt stress and essential oil yield of basil cv. 'Gennaro de Menta'.

2. Materials and methods

The experiment was conducted in a greenhouse at the Universidade Federal do Recôncavo da Bahia (UFRB) in Cruz das Almas - BA (12°40'19" S, 39°06'23" W, and mean altitude 220 m) using basil seeds of the 'Gennaro de Menta' variety obtained from ISLA Sementes®. These were sown in disposable recipients (80 mL) containing coconut fiber and irrigated daily with water from the state sanitation company (EMBASA). From the emergence, the seedlings were irrigated with half-strength nutrient solution (NS) of Furlani (Furlani, 1998) until completing 20 days.

After this period, pretreatments with H_2O_2 in NS were applied. Previous experiments using different concentrations of H_2O_2 and exposure periods showed that the exposure of plants to 1 μM H_2O_2 for 48 h and 1000 μM H_2O_2 for 24 h were the best combinations for basil acclimation to salinity (data not shown). After pretreatment with H_2O_2 , the seedlings were transferred to a hydroponic DFT (Deep Flow Technique) pyramid system (Santos Júnior et al., 2016) with 10-cm spacing in the absence or presence of 80 mM of NaCl.

The experiment was carried out in a completely randomized design, with 6 treatments and 5 replicates. The treatments were: T1 - 0 mM NaCl and 0 μM H_2O_2 (control); T2 - 80 mM NaCl and 0 μM H_2O_2 ; T3 - 80 mM NaCl and 1000 μM H_2O_2 /24 h in the seedling phase; T4 - 80 mM NaCl and 1 μM H_2O_2 /48 h in the seedling phase; T5 - 80 mM NaCl and 1000 μM H_2O_2 /24 h in the seedling phase and in the preflowering; T6 - 80 mM NaCl and 1 μM H_2O_2 /48 h in the seedling phase and in the preflowering. The treatments T1, T2, T3, and T4 consisted of 8 plants per replicate, while T5 and T6 consisted of 4 plants per replicate. Two harvests were performed and evaluated separately, one at 15 days of salt stress (preflowering) using 4 plants per replicate of T1, T2, T3, and T4 treatments, and another at 25 days of salt stress (postflowering) using the other 4 plants of the treatments T1, T2, T3, and T4, and the 4 plants per replicate of T5 and T6.

In each harvest, out of 4 plants per replicate, one was used for nutritional and growth analysis, another for the physiological and biochemical analysis, and 2 for the determination of essential oil. Due to the exuberant

growth of the control plants (T1), after the first harvest the spacing between the plants of this treatment was increased to 20 cm in order to avoid shading.

After harvest, the plants were separated into leaves and stems and the leaf fresh mass (LFM) and leaf area (LA) were determined. The plant material was then oven dried at 65 °C for 72 h for determination of the leaf (LDM), stem (SDM), and shoot dry mass (SHDM). From the data of LFM, LDM, and LA, the leaf succulence (SUC) and sclerophylly index (SI) were calculated, as described by Cova et al. (2016).

For determination of contents of sodium (Na^+), potassium (K^+), and chloride (Cl^-) extracts of plant material were prepared according to the methodologies described by Gondim et al. (2011).

The determination of relative water content (RWC), organic solutes, and pigments (chlorophylls and carotenoids) was performed on the first pair of fully expanded leaves. The RWC was determined according to Barrs and Weatherley (1962). For analysis of organic solutes samples of leaves were collected, immediately frozen, lyophilized, ground to a powder, and maintained in a freezer (-20 °C). The preparation of extracts and the determination of soluble carbohydrates, free amino acids, soluble proteins, and free proline were carried out as described by Sacramento et al. (2014).

The concentration of chlorophyll *a* (CHa), chlorophyll *b* (CHb), and carotenoids (Car) was determined in a spectrophotometer at 649, 664, and 470 nm. Ethanolic extracts were prepared according to the methodology recommended by Lichtenthaler and Buschmann (2001).

The extraction of essential oil from 'Gennaro de Menta' basil was carried out by hydrodistillation of leaves and flower buds following the methodology described by Alves et al. (2015). Preliminary trials with this cultivar confirmed that the stem has no significant content of essential oil. For the extraction, 17 g of LDM were used. The distillation time (2 h) was counted from the moment when the first drop of essential oil was deposited. The final volume of essential oil extracted was verified in the Clevenger collector apparatus. From the data of volume, the oil content and yield per plant were calculated according to Alves et al. (2015). From the data on oil content and yield, oil productivity was calculated and expressed as mL m^{-1} tube of hydroponic profile. This unit is indicated to estimate how much can be produced per linear meter of tube in hydroponic structures of the pyramid type (vertical system), making possible an improvement in utilization of useful area in the greenhouse.

The data were subjected to analysis of variance using the F test, and the means were compared by the Scott-Knott test at 0.05 probability using the SISVAR 4.6 software (Ferreira, 2011).

3. Results and discussion

Salinity decreased the biomass production of basil plants when compared to control treatment (T1) regardless of the period considered (Figure 1). At 15 days of salt stress, T3 and T4 treatments (acclimated with H_2O_2 in the seedling phase) showed, respectively, increases of 27% and 14% in the LDM yield compared to T2 salt treatment without H_2O_2 pretreatment (Figure 1A). However, at 25 days after application of stress, only the T6 treatment (acclimated with H_2O_2 in both seedling and preflowering stages) showed an increase of 31% in relation to T2. Regarding SDM, there was no significant difference among salt treatments for any of the stress times (Figure 1B). Salinity decreased SHDM at 15 days of stress, and this reduction was more pronounced in T2 treatment (44%) than in T3 (29%) and T4 (34%) (Figure 1C). At 25 days of stress, no significant differences were observed among stress treatments, as observed in SDM, but all were significantly inferior to T1 (control).

Considering a combined analysis of biomass (Figures 1A–1C), the results indicate that the application of H_2O_2 mitigates the deleterious effects of salinity, occurring with more intensity at short term and requiring possibly a reapplication of H_2O_2 at a low concentration (1 μ M) in the long term. Our results show, therefore, that the application of H_2O_2 in hydroponic cultivation using brackish water can be a viable strategy for basil yield for in natura consumption purpose.

Although acclimation is a complex phenomenon, the increase of biomass yield suggests that the application of H_2O_2 increased the tolerance of basil to salinity. This increase may be related to the role of H_2O_2 in the signaling process, thereby increasing the expression of genes involved in antioxidant activity (Niu and Liao, 2016). Other authors have also reported similar results in maize (Azevedo Neto et al., 2005) and in *Ocimum basilicum* (Delavari et al., 2014).

Salinity reduced RWC at both 15 days (7%) and 25 days (10%) of stress, compared to control treatment T1 (Figure 1D). Christou et al. (2014) showed that the application of H_2O_2 maintained the RWC in strawberry leaves only in immediately salt-treated plants, reducing the negative effect caused by salt. However, in the present study plants under salt treatment after 7 days of H_2O_2 application did not differ from the treatment without application of H_2O_2 .

Leaf succulence (SUC) of plants under salt treatments was 15% higher than that in control treatment (T1) at 15 days of salt stress. At 25 days, T1 and T2 treatments were not different, but an increase of about 31% was observed in the SUC of plants acclimated with H_2O_2 (Figure 1E), suggesting that the plants under these treatments maintained a greater cell water status, reducing salt-induced water stress. Cova et al. (2016) reported that

salinity induced an increase of SUC in noni (*Morinda citrifolia* Linn) leaves after 40 days of salt stress. According to these authors, plants under salt stress or other abiotic stress may present such mechanisms to survive adverse environmental conditions. This mechanism results in a greater amount of water per unit of leaf area, which may be important for water storage and dilution of the toxic Na^+ and/or Cl^- ions.

In contrast to RWC, salinity induced an increase of 49% and 44% in the sclerophylly index (SI) of salt stressed plants at 15 and 25 days, respectively, when compared to T1 treatment (Figure 1F). SI can be considered as a physiological trait in response to stress conditions, such as salinity. Therefore, this increase in SI indicates a higher leaf thickness, with more layers of photosynthetic tissues, which may be an important factor to reduce the deleterious effects of salinity on plant growth (Cova et al., 2016).

The alteration in the contents of photosynthetic pigments is one of the most common effects of salt stress and is the result of the accumulation of high concentrations of toxic ions in the leaves (Dias et al., 2016). Thus, some authors consider the variation in pigments content as a marker of the effects of salt stress on plants (Singh and Dubey, 1995).

Salinity increased the contents of CHa (17%), CHb (25%), and CHt (20%) in the T2 treatment in comparison to the control treatment (T1) at 15 days of stress (Figures 2A–2C). At 25 days, CHa and CHt contents in T2 remained higher than those of other treatments (Figures 2A and 2C). In the T5 and T6 treatments the levels of CHa decreased by 24% and 12%, respectively, compared to T1 (Figure 2A). In this same period, CHb in T2 treatment was not affected by salt stress; however it decreased by 11% in T3 and T4 and 23% in T5 and T6 (Figure 2B). The increase of CHa and CHb levels in the T2 treatment may represent a greater energy cost for the biosynthesis of these pigments in the plants of this treatment when compared to those that were pretreated with H_2O_2 . Therefore, the data suggest that the maintenance of chlorophyll content may be a good indicator of salt tolerance in basil.

As chlorophylls, carotenoids (Car) are integral constituents of thylakoid membranes and act as accessory pigment in light capture as photoprotective agents, dissipating rapidly absorbed excess light (Taiz and Zeiger, 2003). At 15 days, the Car content was not affected by salinity. However, at 25 days, an increase of 30% was observed in Car content in all salt stress treatments except T5, when compared to control (Figure 2D). These results suggest that in contrast to chlorophylls, Car were not good indicators of salt tolerance induced by H_2O_2 acclimation.

Chlorophyll a to b ratio did not differ among treatments at 15 days. However, at 25 days, there was an increase of CHa/CHb ratio in T3, T4, and T6 treatments (Figure

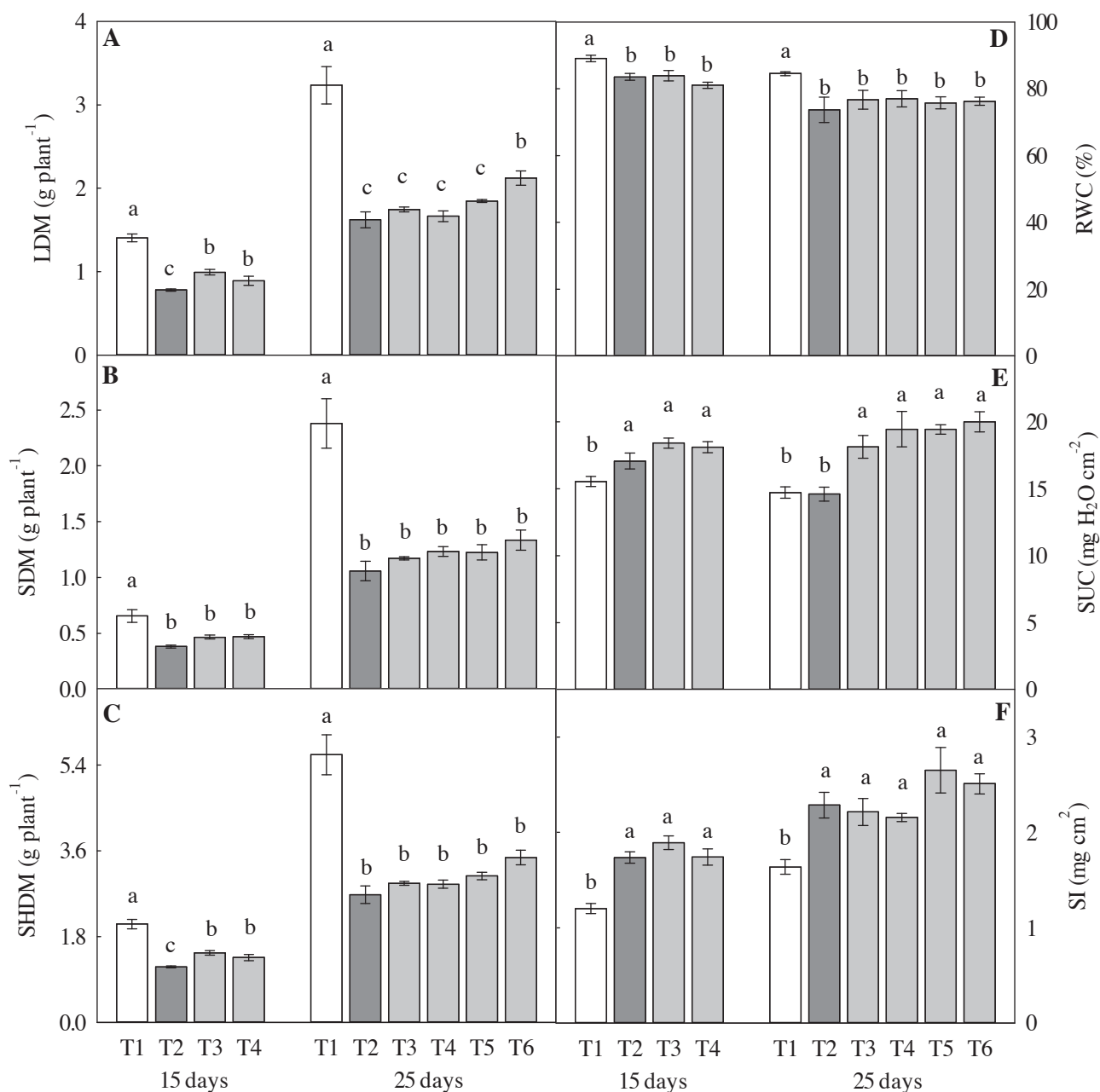


Figure 1. Leaf dry mass – LDM (A), stem dry mass – SDM (B), shoot dry mass – SHDM (C), relative water content – RWC (D), leaf succulence – SUC (E), and sclerophylly index – SI (F) of ‘Gennaro de Menta’ basil plants in hydroponic DFT system. Means followed by the same letters do not differ from each other by the Scott–Knott test at 0.05 probability. Treatments tested: T1 – 0 mM NaCl and 0 μM H_2O_2 (control); T2 – 80 mM NaCl and 0 μM H_2O_2 ; T3 – 80 mM NaCl and 1000 μM H_2O_2 /24 h in the seedling phase; T4 – 80 mM NaCl and 1 μM H_2O_2 /48 h in the seedling phase; T5 – 80 mM NaCl and 1000 μM H_2O_2 /24 h in the seedling phase and in preflowering; T6 – 80 mM NaCl and 1 μM H_2O_2 /48 h in seedling phase and in preflowering. Values indicate the mean \pm standard error (SE).

2E). At 15 days of stress, salinity decreased the CHt/Car ratio in T3 and T4 treatments only. At 25 days, CHt/Car ratio decreased in all salt treatments, but this reduction was more pronounced in treatments whose plants were acclimated with H_2O_2 (Figure 2F).

Salinity increased the Na^+ and Cl^- contents in the leaves and stem (Figures 3A–3D). At 15 days, Cl^- content in leaves and stem did not differ among salt treatments. However, at 25 days, the leaf Cl^- content in the T5 treatment was 22% higher than in the other salt treatments (Figure 3A). In the

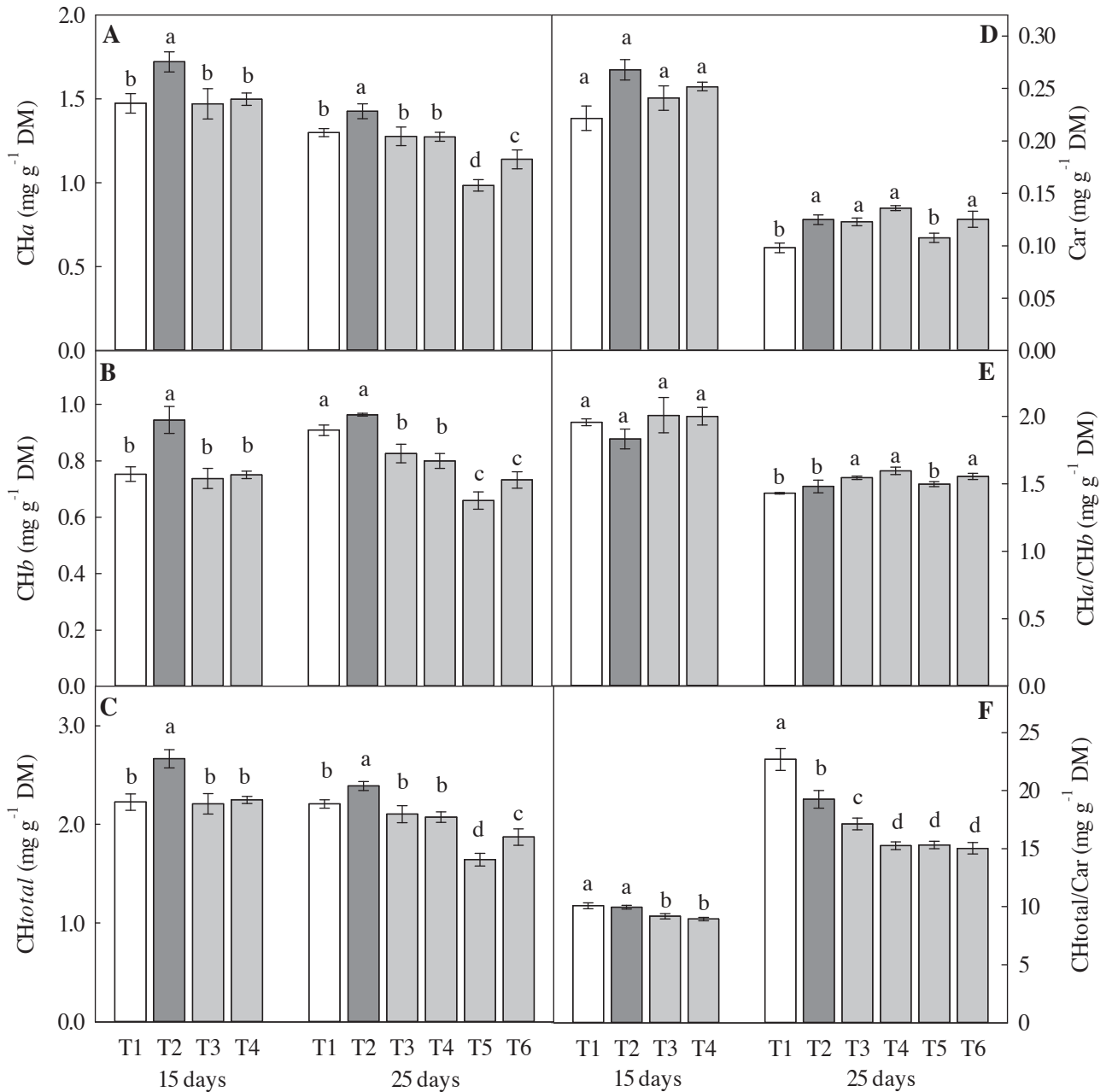


Figure 2. Chlorophyll *a* – CHa (A), chlorophyll *b* – CHb (B), total chlorophyll – CHt (C), carotenoid – Car (D), chlorophyll *a*/chlorophyll *b* – CHa/CHb (E), and total chlorophyll/carotenoids – CHt/Car (F) of ‘Gennaro de Menta’ basil plants in hydroponic DFT system. Values indicate the mean ± standard error (SE). Additional details as in Figure 1.

stem, for the same period, the Cl⁻ content in T4, T5, and T6 treatments was 17% higher than in T2 and T3 (Figure 3B).

At 15 days, the Na⁺ content in leaves of plants under salt treatments did not differ among them; however, at 25 days, the content in T4, T5, and T6 treatments was higher than in T2 and T3 (Figure 3C). In the stem results were similar in both harvests (Figure 3D). Thus, Na⁺ content in T2 and T3 treatments was similar but lower than in T4, T5, and T6

treatments. It can also be observed that Na⁺ content in the stems of the salt stressed plants was substantially higher than that observed in the leaves (Figures 3C–3D). This retention of Na⁺ in stem tissues represents a mechanism of salt tolerance, alleviating the toxicity of this ion in the photosynthetically active tissues of leaves.

The accumulation of Na⁺ and Cl⁻ in the tissues of plants exposed to salt stress represents one of the main factors

responsible for salt induced metabolic disturbances (Dias et al., 2016). Thus, excessive uptake of these ions can affect cell membrane stability and plant growth (Barrs and Weatherley, 1962).

Salt stress treatments that showed the highest Na⁺ and Cl⁻ contents (T5 and T6) also had higher SUC and LDM. These results suggest that higher SUC induced by application of H₂O₂ allowed an increased Na⁺ and

Cl⁻ contents in the leaves, reducing water potential and improving water uptake (Silveira et al., 2009; Azevedo Neto et al., 2015).

In general, salinity did not affect the K⁺ contents at 15 days in the leaves but decreased by about 28% in the stem. At 25 days, salinity decreased the K⁺ content in the leaves and stem, but this reduction was more conspicuous in the H₂O₂ acclimated plants (Figures 3E-3F). The Na⁺

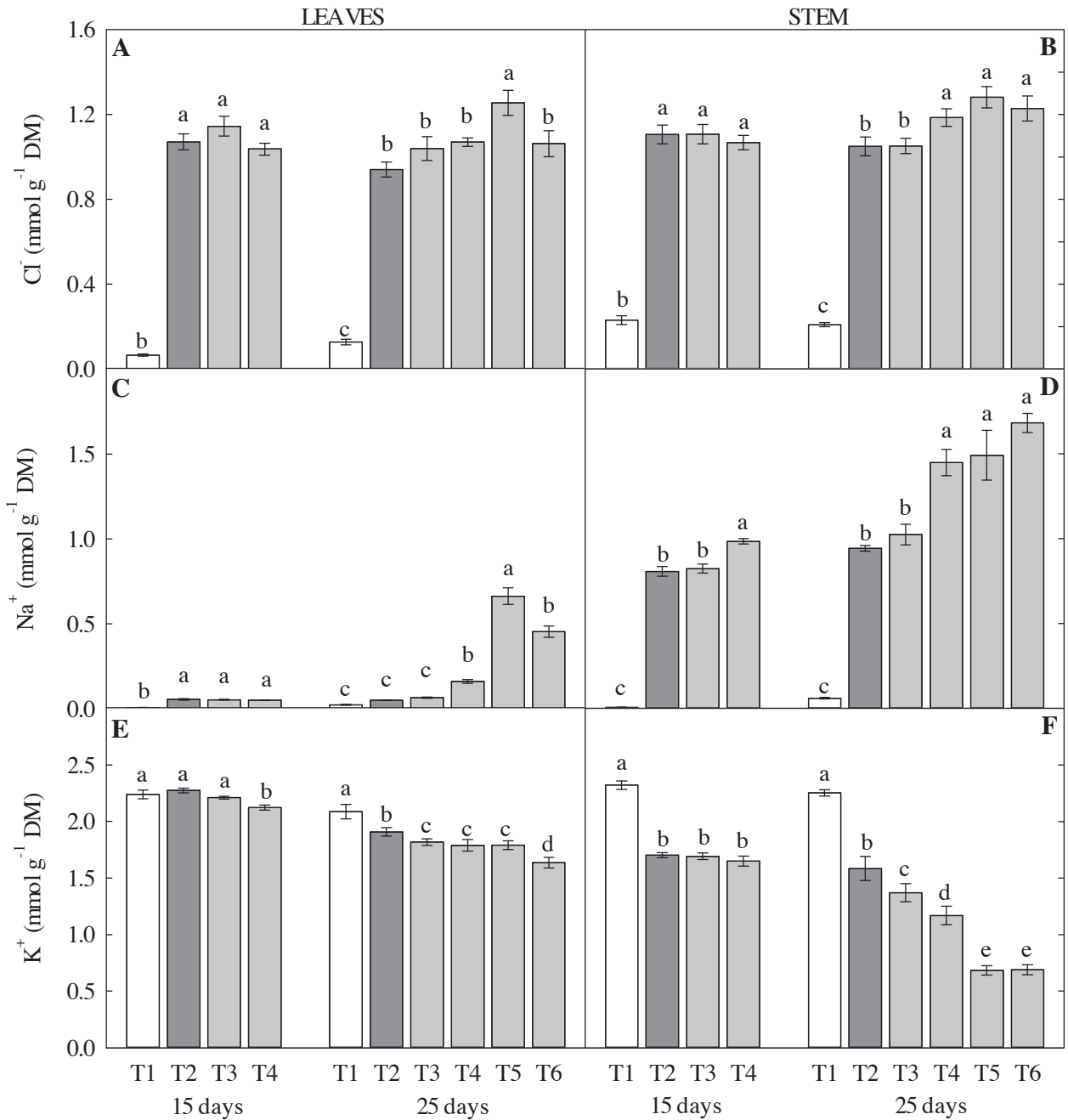


Figure 3. Cl⁻ (A, B), Na⁺ (C, D), and K⁺ (E, F) content in leaves and stem, respectively, of 'Gennaro de Menta' basil plants in hydroponic DFT system. Values indicate the mean ± standard error (SE). Additional details as in Figure 1.

induced decrease in K^+ content was reported by other authors (Gondim et al., 2011; Cova et al., 2016). This reduction is related to the antagonism between these two ions, since Na^+ ions uptake by root cells occurs through nonselective K^+ channels and high affinity K^+ transporters due to physicochemical similarities between Na^+ and K^+ (Gondim et al., 2011; Miranda et al., 2017). Despite the reduction in leaf K^+ content in all salt treatments, these are above the normal range (0.81–1.18 mmol g^{-1} DM) for vegetable cultivation (Mengel et al., 2007), indicating that the reduction in dry mass production induced by salt stress was not due to disturbances in K^+ nutrition.

Salinity increased the leaf organic solutes content as compared to T1 treatment (Figure 4), but this increase was more conspicuous in the plants treated with H_2O_2 (T3, T4, T5, and T6) than in nontreated ones (T2). At 15 days, the soluble carbohydrates content in the plants under salt treatments increased by about 112% compared to T1 treatment. At 25 days, the T2 treatment did not differ from the control (T1); however the soluble carbohydrates in salt treatments that received one (T3 and T4) or two (T5 and T6) applications of H_2O_2 were, respectively, 56% and 159% higher than in T2 (Figure 4A).

The free amino acid content in the plants treated with H_2O_2 was higher than in T1 and T2 treatments in both

harvests. Thus, at 15 days free amino acids content in T3 and T4 increased, respectively, by 24% and 71% compared to T2. At 25 days, the increase observed was around 42% in T3, T4, and T6 treatments and 72% in T5 treatment (Figure 4B). The increase of these compounds in plants grown under salt stress was also reported by Heidari (2012) and Sacramento et al. (2014).

At 15 days, the soluble protein content in the plants of T4 treatment increased 36% in comparison to T2 treatment. At 25 days, the highest concentrations were observed in the T4 and T5 treatments, with an increase of 60% when compared to T2 treatment (Figure 4C).

Under salt conditions, plants often accumulate low molecular mass proteins (Azevedo Neto et al., 2009), also called stress proteins, whose main function appears to be the protection of cellular and subcellular structures against oxidative damage and dehydration, as well as signaling responses to salinity (Wahid et al., 2007). In addition, its accumulation can serve as a source of nitrogen storage that can be mobilized after alleviation or removal of stress (Azevedo Neto et al., 2009).

In salt conditions, proline content in plants treated with H_2O_2 increased by 90% (T3) and 33% (T4) at 15 days, and by around 111% (T4 and T5) at 25 days when compared to T2 treatment (Figure 4D). Although the role of proline

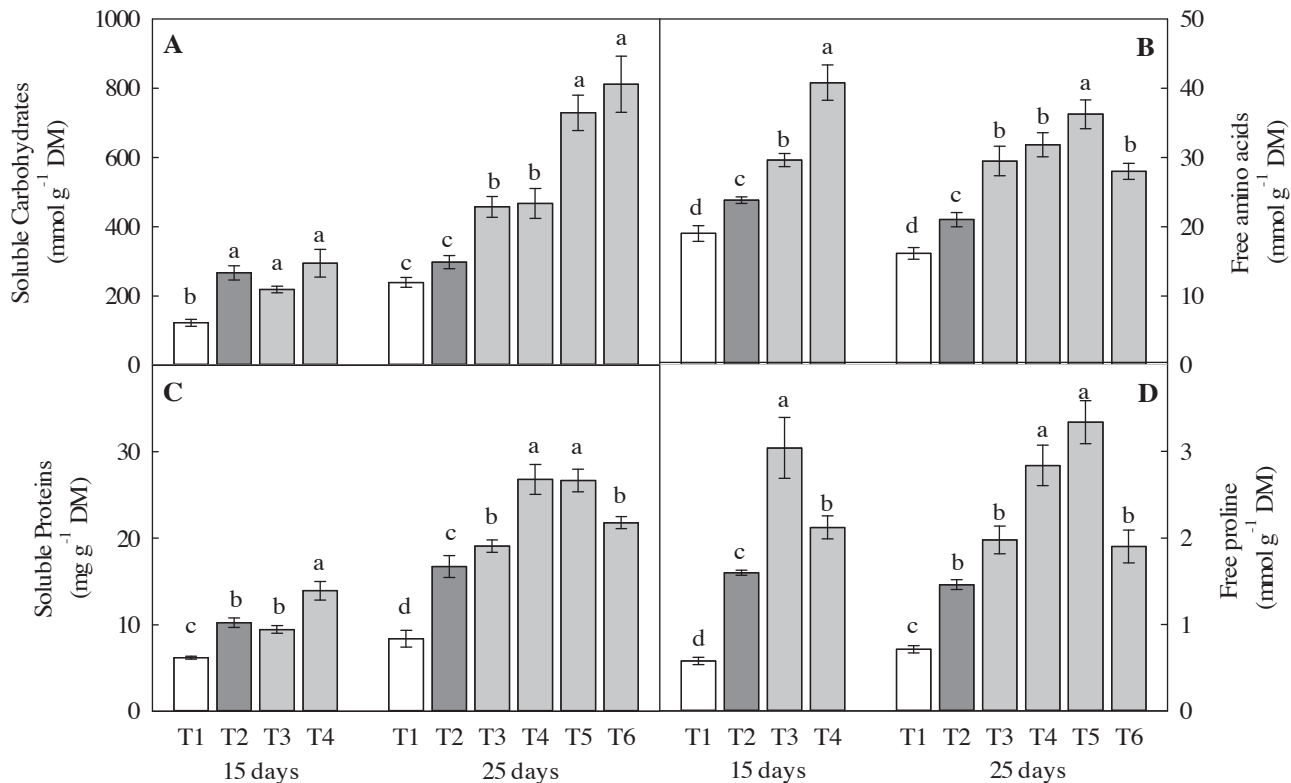


Figure 4. Soluble carbohydrate (A), free amino acid (B), soluble protein (C), and free proline (D) contents of ‘Gennaro de Menta’ basil plants in hydroponic DFT system. Values indicate the mean \pm standard error (SE). Additional details as in Figure 1.

Table. Means of content, yield, and productivity of essential oil of ‘Gennaro de Menta’ basil plants in hydroponic DFT system, as a function of treatments used**.

Treatments	Oil content (% LDM)	Oil yield (mL planta ⁻¹)	Oil productivity (mL m ⁻¹ tube)
T1	0.308 ± 0.028	0.011 ± 0.0012	0.050 ± 0.005
T2	1.041 ± 0.135	0.017 ± 0.0019	0.354 ± 0.019
T3	1.207 ± 0.048	0.021 ± 0.0023	0.412 ± 0.023
T4	0.754 ± 0.097	0.013 ± 0.0014	0.245 ± 0.014
T5	0.887 ± 0.017	0.016 ± 0.0018	0.303 ± 0.018
T6	1.509 ± 0.135	0.031 ± 0.0034	0.479 ± 0.034

* Mean ± standard deviation (SD).

** Treatments: T1 – 0 mM NaCl and 0 μM H₂O₂ (control); T2 – 80 mM NaCl and 0 μM H₂O₂; T3 – 80 mM NaCl and 1000 μM H₂O₂/24 h in the seedling phase; T4 – 80 mM NaCl and 1 μM H₂O₂/48 h in the seedling phase; T5 – 80 mM NaCl and 1000 μM H₂O₂/24 h in the seedling phase and in preflowering; T6 – 80 mM NaCl and 1 μM H₂O₂/48 h in the seedling phase and in preflowering.

in response to salinity has been reported by other authors (Azevedo Neto et al., 2009; Sacramento et al., 2014), its role in acclimation to salt stress is still controversial (Azevedo Neto et al., 2009). In this study, the higher proline content observed suggests a beneficial effect of this organic solute on basil acclimation to salt stress.

Similar to that observed in inorganic solutes, the increase of organic solutes content in plants under salt stress and treated with H₂O₂ induces an increase in water uptake, SUC, dilution of Na⁺ and Cl⁻, and consequently, reduction of the toxic effects of these ions. The observation that among salt treatments the highest production of LDM was obtained in plants with higher SUC and higher inorganic and organic solutes contents supports this hypothesis.

Salinity increased the content, yield, and productivity of essential oil (Table). The oil content of T2 treatment (1.041%) was 3.38-fold higher than the control treatment (0.308%). Comparing the salt treatments, it can be verified that the oil contents of treatments T3 (1.207%) and T6 (1.509%) were 16% and 45% higher than those obtained in T2 (Table 1). Several secondary compounds have been shown to scavenge reactive oxygen species (Selmar, 2008). Thus, the increase in secondary compounds content in basil may represent an important mechanism for salt stress tolerance.

Yield and productivity of essential oil followed similar behavior as oil content. There was an increase of 54% in oil yield of plants in T2 treatment, compared to T1. However, yields in T3 and T6 treatments were, respectively, 25% and 83% higher than in T2 (Table). Salt stress reduced the production of shoot biomass by 52%; therefore the

population density used in salt treatments was twice that of the control (see materials and methods). Under these conditions, oil productivity in T2 treatment was 208% higher than in T1, while the productivities in T3 and T6 were 25% and 83% higher than in T2 (Table).

Data on the influence of salt stress on the enhancement of secondary metabolites is dependent on the genotype used. Bernstein et al. (2010) verified an increase in essential oil content using seedlings of basil, cultivar ‘Perrie’, under salt conditions. In contrast, Alves et al. (2015) reported a decrease in oil yield in basil (unidentified cultivar), with further increases of salinity. In our work, the results clearly show that, in the ‘Gennaro de Menta’ cultivar, the essential oil content, yield, and productivity are increased by both salt stress and H₂O₂ application.

In conclusion, the application of 1 μM H₂O₂/48 h in the nutrient solution in the seedling phase and its reapplication in the preflowering phase is the most efficient strategy to increase salt tolerance of basil cv. ‘Gennaro de Menta’, either for biomass or oil production. This H₂O₂ induced increase in salt tolerance is related, at least in part, to an increase in leaf content of inorganic and organic solutes, leading to higher SUC, dilution of Na⁺ and Cl⁻, and consequently reduction of the toxic effects of Na⁺ and Cl⁻ ions.

With the problem faced in arid and semiarid regions, where brackish waters are often used in irrigation, the hydroponic cultivation stands out as an important alternative for agriculture in these regions. The data from this study indicate that the application of H₂O₂ in nutrient solution in hydroponic systems using brackish water is a viable technique for basil cultivation for both in natura consumption and essential oil production in these regions.

Acknowledgments

We thank the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), the Conselho Nacional de

Desenvolvimento Científico e Tecnológico (CNPq), and the Universidade Federal do Recôncavo da Bahia (UFRB) for their financial support over the years.

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