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Comparative Effects of Salicylic Acid and/or Trehalose on Osmotic Adjustment and Solutes Allocation of Two Droughted Wheat (*Triticum aestivum L.*) Cultivars

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Abstract

The comparative effects of salicylic acid and/or trehalose on osmotic adjustment and solutes accumulation of two droughted wheat cultivars were examined. Studies were carried out with two wheat (*Triticum aestivum L.*) Gemmieza-7 (drought sensitive cultivar) and Sahel-1 (drought tolerant cultivar cultivars during grain-filling. Water stress was found to induce a marked increase in osmotic pressure, total soluble sugars, total soluble nitrogen, proline, organic acids as well as ions content (Na^+, K^+, Ca^{2+} and Cl^-) and Na^+/ K^+ ratio (except K^+ appeared to non-significantly affected in case of sensitive cultivar) in flag leaf of both cultivars during grain-filling. SA and/or Tre caused significant increase in these osmolytes contents. For osmolytes, it appeared that osmotic pressure was positively correlated with TSS, TSN, proline, keto-acids, citric acid, Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^-, Na^+ / K^+ ratio for both Gemmieza-7 and Sahel-1 respectively.

Keywords: Wheat; Drought; Osmotic Adjustment; Compatible Solutes

Abbreviations: ANOVA: Analysis of Variance; OP: Osmotic Pressure; TSS: Total Soluble Sugar; TSN: Total Soluble Nitrogen; WS: Water Stress; SA: Salicylic Acid.

Introduction

Water stress is likely the most important factor that adversely affects plant growth and development [1,2]. Drought is the first most wide spread problem for wheat production. Therefore, enhancing drought tolerance is of particular interest for sustainable wheat production. Of several strategies, selection of species with drought resistance is an economic and efficient means of alleviating the problem [3]. Drought tolerance is the ability of plant to grow, flower and display economic yield under suboptimal water supply [4] and wheat is known as moderately drought tolerant crop [5]. Plants are constantly bombarded with various environmental signals, some of which cause stress and restrict plant growth and development. In response to those adversities, plants have developed a number of strategies that increase their tolerance or adaptation to stress conditions [6]. One such mechanism that is ubiquitous in plants is the accumulation of certain organic metabolites of low molecular weight that are known collectively as compatible solutes [7,8]. These osmolytes were reported

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to play a pivotal role in cellular osmotic adjustment in response to water stress [9].

As a consequence to osmotic regulation exerted by water-stressed plants, the osmotic pressure of their extract is expected to be higher than that when they are unstressed. In agreement with this concept, Aldesuquy et al. [10] recorded that wheat plants exhibited higher values of osmotic pressure of their leaf-water extract as a result of physiological water stress. Similarly, Aranjuelo *et al.* [11] recorded significant increase in leaf osmotic potential in droughted alfalfa plants. Sugars contribute up to 50% of the total osmotic potential in glycophytes subjected to water stress [12]. The accumulation of soluble carbohydrates in plants has been widely reported as a response to water shortage despite the significant decline in the net CO_2 assimilation rate [13]. In this respect Xue *et al.* [14] found that water deficit in wheat leaves caused high synthesis of total soluble sugars. Furthermore, drought induced marked increase in total soluble sugars of wheat flag leaves at heading and anthesis stages as compared to control values [15]. Nitrogen-containing compounds were also regarded to be involved as main osmolytes contributing to the plant osmotic regulation under water stress. In relation to control values, the amount of total soluble nitrogen significantly increased in the leaf-water extract of different plants suffering from water stress conditions [16,10].

Proline was considered as a major organic osmolyte accumulating in a variety of plant species in response to environmental stresses such as salinity, drought, extreme temperatures, ultraviolet radiation and heavy metals [17]. Accumulation of proline in many stressed plant species has been correlated with stress tolerance, and its concentration has been shown to be generally higher in stress-tolerant than in stress-sensitive plants [18]. Increased proline in water-stressed plants has been documented in chickpea [19] and wheat [10]. The increase in organic acids has been observed under various a biotic stress conditions [20,21]. In addition, a key feature pertaining to osmotic adjustment is the ability to accumulate various ions in the plant vacuoles [22]. The accumulation of organic acids (represented in citric and keto acids) as well as different ions (represented in Na^+, K^+, Ca^{++}, Mg^{++} and Cl^-) in response to water stress was reported by Ghanem [23].

Materials and Methods

Plant material and growth conditions

Pure strains of *Triticum aestivum L*. Gemmieza-7 (drought sensitive cultivar) and Sahel-1 (salt tolerant cultivar) were kindly supplied by the Agricultural Research Center, Ministry of Agriculture, Giza, Egypt.

For soaking experiment, a homogenous lot of *Triticum aestivum L*. (i.e. either sensitive or tolerant cultivar) grains were selected. The grains were separately surface sterilized by soaking in 0.01 M $HgCl_2$ solution for three minutes, then washed thoroughly with distilled water. The sterilized grains from each cultivar were divided into two sets (≈ 500 g per set for each cultivar). Grains of the 1st set were soaked in distilled water to serve as control, while those of the 2nd were soaked in salicylic acid (3 mM) each for about 6 hours.

After soaking, thoroughly washed grains were drilled in 20 November 2011 and 2012 in plastic pots (20 cm in diameter) filled with 5.5 kg soil (clay/sand 2/1, v/v), where fifteen grains were sown in each pot. The pots were then kept in a greenhouse at Botany Department, Faculty of Science, Mansoura University, Egypt. The plants were subjected to natural day/night conditions (minimum/maximum air temperature and relative humidity were 15/25°C and 35/45%; respectively) at mid-day during the experimental period. The plants in all sets were irrigated to field capacity by tap water. After two weeks from sowing, thinning was started so that five uniform seedlings were left in each pot for the subsequent studies.

On the day 65 after planting (at the beginning of heading) the pots of the 1st set was allocated to four groups (20 pots per each group) as follows: control (cont.), water stress (WS), trehalose control, trehalose + water stress (trehalose + WS). The 2nd set group was allocated to four groups as follows: salicylic acid control (SA**)**, salicylic acid + water stress (SA+WS), control trehalose + salicylic acid (SA + trehalose) and salicylic acid + trehalose + water stress (SA+ trehalose +WS). For + trehalose (1.5mM) treatment, the plants were sprayed by trehalose 48 hrs before starting the stress period and weekly during the stress period.

Water deficit was imposed by withholding water at the reproductive stage for 30 days within two periods: on the day 65 from planting (heading stage) and the day 80 from planting (anthesis stage).

Each droughted pot received 500 ml water at the end of 1st stress period. At the end of stress periods, rewatering to the field capacity was carried out. The undroughted (control) plants were irrigated to the field capacity during the stress period, and all plants were left to grow until grain maturation under normal irrigation with tap water. After thinning and at heading, the plants received 36 kg N

ha^{-1} as urea and 25 kg P ha^{-1} as super-phosphate. For osmolytes analyses during grain-filling (21 days post-anthesis) (i.e. 106 days after sowing) only three samples were taken from each treatment. Data were obtained and the mean values (per plant) were computed for each treatment.

Determination of osmotic pressure

Preparation of plant extract: The leaf plant extract was prepared according the procedure adopted by El-Sharkawi & Salama [24]. The flag leaves were oven-dried for two days at 80^0C and then powdered. Powder was taken in a test tube, to which 10 ml of distilled water was added, and heated at 90^0C in a water bath for one hour. The tubes were shaken every 5 minutes during heating. In a centrifuge tube, the suspension was quantitatively transferred and centrifuged at 7000 xg for 15 minutes. The supernatant was then transferred to 25 ml Erlenmeyer flask. The precipitate was transferred back to the same test tube used before, by perfect decantation with distilled water, and the same extraction procedure was repeated. After the second centrifugation, the supernatant extract was added to the previously prepared portion of extract and the volume was completed to 25 ml.

Determination of osmotic pressure of the extract: The cryscopic method of Walter [25] was used in this determination. The freezing point of the extract was determined cryscopically by using a special Beckmam differential thermometer (calibrated to 0.01°C) as illustrated by Slatyer & Mcilroy [26]. The osmotic pressure of the sap was then calculated as described by El-Sharkawi & Abdel Rahman [27].

Determination of soluble osmotically active metabolites: The analysis of soluble osmotically active metabolites included the determination of total soluble sugars (as carbon metabolites), total soluble nitrogen and proline (as nitrogen metabolites), citric and keto acids (as organic acids) as well as some ions (Na^+, K^+, Ca^{2+}, Mg^{2+} and Cl^-). These four types of compounds represent the major fractions of osmotically active metabolites that can contribute to the osmotic adjustment in plants.

Estimation of total soluble-N (TSN): The total soluble nitrogen was determined by the conventional semi micro-modification of Kjeldahl method [28]. An aliquot of the extract was taken into a digestion flask and heated for at least 8 hours with 0.5g catalyst (80g, K_2SO_4; 20g, $CuSO_4.5H_2O$ and 0.3g, SeO_2), 2 ml of ammonia free concentrated H_2SO_4 and 1 ml of distilled water. The solution was treated with 15 ml of 40% NaOH and steam distilled in the conventional manner into 5 ml of 0.05 N H_2SO_4. The distillate was then made up to volume and used for estimation of total soluble nitrogen by estimating ammonia. Ammonia-N was estimated spectrophotometrically by the method adopted by Delory [29]. Using Nessler's reagent as modified by Naguib (1964). An aliquot of the extract was mixed with 1 ml of 1 N NaOH and 0.5 ml of 0.5% $ZnSO_4$. The mixture was made up to 14 ml with distilled water before 1 ml of Nessler's reagent was added, shaken well and allowed to stand for 5 minutes and the optical density was then measured at 450 nm.

Preparation of Nessler's reagent

Thirty five g of KI were dissolved in 100 ml of water and then 4% $HgCl_2$ solution were added with continuous stirring till persistent slight red precipitate was obtained (about 325 ml is required). While stirring, solution of 120 g NaOH in 250 ml of distilled water was introduced and the mixture was made up to 1 L with distilled water. Slight excess of $HgCl_2$ solution was added till permanent turbidity. The mixture was allowed to stand for one day and then filtered. It was always kept in a stopper dark brown reagent bottle in a refrigerator but not more than 14 days. The standard curve was made using a series of standard ammonia-N concentrations, where a linear relationship being obtained between these concentrations and the optical density readings.

Estimation of proline: The method adopted, for the estimation of proline was essentially that described by Snell & Snell [30]. As a reagent, mix 4 ml of 1:1 dilution of syrupy phosphoric acid and 6 ml of glacial acetic acid. Add 0.25 g of ninhydrin and heat to 70^0C to obtain complete solubility. To 1 ml of the concentrated water extract in quick fit tubes, 1 ml of glacial acetic acid and 1 ml of the reagent were added. At the same time, a sample blank, using the acid mixture without ninhydrin, was prepared. The samples and blanks were then heated at 100 ºC for 60 minutes, with caps in place. One ml of glacial acetic acid was added to each then cool to room temperature. The volume in each was adjusted to 5 ml with glacial acetic acid. The optical density of the color developed was measured within one hour at 515 nm using spectrophotometer. The concentration of proline in the extracted samples was calculated using calibration curve.

Estimation of keto-acids: For the estimation of keto-acids, the method of Friedman & Haugen [31] was used. This method depends on the formation of the respective hydrazones, purification of such hydrazones by extraction with suitable organic solvents and re-elution of these hydrazones in sodium carbonate solution which on alkalization would give a urine red colour which can be measured spectrophotometrically?

Procedure

In a stoppered test tube, 3 ml of the plant extract was mixed with 1 ml of a freshly prepared 0.2% 2, 4-dinitrophenylhydrazine in 2N HCl. The mixture was thoroughly shaken and kept standing at room temperature for 30 minutes, 8-10 ml ethyl acetate were added and the mixture were vigorously shaken several times, until the aqueous layer became colorless. After complete separation, the aqueous layer was quantitatively pipetted off and discarded. To the remaining ethyl acetate extract, 6 ml of 10% Na_2CO_3 solution was added, and the mixture was further shaken until the water layer attained a constant yellow tint, whereby the keto-acid hydrazine was taken in the carbonate solution. Upon complete separation, 5 ml of the aqueous extract were quantitatively transferred to another dry test tube containing 5 ml of 2N NaOH solution. The mixture was shaken and kept for 15 minutes before being estimated spectrophotometrically at 510 nm. This method gave linear relation between the concentration of pyruvate and α-ketoglutarate and the respective optical density readings at sample range of α-ketoglutaric and pyruvic acids.

Estimation of citric acid: The method adopted for determining citric acid was essentially that described by Snell & Snell [32].

Procedure

To an aliquot of water extract, 15 ml of a deproteinizing solution was added. This solution was prepared by dissolving 3g of each of mercuric chloride and zinc sulphate heptahydrate in water and the solution was completed to 100 ml. Filtration was carried out, and to 5 ml aliquot of the aliquot of the filtrate, 4 ml of 1:1 10 N HCl and 1 ml of 6.2 % ferric chloride solution were added. The mixture was diluted with distilled water to 10 ml and the color developed was read at 445 nm against a reagent blank, using spectrophotometer. This method gave a linear relation between the concentration of citric acid (2-12 mg) and the optical density of the color developed.

Estimation Na^+, K^+ and Mg^{2+}: A known weight of oven-dried flag leaves was digested in concentrated HNO_3 then filtration was carried out and filtrate was made up to known volume with distilled water. Flame spectrophotometry was used for determining Na^+ and K^+, while Mg^{2+} was measured by atomic absorption spectrophotometry according to the method described by Chapman & Pratt [33].

Estimation of Cl^-: According to Hansen & Munns [34] Chloride levels were determined by volumetric titration using N/ 35.5 $AgNO_3$ and 5% $K_2Cr_2O_4$ as an indicator.

Statistical analysis

It should be mentioned that the sample numbers which were taken for investigation were as follows: ten for growth parameters, ten for agronomic traits and three for all chemical analyses and only the mean values were represented in the respective figures. The data were subjected to one-way analysis of variance (ANOVA), and different letters indicate significant differences between treatments at $p \leq 0.05$, according to CoHort/ CoStat software, Version 6.311.

Results

Osmolytes in relation to osmotic adjustment

This experiment was planned to investigate the positive effect of grain priming with salicylic acid and spraying the wheat plants with Tre on osmolytes and its relation to osmotic adjustment of water-stressed wheat cultivars by determining osmotic pressure, some organic osmolytes as well as mineral ions. The experimental design was previously mentioned in materials and methods (Chapter 2). Measurements were carried out during grain-filling (21days post-anthesis). Data were obtained and the mean values were computed for each treatment.

Changes in osmotic pressure (OP)

Water stress induced non-significant increase in OP in flag leaf of both cultivars during grain-filling as compared to control values (Figure 1). The tolerant plants showed higher OP values than the sensitive one under stress conditions. Treatments with SA and/or Tre caused non-significant increase in the values of osmotic pressure. Furthermore, SA and Tre treatment had the most noticeable effect in increasing osmotic pressure under stress conditions.

Changes in total soluble sugar (TSS)

Water stress induced a marked increase ($p \leq 0.05$) in TSS in flag leaf of both cultivars during grain-filling as compared to control values. It is clear that the sensitive plants accumulated more TSS than the tolerant one under water stress (Figure 1). Application of SA and/or Tre induced additional increase in TSS. In comparison to all treatments, the effect of SA and Tre on TSS of wheat flag leaf was the most pronounced treatment.

Changes in total soluble nitrogen (TSN)

In relation to control values, water stress induced noticeable increase ($p \leq 0.05$) in TSN in flag leaf of sensitive cultivar and non-significant increase in tolerant cultivar. Hence, the sensitive plants accumulated more TSN than the tolerant one (Figure 1). SA and/or Tre resulted in additional increases ($p \leq 0.05$) in TSN in sensitive cultivar and non significant increase in tolerant cultivar.SA and Tre treatment recorded the highest values in stressed and unstressed wheat plants.

Changes in proline

As compared to the control values, water stress caused apparent increase ($p \leq 0.05$) in proline concentration in flag leaf of both cultivars during grain-filling (Figure 2). Comparing both cultivars, under water stress conditions, Sahel-1 had more proline level than Gemmieza-7. SA and/or Tre caused additional increase ($p \leq 0.05$) in proline content. The highest values were recorded with SA treatment in sensitive cultivar and SA and Tre treatment in tolerant cultivar under stress conditions.

Changes in organic acids

Changes in keto-acids: In relation to control values, keto-acids accumulated in response to water stress was more pronounced ($p \leq 0.05$) in flag leaf of tolerant cultivar during grain-filling where water stress induced non-significant increase in keto-acids in sensitive wheat plants (Figure 2). Significant as well as additional increase ($p \leq 0.05$) was recorded when the stressed tolerant plants were treated with SA and/or Tre. On the other hand, non-significant increase was also recorded when the stressed sensitive plants were treated with SA and/or Tre. The effect was more pronounced with SA and Tre treatment.

Changes in citric acid: Water stress caused a marked increase ($p \leq 0.05$) in citric acid in flag leaf of tolerant cultivar and non-significant increase in flag leaf of sensitive wheat plants comparing with the control plants (Figure 2). Moreover, the tolerant plants accumulated more citric acid than the sensitive one. Significant as well as additional increase ($p \leq 0.05$) was recorded when the stressed tolerant plants were treated with SA and/or Tre. Moreover, non-significant increase was also recorded when the stressed sensitive plants were treated with SA and/or Tre. The effect was more pronounced with SA and Tre treatment.

Changes in ionic content: In relation to control values, water stress generally, induced significant increase ($p \leq 0.05$)in ions content (Na^+, K^+, Ca^{2+} and Cl^-) and Na^+/ K^+ ratio (except K^+ appeared to non-significantly affected in case of sensitive cultivar) in flag leaf of both cultivars during grain-filling (Figures 3,4). SA and/or Tre caused significant increase ($p \leq 0.05$) in these ionic contents (except K^+ appeared to non-significantly affected in case of sensitive cultivar) as well as Na^+/ K^+ ratio. SA and Tre treatment had the most pronounced effect.

For osmolytes, it appeared that osmotic pressure was positively correlated with TSS (r = 0.94, 0.85), TSN (r = 0.89, 0.86), proline (r =0.73, 0.94), keto-acids (r = 0.91, 0.93),citric acid (r = 0.84, 0.89),Na^+ (r = 0.63, 0.93), K^+ (r = 0.55, 0.87), Ca^{2+} (r = 0.81, 0.87), Mg^{2+} (r = 0.63, 0.82), Cl^- (r = 0.63, 0.95), Na^+ / K^+ ratio (r = 0.94, 0.85) for both Gemmieza-7 and Sahel-1 respectively.

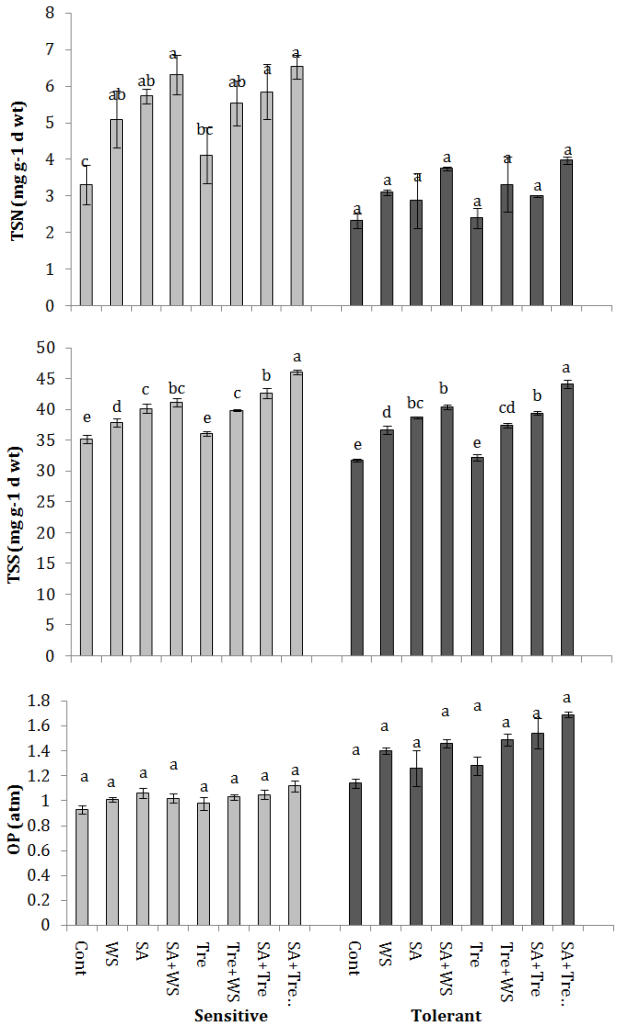

Figure 1: Effect of salicylic acid, trehalose and their interaction on osmotic pressure (OP) (atm), TSS and TSN (mg g^{-1} d wt) in the extract of the flag leaf of droughted wheat cultivars during grain-filling. Vertical bars represent standard error of the mean (n=3). Different letters indicate significant differences between treatments at $p \leq 0.05$, according to CoHort/ CoStat software, Version 6.311.

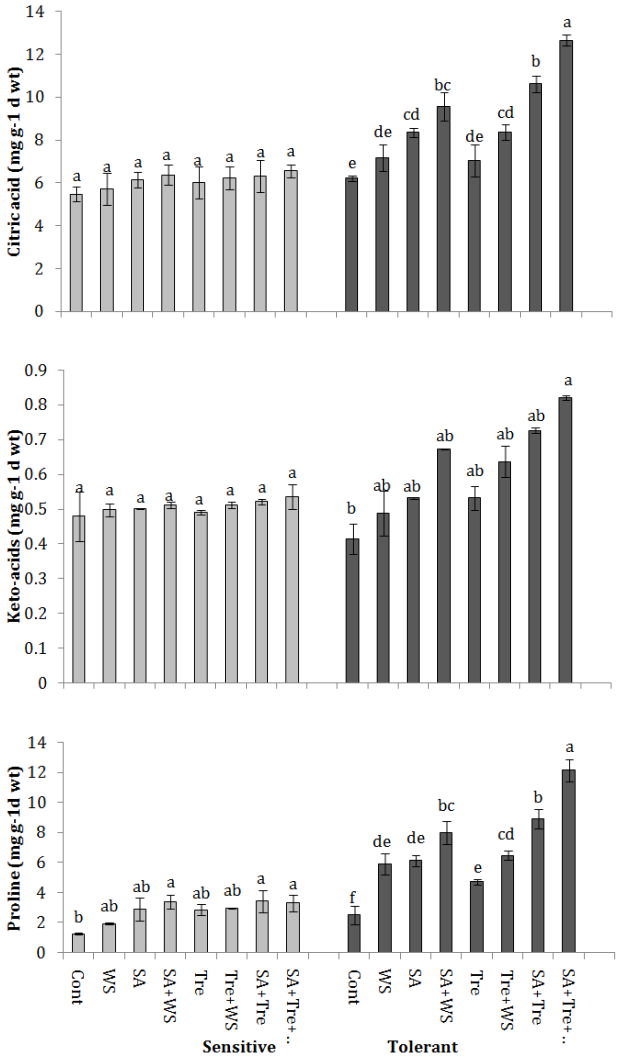

Figure 2: Effect of salicylic acid, trehalose and their interaction on proline and organic acids (mg g^{-1} d wt) in the extract of the flag leaf of droughted wheat cultivars during grain-filling. Vertical bars represent standard error of the mean (n=3). Different letters indicate significant differences between treatments at $p \leq 0.05$, according to CoHort/ CoStat software, Version 6.311.

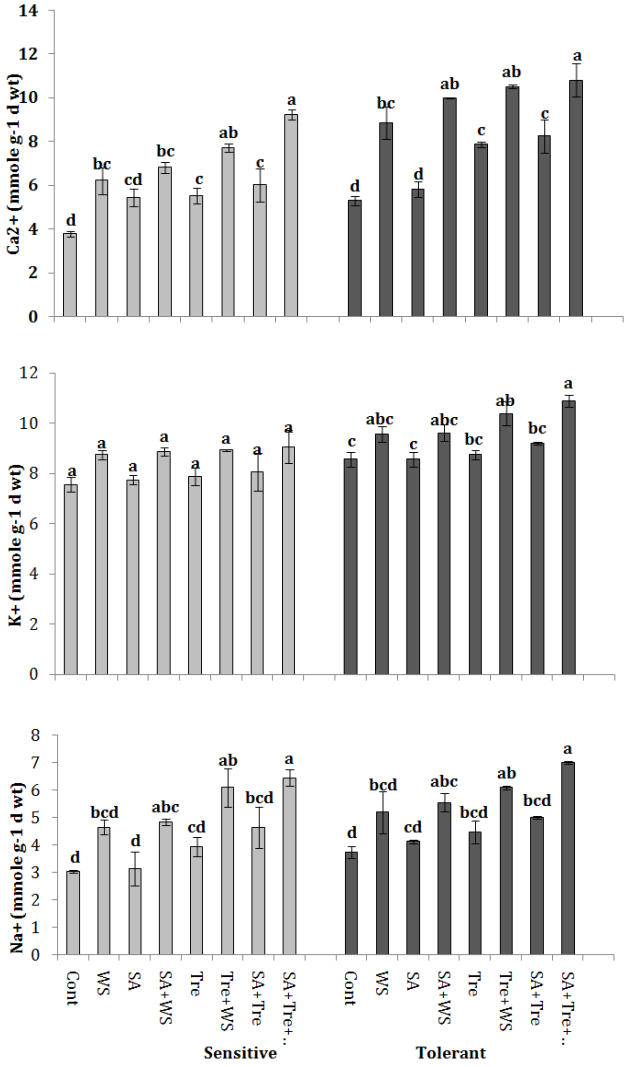

Figure 3: Effect of salicylic acid, trehalose and their interaction on Na^+, K^+ and Ca^{2+} (mmol g^{-1} d wt) in flag leaf extract of droughted wheat cultivars during grain-filling. Vertical bars represent standard error of the mean (n=3). Different letters indicate significant differences between treatments at $p \leq 0.05$, according to Cohort/Costa software, Version 6.311.

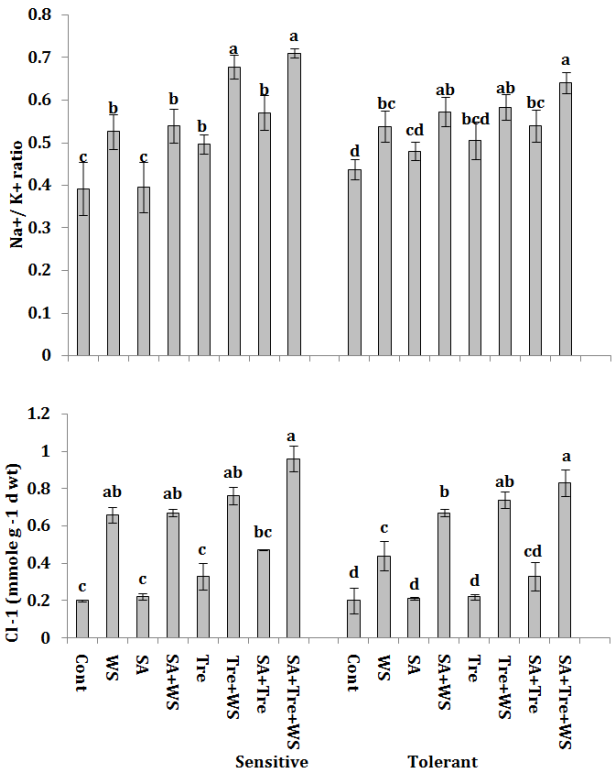

Figure 4: Effect of salicylic acid, trehalose and their interaction on Cl^-(mmol g^{-1} d wt) and and Na^+/ K^+ ratio in flag leaf extract of droughted wheat cultivars during grain-filling. Vertical bars represent standard error of the mean (n=3). Different letters indicate significant differences between treatments at $p \leq 0.05$, according to Cohort/ Costa software, Version 6.311.

Discussion

In order to understand the physiological adaptation of wheat to stress induced by water stress, osmotic pressure, proline, total soluble sugar (TSS), total soluble nitrogen (TSN), organic acids and ions content in flag leaf were studied particularly during grain filling (21 days post-anthesis). One of the mechanisms that plants use to combat the detrimental effects of water loss is to synthesize compatible solutes [35]. The compatible solutes or the osmolytes are involved in signaling and regulating the plant responses to stress conditions [36]. The compatible solutes are non-toxic molecules, do not interfere with normal metabolism and accumulate predominantly in the cytoplasm at high concentrations under osmotic stress [37].

The results obtained from the present investigation revealed that drought increased the osmotic pressure as well as the amount of organic osmotica (i.e. TSS, TSN, proline, keto acids and citric acid). Regarding the inorganic osmoprotectants in the form of Na^+, K^+, Ca^{2+}, Cl^- and Na^+/ K^+ ratioin flag leaf of both droughted wheat cultivars during grain-filling (Figures 1-4).

These findings are in agreement with those obtained by Aldesuquy *et al.* [10]. Who reported that salinity stress by seawater caused marked increase in the amount of various organic (total soluble sugars, total soluble nitrogen, proline, keto acids and citric acid) and inorganic (Na^+, K^+, Ca^+ and Cl^-) osmolytes with concomitant increase in osmotic pressure of different wheat cultivars.

Increased concentrations of compatible solutes in plant cells can contribute to reduced water potential in cytoplasm by balancing the decreased water potential in vacuole and the extracellular volume. These compounds can also alleviate the inhibitory effects of water stress on enzymatic activity without interfering with protein structure and function. Moreover, they may act as antioxidants in scavenging free radicals, and can also help in stabilizing membranes [38]. Additionally, the accumulation of osmolytes has also been claimed to facilitate better translocation of carbohydrate reserves to different plant parts [39]. To accommodate the ionic balance in the vacuoles, cytoplasm accumulates low-molecular mass compounds, the compatible solutes; because they do not interfere with normal biochemical reactions rather they replace water in these reactions [40].

Measuring the osmotic pressure in the cell sap of seawater-stressed plants could provide clear indication for the water status of the plant cells, where the tolerance to stressful conditions is thought to involve raising up the internal osmotic pressure, *via* the accumulation of either organic or inorganic osmotica, to exceed that of the external growing medium [10]. In the present study, osmotic pressure of leaf water extract was found to increase in stressed wheat plants (Figure 1). In consistence with these results, Ottow *et al.* [41] cleared that stressed poplar plants were able to adjust the osmotic pressure of their leaves to levels just exceeding those of the nutrient solution, which is important to maintain water uptake and to prevent dehydration. In this connection, Blum [42] concluded that osmolytes accumulation in plant cells could result in a decrease of the cell osmotic potential and thus maintenance of water absorption and cell turgor pressure, which might contribute to sustain physiological processes.

The accumulation of soluble carbohydrates in plants has been widely reported as a response to water stress inspite

of the significant decrease in the net CO_2 assimilation rate [13]. In the present work, drought caused marked increase in total soluble sugars in water extract of wheat flag leaf when compared with the control plants (Figure 1). Similarly, Murphy *et al.* [43] suggested that soluble carbohydrates could act as compatible solutes under water deficit conditions. It was assumed that sugars act for osmotic adjustment and/or protect specific macromolecules and contribute to the stabilization of membrane structures, where sugars are thought to interact with polar head groups of phospholipids in membranes so that membrane fusion is prevented [44].

The observed accumulation of soluble sugars in stressed wheat plants may reveal an enhanced ability of a tolerant cultivar (Sahel-1). In this regard, growth arrest resulted from water deficit was considered as a possibility to preserve carbohydrates for sustained metabolism, prolonged energy supply and better recovery after stress relief [44]. For instance, hexose accumulation was shown to account for a large proportion of osmotic potential in water-stressed maize plants [45].

The increase in the soluble nitrogen compounds is also of great importance to plant osmo regulation in response to water deficit conditions [15]. In the present work, stressed wheat plants had higher total soluble nitrogen content in their leaf extract when compared with their unstressed relatives (Figure 1). In agreement with these results, Aldesuquy *et al.* [15] indicated that drought treatment induced marked accumulation of total soluble nitrogen in two different wheat cultivars.

The effect of water stress on the amount of different nitrogenous compounds is fairly well known [46]. The accumulation of these nitrogenous compounds may mainly result from a sharp increase in total free amino acids, total soluble proteins and glycine betaine [16]. However in other cases, reversed change in nitrogen content may be related to the inhibition of translocation from root to shoot, inhibition of protein synthesis or the increase in protease activity [47].

Proline accumulation is one of the common characteristics in many plants exposed to water stress [12]. Therefore, proline could be used as a good parameter to evaluate plant tolerance or sensitivity to stress [48]. Thus, water shortage increased proline content in the leaf extract of wheat plants (Figure 2). These results indicate that the increase in proline levels might be one of the metabolic responses triggered in the translocation pathway that links the perception of many environmental stresses to the elicitation of physiological responses at the cellular level [49]. Consistent with these results, lettuce plants affected by water stress were characterized by increased proline concentration than their control comparatives [50]. Also, Misra & Saxena [51] cited that lentil plants grown under stress conditions could accumulate proline to a level higher than that in the unstressed plants. Similar trend was also recorded for different plant species in other studies [52,53].

Water deficit enhances the accumulation of proline in many plant species, where proline is probably the most widely-distributed osmolyte [54]. Various roles of proline have been proposed but the main roles could be the osmotic adjustment in osmotically-stressed plant tissues, the protection of plasma membrane integrity [55] and being a source for carbon and nitrogen [56]. De-Lacerda *et al.* [57] and Kavi *et al.* [58] reported that proline could accumulate in response to several environmental types of stress to protect the cell by balancing the osmotic strength of cytosol with that of the vacuole and external environment. Proline accumulation could be a protective response, not only due to the osmo protectant role of proline that prevents stress-induced water deficit, but also for its radical scavenger and protein stabilizing properties [59]. Moreover, Ford & Wilson [60] proposed that proline could play an indirect role in osmo regulation by increasing the water-binding capacity of plant cell walls to maintain the hydration of protoplasm and to increase membrane permeability.

Organic acids, as micro molecular organic compounds, not only participate in maintaining ionic balance as anions but also cause osmotic adjustment as cell osmolytes. Therefore, the accumulation of organic acids appears to be a specific physiological response of plants to water stress [22]. In the present investigation, the content of organic acids (mainly citric and keto acids) in the leaf sap of stressed wheat plants was higher than that in the unstressed ones (Figure 2). The regulation of organic acid metabolism plays a key role when plants encounter unfavorable conditions [22].

In agreement with these findings, when *Puccinellia Tenuiflora* seedlings were water stressed, the total concentration of organic acids in the shoots was found to increase strongly with increasing the stress level [22]. The organic acids accumulated were suggested to be not only an important organic osmotic regulator, but also an important negative charge contributor, playing important roles in ionic balance and pH adjustment [22]. Excess or deficiency of any mineral nutrient is crucial for the reason that the plant growth depends on supply of inorganic nutrients [61]. Although organic compounds are the major compounds of osmo regulation in plant cells during water deficit stress, inorganic ions would also contribute

to the osmotic adjustment [22]. In addition, synthesis and accumulation of organic solutes consume more energy than uptake of inorganic ions [62].

The concentration of mineral elements in plants is usually influenced by various agronomic and environmental factors such as water stress [63]. The most surprising result of the present study is that, water stress in the absence of salinity in the root zone, induced a conspicuous increase in ions content (Na^+, K^+ (non significant increase in sensitive cultivar), Ca^{2+}, and Cl^-)and Na^+/ K^+ ratioin flag leaf of both cultivars during grain-filling (Figures 3,4). In agreement with our results, research reports indicated that water stress could increase sodium level in various plant parts [64]. Also, potassium content was reported to be increased due to water stress [65]. Moreover, water stress has been shown to cause significant increase in calcium content of different plant species [66,65]. In this regard, Pitman [67] stated that plants have two strategies for maintenance of osmotic content; one is to absorb ions from the soil, if possible, and the other is to form organic solutes. Also, Alam [68] reported that water stress favored an increase in Na^+, K^+ and Ca^{2+} amounts.

An important role of Na^+ in plants is related to its involvement in photosynthesis. It was reported that an increased Na^+ concentration in plants experiencing water stress may be related to an increase in the metabolic requirement of Na^+ to sustain photo synthesis under these conditions [69]. In addition, Na^+ has been reported to be involved in the maintenance of mesophyll chloroplast structure, mainly in relation to granal stacking and thus, Na^+-deficient plants exhibit a wide range of chlorophyll a fluorescence perturbations [70]. Moreover, Na^+ may also be involved in their generation of phosphorenolpyruvate in mesophyll chloroplasts because Na^+ gradient across the envelope could be an alternative energy source for the active transport of pyruvate [71]. An increase in Na^+ uptake could then be the consequence of a stress-induced decrease in the efficiency of the Na^+/ pyruvate co transport system [72].

Potassium is generally thought to be the most important cation in mesophytes under drought stress [73]. Sinha [74] reported that drought-tolerant wheat varieties can accumulate more K^+ than the susceptible varieties, and plants well supplied with K^+ had higher stomatal resistance, which results in low transpiration rate. K^+ is known to function in osmotic adjustment in the guard cell controlling the stomatal movements and thus CO_2 assimilation in photosynthesis [75, 76]. Similarly, changes in the potassium content may contribute substantially to osmo regulation [77] and may occur in concert with changes in sugars and amino acids [78]. Calcium is known to play an important role in processes that preserve the structural and functional integrity of plant membranes, regulate ion transport and control activities of cell wall enzymes [79]. Moreover, many researches showed that calcium signals decoding elements are involved in ABA-induced stomatal closure and plant adaptation to abiotic stresses and some new studies show that Ca^{2+} is dissolved in water in the apoplast and transported primarily from root to shoot through the transpiration stream [80]. Nayyar [81] found that Ca^{2+} appeared to reduce the devastating effects of stress on wheat plants by elevating the content of proline and glycine betaine, thus improving water status and growth of the stressed plants and minimizing the injury to membranes. The increase in Ca^{2+} level as a result of water stress may enhance the tolerance of wheat plants to drought stress since Ca^{2+} is a non-toxic inorganic nutrient and has a function of detoxification under saline medium [82]. Moreover, plants respond directly and specifically to Na^+ within seconds by increasing cytosolic Ca^{2+} [83].

The recorded increase in sodium, potassium and calcium content in response to drought stress may be an adaptive feature of a tolerant variety (Sahel-1) in a trial to increase the cellular osmotic pressure that would help to maintain more water. This induction in Na^+/ K^+ ratio in wheat leaves with water stress might be due to competition of Na^+ with K^+ where this competition could be at uptake level and/or transport level. Since maintenance of a low cytosolic Na^+/ K^+ ratio is a key feature of plant tolerance, as indicated by Cuin *et al.* [84] and Kronzucker *et al.* [85]. Additionally, intracellular K^+ and Na^+ homeostasis bears importance for the activities of many cytosolic enzymes, maintaining membrane potential and a suitable osmoticum for cell volume regulation [77, 83].

Conclusion

The present results indicated that SA and/or Tre induced additional increase in the measured osmotic pressure and osmolytes concentration (proline, total soluble nitrogen, total soluble sugars, ions content (Na^+, K^+, Ca^{2+}, and Cl^-)as well as Na^+/K^+ in wheat plants subjected to water deficit. In fact, salicylic acid caused an additional increase in wheat osmotic pressure as well as osmolytes and these results are similar to those obtained by Chinnusamy & Zhu [86]. Similar results were observed by ShiraniBidabadi *et al.* [87] who reported that proline content of banana shoot tips were significantly increased by the application of SA under drought stress conditions. An increase in proline concentration in SA-treated plants under normal and stress conditions was also observed. Proline can thus be considered as an important

component in the spectra of SA-induced ABA-mediated protective reactions of wheat plants in response to drought, contributing to a reduction in the injurious effects of drought and an acceleration of the reparation processes following stress, evidencing the protective action of SA on wheat plants [88]. However, exogenously applied Tre is readily accumulated and transported by leaf or roots tissues and displays significant roles as osmo protectant [89]. Previous studies proved trehalose as efficient protectant against drought stress or under water deficit conditions [90,91]. Based on these results, we conclude that the drought exposure imposed evident on osmolytes. On the other hand, the exogenous application of salicylic acid (SA), trehalose (Tre) or their interaction appeared to mitigate this damage effect of drought with different magnitude through accumulation of osmolytes in flag leaf of both wheat cultivars (i.e. total soluble sugar, total soluble nitrogen, proline, keto acids, citric acid as well as ionic content (i.e. Na^+, K^+, Ca^{2+}, Cl^-, Na^+/K^+ ratio)) in relation to osmotic adjustment.

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