ANALYSIS OF SECONDARY METABOLISM AND TOTAL CHLOROPHYLL CONTENT PROVIDES NEW INSIGHTS INTO THE ROLE OF A-TOCOPHEROL FOR WILD TYPE AND VTE4 MUTANT Arabidopsis thaliana UNDER DIFFERENT ABIOTIC STRESSES

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Plants experience different abiotic stresses under natural conditions including salinity, water deficit, low temperature and high light. Once plants are exposed to these stresses they might have a variety of responses physiologically and biochemically. In this study, we test this hypothesis in wild type Col-0 and vte4 mutant of Arabidopsis thaliana by measuring major secondary metabolites alongside with total chlorophyll content under different abiotic stresses namely salt stress, water stress and prolonged water deficiency. These stresses were imposed to the plants in separate experiments in which each treatment was replicated three times in a complete randomized design with factorial arrangement. It was concluded that under all abiotic stresses wild type Col-0 Arabidopsis plants showed stronger performance in terms of all major metabolites

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compared to \textit{vte}_4 mutant. \(\alpha\)-tocopherol deficiency in \textit{vte}_4 mutant plants led to lower accumulation of proline, total protein and total amino acids as well as starch and total sugars in comparison with wild type \textit{A.thaliana}. Furthermore, all five secondary metabolites obtained the highest value under 100mM NaCl concentration (Salt stress), under 50% of field capacity (water stress) and under 8 days of water withholding (prolonged water deficiency). Wild type Col-0 resulted in higher level of total chlorophyll content under all abiotic stresses compared to mutant plants. Therefore, our results suggested that the loss of \(\alpha\)-tocopherol in \textit{vte}_4 mutant \textit{A.thaliana} under different abiotic stresses affected the efficiency and the stability of central metabolism and photosynthetic apparatus.

\textit{Keywords:} Arabidopsis thaliana, abiotic stresses, mutantm secondary metabolites, Wild type

INTRODUCTION

Abiotic stresses such as water deficit and salinity are the most common environmental stresses limiting plant productivity (SHAFIEI-ZARGAR \textit{et al}., 2013). It is intrinsic to most abiotic forms of stress, not only during drought, but also when the temperature is low and soil contains very high concentration of ions. The severity of drought is unpredictable as it depends on many factors such as occurrence and distribution of rainfall, evaporative demands and moisture storing capacity of soils (WERY \textit{et al}., 1993). Water stress situations can be classified as either terminal or intermittent. During terminal drought, the availability of soil water decreases progressively and this leads to premature plant death. Intermittent drought is the result of finite periods of inadequate irrigation occurring at one or more intervals during the growing season and is not necessarily lethal (NEUMANN, 2008). The effect of water stress range from morphological, physiological to molecular levels and are evident at all physiological stages of plant growth at whatever stage the water deficit takes place.

Salt stress is a condition in which excessive amount of salts in soil solution can cause inhibition of plant growth. It has been stated that, no other toxic substance inhibits plant growth more than salt. Salt stress indicates an enhancing threat to agricultural products. Many studies have shown that among the different sources of soil salinity, poor drainage alongside with irrigation is the most serious factor, due to the fact that it represents productivity losses of agricultural land. The stresses caused by a high concentration of salt in the soil solution are 2-fold. Initially, many of the salt ions at high concentrations are toxic to plant cells whether it is external or internal. Typically, sodium chloride constitutes the majority of the salts. For most plants sodium ions are toxic, and some plants are affected by high concentrations of chloride ions as well. Moreover, high salt indicates an osmotic stress or water deficit as the result of diminished osmotic potential in the soil solution (ZHU, 2007).

In order to produce plants with tolerant or resistant response to different abiotic stresses such as water deficit and salt stress, various methods have been used. The breeding techniques alongside with novel genetic approaches have provided the paradigm to achieve this goal (KHALATBARI \textit{et al}., 2007; NAROUJI-RAD \textit{et al}., 2013). The use of forward genetics screens has led to the cloning and identification of a spectrum of genes that play central rules in such processes as intra- and intercellular communication and the responses of plant to the environment regarding central metabolism. The feat, which results in the largest so-called “knock out” gene
collection of a complex multi-cellular organism, now allows researchers to study the function of each of those genes individually or together. Knocking out a gene or a group of genes allows scientists to observe what goes wrong in the mutant plant and determine what functions the inactivated gene(s) had in the plant (MEINKE et al., 2003). Recent studies using Arabidopsis thaliana mutant vte4 have provided experimental evidence supporting the protective role of tocopherols against oxidative stress (KANWISCHER et al., 2005) as well as beneficial effects on seed longevity and germination as a result of lipid stabilization by tocopherols in plants (SATTLER et al., 2006). The analysis of Arabidopsis wild type and vte4 mutant (lack the α-tocopherol) growth, development and secondary metabolites can produce a methodology in order to identify and interpret phenotypic differences in plants caused by genetic variation and environmental stress (BOYES et al., 2001). The objectives of this study were: 1) to examine the changes in major secondary metabolites and total chlorophyll content in wild type and vte4 mutant Arabidopsis under different abiotic stresses including water stress, prolonged water deficiency and salt stress 2) to investigate the mechanism of α-tocopherol function in terms of water and salt stress attributes 3) to evaluate the effect of plant type including vte4 mutant and wild type Col-0 on biochemical and physiological traits.

MATERIALS AND METHODS

Plant Material, Growth Conditions, and Harvests

Seeds of A.thaliana, genotype Colombia-0 and vte4 mutant, were used in this experiment (The seeds were a gift from Dean DellaPenna, Department of Biochemistry, Michigan State University), and rinsed in running tap water. Seeds were sterilized in 70% (v/v) ethyl alcohol for 1 min, followed by rinsing twice with sterile deionized water. After thorough rinsing in sterilized water, the surface-sterilized seeds were sown in soil mix of commercial potting soil/vermiculate (2:1) kept under a 12 h photoperiod at 20°C under standard cool white fluorescent bulbs at a photon flux density of 120-150 μmol m^-2 s^-1 in growth chamber. Germination started within 2 to 4 days of sowing. The range of humidity was 50-75%.

During the first experiment (different water regimes including 50%, 75% and 100% of field capacity), for secondary metabolites and total chlorophyll content analyses, harvest was performed by day 20 after onset of water stress at the end of the photoperiod. For the second experiment (different water frequency including 4 days of water withholding, 8 days of water withholding and control condition) harvest was performed after 24 days of water deficit initiation and for the third experiment (Different NaCl concentrations including 50mM, 100mM and control condition) harvest was performed by day 11 of salt stress onset at the end of the photoperiod.

Water and Salt Stress treatments

For the first experiment, controlled watering was imposed to all plants until stage 1.04 (4th leaf is approximately 1 mm in size), after which watering continued for control plants, but was stopped for stressed plants until soil relative water content reached a target value corresponding to a moderate water deficit (0.25 g water g-1 dry soil; corresponding to a predawn water potential of −0.6 MPa) or to a severe water deficit (0.18 g water g-1 dry soil; corresponding to a predawn water potential of −1.1 MPa) (HUMMEL et al., 2010). Once the target soil water content was reached, after 3 to 5 d, it was kept constant by daily watering until plant harvest.
For the second experiment, Controlled watering was imposed to all plants until stage 1.04 (4th leaf is approximately 1 mm in size), after which watering continued for control plants (daily watering with soil relative water content of 0.35-0.45 g water/g dry soil; corresponding to a predawn water potential of -0.35 MPa), but then 4 days and 8 days interval was imposed to wild type and vte4 mutant plants with the same soil water content of control plants (HUMMEL et al., 2010).

For the third experiment, Controlled watering was imposed to all plants until stage 1.04 (4th leaf is approximately 1 mm in size), after which watering continued for control plants (daily watering with soil relative water content of 0.35-0.45 g water/g dry soil; corresponding to a predawn water potential of -0.35 MPa), but then two NaCl concentrations of 50mM and 100mM were added to wild type and vte4 mutant plants with the same controlled watering (HUMMEL et al., 2010).

**Metabolite Measurements**

For metabolite analysis four independent randomly picked control wild-type and vte4 mutant plants were used per treatment for each replication. Harvest of whole rosettes for the experiments was performed and quickly was frozen under liquid nitrogen for the analysis. The wild type and vte4 mutants were analysed for total soluble sugars, starch, total soluble protein, proline and total amino acids via spectrophotometric analysis. Arabidopsis leaf tissues were ground in liquid nitrogen then metabolites were extracted twice with 80% ethanol and once with 50% ethanol (CROSS et al., 2006).

Reaction mix for starch included 0.6 mg of extract, 80 mM HEPES, pH 7.0, 2.5 mM magnesium chloride, 0.95 mg/mL NADP, 1.6 mg/mL ATP, and 0.6 units Glc6P dehydrogenase grade I in a final volume of 200 mL. Optical density was read at 535 nm (HENDRIKS et al., 2003). One reaction for total sugar measurements contained 1.14 mg of extract, 3 mM magnesium chloride, 1.25 mM ATP, 0.75 mM NADP, 0.7 units of Glc6P dehydrogenase grade I, 0.5 mM MTT, 0.25 units of diaphorase, and 0.1% Triton X in a total volume of 200 mL. Optical density was read at 570 nm (HENDRIKS et al., 2003). Assay of total amino acids is based on the reaction of amino acids described by POLAK et al. (2001). Reaction mix harbor 0.038 mg of extract, 7.2 mM sodium borate, pH 8.0 in a final volume of 207 mL. Optical density was read at 595 nm. Protein amounts were assessed as described by BRADFORD (1976). Reaction mix for protein contained Coomassie Brilliant Blue G-250 (100 mg) dissolved in 50 ml 95% ethanol. To this solution 100 ml 85% (w/v) phosphoric acid was added. Optimal density was read at 595 nm. Proline content was determined using ninhydrin in acidic reaction (BATES et al., 1973). Acid-ninhydrin was prepared by warming 1.25 g ninhydrin in 30 ml glacial acetic acid and 20 ml 6mM phosphoric acid, with agitation, until dissolved. Optimal density was read at 520 nm.

**Chlorophyll Content**

To estimate the chlorophyll content of rosettes a SPAD-502 chlorophyll meter (Minolta Co., Ltd.) was used. The SPAD-502 measures peak chlorophyll absorbance at 650 nm and non-chlorophyll absorbance at 940 nm. The mechanism of SPAD is a microprocessor that calculates the relative optical density, based on the ratio of absorbencies of the two wavelengths. The area measured by the equipment is very small (6mm²), thus 4 readings of each rosette from each sample of per treatment and replication were taken and then averaged (KANWISCHER et al., 2005).
Experimental design and Statistical analysis

Three watering regimes (representing 100% of field capacity, 75% of field capacity and 50% of field capacity), three water frequencies (control condition, 4 days and 8 days of water withholding) and three different salt treatments (including 50mM, 100mM of NaCl concentration along with control condition) were imposed on the plants. Each treatment was replicated three times in a complete randomized design (CRD) with factorial arrangement. Data was analysed based on simple ANOVA, using SAS computer package (SAS Institute Inc., 2007). Duncan New Multiple Range Test (DNMRT) was used for comparison of means of quantitative traits. For metabolite analysis four independent randomly picked control wild-type and vte4 mutant plants were used per treatment for each replication.

RESULTS

Different abiotic stresses including water stress, prolonged water deficiency and salt stress showed significant effects on both wild type and vte4 mutant Arabidopsis plants. Apparent differences were observed in terms of five major secondary metabolites and chlorophyll content in both wild type Col-0 and vte4 mutant A.thaliana. However, no interaction was observed between different abiotic stresses and plant type in this study (Table 1, 2 and 3).

Table 1: ANOVA factorial arrangement based on complete randomized designed (CRD) for secondary metabolites parameters of vte4 mutant and wild type Arabidopsis thaliana after 20 days of water stress onset.

<table>
<thead>
<tr>
<th>SOV</th>
<th>d.f</th>
<th>Proline</th>
<th>Total Protein</th>
<th>Total Amino acids</th>
<th>Total sugars</th>
<th>Starch</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>2</td>
<td>3609.38**</td>
<td>1774.88**</td>
<td>228.16**</td>
<td>92.17**</td>
<td>1451.16**</td>
</tr>
<tr>
<td>B</td>
<td>1</td>
<td>10.88**</td>
<td>32.00**</td>
<td>34.72**</td>
<td>18.00**</td>
<td>43.55**</td>
</tr>
<tr>
<td>AB</td>
<td>2</td>
<td>0.05ns</td>
<td>0.66ns</td>
<td>0.05ns</td>
<td>0.16ns</td>
<td>0.05ns</td>
</tr>
<tr>
<td>CV</td>
<td>2.85</td>
<td>3.05</td>
<td>5.47</td>
<td>7.52</td>
<td>2.31</td>
<td></td>
</tr>
</tbody>
</table>

**, Significant at (P < 0.01), *, Significant at (P < 0.05), ns: not significant
The Impact of Water Stress on Different Secondary Metabolites in Arabidopsis Plants

The concentrations of 5 major secondary metabolites including proline, total protein, total amino acids, total sugars and starch were studied (Table 4). Analysis of variance showed the accumulation of proline under water stress in which the highest concentration was observed for both wild type and vte4 mutant plants under 50% of field capacity with the value of 58.66 µmol/gFW rosette after 20 days of water stress onset. Moderate-stressed plant (75% field
capacity) obtained 44.16 µmol/gFW rosette in which the lowest score belonged to control condition (10.83 µmol/gFW). When total protein was measured it scored the lowest concentration of 14.34 µmol/gFW rosette under 100% of field capacity. It should be noted that when severe stress (50% field capacity) was experienced by plants, total protein indicated 48.67 µmol/gFW rosette by day 20 of stress initiation (Table 4).

Table 4: Impact of three water regimes on secondary metabolite parameters of wild type and vte mutant Arabidopsis thaliana after 20 days of water stress onset.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Water treatments (%) of field capacity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(50%)</td>
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<tr>
<td>Proline (µmol/g FW rosette)</td>
<td>58.6a</td>
</tr>
<tr>
<td>Total Protein (µmol/g FW rosette)</td>
<td>48.6a</td>
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<tr>
<td>Total Amino acids (µmol/g FW rosette)</td>
<td>18.84a</td>
</tr>
<tr>
<td>Total Sugars (µmol/g FW rosette)</td>
<td>11.00a</td>
</tr>
<tr>
<td>Starch (µmol/g FW rosette)</td>
<td>49.17a</td>
</tr>
</tbody>
</table>

Means in the same row followed by different letters indicate a significant difference according to DMRT (Pc 0.05).

FW fresh weight

In accordance with the results gained for both proline and total protein, other selected metabolites (total amino acids, total sugars and starch) accumulated under water stress (Table 4). Total amino acids indicated an increasing trend as stress intensity mounted. The highest score went to 50% of field capacity with 18.84 µmol/gFW rosette after 20 days of water stress onset (Table 4). Under 70% field capacity, the accumulation of 11 µmol/gFW rosette was scored while control condition showed the lowest value of 6.67 µmol/gFW rosette. The measurement of total sugars was done where it obtained the scores of 11, 6.84 and 3.17 µmol/gFW rosette under severe stress, moderate stress and control condition by day 20 of stress onset, respectively (Table 4). To estimate the effect of water stress on carbohydrate components such as starch, its concentration was determined (Table 4). Starch concentration enhanced as the plants were subjected to different water regimes. The highest score belonged to severe stress with 49.17 µmol/gFW rosette. The moderate-stressed (75% field capacity) and well-watered plants (100% field capacity) gained 38.33 and 18.50 µmol/gFW rosette after 20 days of stress initiation, respectively (Table 4).

In order to see whether the plant types have an impact on metabolites under water stress or not, vte4 and wild type plants were examined (Table 5). When the stress was imposed to plants, wild type plant presented higher concentration of proline (38.66 µmol/gFW rosette) compared to vte4 mutant (37.11 µmol/gFW rosette) after 20 days of stress initiation (Table 5). The similar outcome was obtained for total protein where it scored the values of 32.22 and 29.55 µmol/gFW rosette for wild type and mutant plants, respectively. The concentrations of amino acids were recorded in which vte4 mutant gained 10.78 µmol/gFW rosette. In comparison with
mutant plant, wild type Arabidopsis thaliana indicated the higher level of accumulation in total amino acids with the value of 13.59µmol/gFW rosette (Table 5). The concentrations of total sugars and starch were determined in order to observe the effect of plant type on these two parameters (Table 5). By day 20 of stress initiation, the higher level of accumulation in total sugars belonged to wild type Col-0 which it scored 8 µmol/gFW rosette while mutant plant obtained the value of 6 µmol/gFW rosette. Following the same pattern, concentration of starch for wild type and vte4 mutant Arabidopsis thaliana were 36.88 and 33.77 µmol/gFW rosette after 20 days of stress onset, respectively (Table 5).

Table 5: Impact of plant types (wild type and vte4 mutant) on secondary metabolites parameters of Arabidopsis thaliana after 20 days of water stress onset.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Arabidopsis thaliana plant type</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild type</td>
</tr>
<tr>
<td>Proline (µmol/g FW rosette)</td>
<td>38.66a</td>
</tr>
<tr>
<td>Total Protein (µmol/g FW rosette)</td>
<td>32.22a</td>
</tr>
<tr>
<td>Total Amino acids (µmol/g FW rosette)</td>
<td>13.59a</td>
</tr>
<tr>
<td>Total Sugars (µmol/g FW rosette)</td>
<td>8.00a</td>
</tr>
<tr>
<td>Starch (µmol/g FW rosette)</td>
<td>36.88a</td>
</tr>
</tbody>
</table>

Means in the same row followed by different letters indicate a significant difference according to DMRT (P≤0.05).

FW fresh weight

The Impact of Prolonged Water Deficiency on Major Metabolites in Arabidopsis Plants

In this study, the main metabolites (proline, total protein, total amino acids, total sugars and starch) in central metabolism were examined (Table 6). Analysis of variance indicated the increase in proline under prolonged water deficiency (water frequency) in which the highest concentration was obtained for both wild type and vte4 mutant plants from 8 days of water withholding with the value of 63.33 µmol/gFW rosette after 24 days of water deficit onset. Moderate-stressed plant (4 days of water withholding) gained 45 µmol/gFW rosette in which the lowest score belonged to control condition which scored 12.34 µmol/gFW. When total protein was measured it scored the lowest concentration of 12.67 µmol/gFW rosette under control condition. Furthermore, when 8 days of water withholding was imposed to plants, total protein recorded 64.42 µmol/gFW rosette by day 24 of stress onset (Table 6). Following the same trend obtained by both proline and total protein, total amino acids, total sugars and starch accumulation were provoked under water deficiency (Table 6). Total amino acids demonstrated an enhancing trend while stress intensified. The highest value went to 8 days of water withholding with 24.16 µmol/gFW rosette after 24 days of water deficit initiation (Table 6). When 4 days of water withholding was imposed, the accumulation of 14 µmol/gFW rosette was obtained in which control condition indicated the lowest value of 9 µmol/gFW
rosette. Total sugars was measured where it gained the scores of 13, 7.16 and 4.18 µmol/gFW rosette under 8 days of water withholding, 4 days of water withholding and control condition by day 24 of stress onset, respectively (Table 6). To determine the effect of prolonged water deficiency on concentration of starch, plants were studied regarding this matter (Table 6). Starch concentration escalated as the plants experienced different water regimes. The highest score went to severe stress (8 days of water withholding) with 63.20 µmol/gFW rosette. The moderate-stressed (4 days of water withholding) and well-watered plants (control condition) obtained 49.34 and 20.39 µmol/gFW rosette by day 24 of water deficit onset, respectively (Table 6).

Table 6: Impact of three water frequency (prolonged water deficiency) on secondary metabolites parameters of wild type and vte₄ mutant Arabidopsis thaliana after 24 days of water deficit onset.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Water treatments (frequency)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(8 days)</td>
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<tr>
<td>Proline (µmol/g FW rosette)</td>
<td>63.33¹</td>
</tr>
<tr>
<td>Total Protein (µmol/g FW rosette)</td>
<td>64.42¹</td>
</tr>
<tr>
<td>Total Amino acids (µmol/g FW rosette)</td>
<td>24.16²</td>
</tr>
<tr>
<td>Total Sugars (µmol/g FW rosette)</td>
<td>13.00²</td>
</tr>
<tr>
<td>Starch (µmol/g FW rosette)</td>
<td>63.20¹</td>
</tr>
</tbody>
</table>

Means in the same row followed by different letters indicate a significant difference according to DMRT (P≤ 0.05).
FW=dry weight

To study the effect of plant types on secondary metabolites under prolonged water deficiency, vte₄ and wild type plants were examined (Table 7). When the stress was experienced by plants, wild type plant showed higher concentration of proline with 41.55 µmol/gFW rosette in comparison with vte₄ mutant (38.89 µmol/gFW rosette) after 24 days of stress onset (Table 7). The same result was gained for total protein in which it scored the values of 37.90 and 33.55 µmol/gFW rosette for wild type and vte₄ mutant plants, respectively (Table 7). The concentrations of total amino acids were determined where mutant obtained 14.55 µmol/gFW rosette. Compared to vte₄ mutant plant, wild type Arabidopsis thaliana demonstrated the higher level of accumulation in total amino acids with the score of 16.91 µmol/gFW rosette (Table 7). The concentrations of total sugars and starch were studied to determine the impact of plant type on these parameters (Table 7). By day 24 of water deficit onset, the higher level of accumulation in total sugars belonged to wild type Col-0 where it obtained 9.11 µmol/gFW rosette while mutant plant recorded the value of 7.12 µmol/gFW rosette. Concentration of starch for wild type and vte₄ mutant Arabidopsis thaliana indicated the similar pattern with 45.77 and 42.80 µmol/gFW rosette after 24 days of stress initiation, respectively (Table 7).
The Impact of Salt Stress on Different Secondary Metabolites in Arabidopsis Plants

To investigate the impact of salt stress on central metabolism of *Arabidopsis thaliana*, major secondary metabolites (proline, total protein, total amino acids, total sugars and starch) were studied for wild type Col-0 and *vte4* mutant plants (Table 8). Analysis of variance showed the accumulation of proline under salinity in which the highest concentration was observed for both wild type and *vte4* mutant plants under severe stress (100mM NaCl) with the value of 67.17 µmol/gFW rosette after 11 days of salt stress onset. Moderate-stressed plant (50mM NaCl) gained 49.19 µmol/gFW rosette whereas the lowest value of 11.50 µmol/gFW rosette belonged to control condition. When total protein was examined, it obtained the lowest concentration of 12.70 µmol/gFW rosette under control condition. In addition, when severe stress (100mM NaCl) was experienced by plants, total protein indicated 67.50 µmol/gFW rosette by day 11 of stress initiation (Table 8). Total amino acids showed an enhancement in its concentration when plants were exposed to different levels of salinity. The highest value went to severe stress (100mM NaCl) with 26.83 µmol/gFW rosette after 11 days of salt stress onset (Table 8). With 50Mm NaCl, the accumulation of 16.33 µmol/gFW rosette was scored whereas control condition showed the lowest value of 9.41 µmol/gFW rosette. Furthermore, total sugars concentration was measured where it recorded the scores of 13.84, 8.85 and 3.36 µmol/gFW rosette under severe stress, moderate stress and control condition by day 11 of salt stress onset, respectively (Table 8). Starch as one of the main carbohydrate components was studied in order to determine the impact of salinity on this parameter in wild type and *vte4* mutant *Arabidopsis* plants (Table 8). The highest value was recorded for severe stress (100mM NaCl) with 73.50 µmol/gFW rosette. The moderate-stressed (50mM NaCl) and controlled plants gained 53.71 and 21 µmol/gFW rosette after 11 days of salt stress onset, respectively (Table 8).

Table 7: Impact of plant types (wild type and *vte4* mutant) on secondary metabolites parameters of *Arabidopsis thaliana* after 24 days of water deficit onset (prolonged water deficiency).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Arabidopsis thaliana plant type</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild type</td>
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<tr>
<td>Proline (µmol/g FW rosette)</td>
<td>41.55b</td>
</tr>
<tr>
<td>Total Protein (µmol/g FW rosette)</td>
<td>37.90b</td>
</tr>
<tr>
<td>Total Amino acids (µmol/g FW rosette)</td>
<td>16.91b</td>
</tr>
<tr>
<td>Total Sugars (µmol/g FW rosette)</td>
<td>9.11b</td>
</tr>
<tr>
<td>Starch (µmol/g FW rosette)</td>
<td>45.77b</td>
</tr>
</tbody>
</table>

Means in the same row followed by different letters indicate a significant difference according to DMRT (*P*≤0.05). FW=fresh weight.
In order to investigate the impact of plant types on secondary metabolites under salt stress, vte4 and wild type plants were studied (Table 9). When salt stress was imposed to plants, wild type plant obtained higher concentration of proline (43.55 µmol/gFW rosette) compared to vte4 mutant (41.66 µmol/gFW rosette) after 11 days of stress initiation (Table 9). Interestingly, the result was same for total protein where it gained the values of 40.89 and 38.44 µmol/gFW rosette for wild type and mutant plants, respectively. The concentrations of total amino acids showed that vte4 mutant gained the lower value of 16.22 µmol/gFW rosette. It was wild type Arabidopsis thaliana which performed the higher level of accumulation in total amino acids where it scored 18.78 µmol/gFW rosette (Table 9).

Additionally, the accumulation rate of other two metabolites including total sugars and starch were observed in terms of plant type in Arabidopsis thaliana (Table 9). After 11 days of stress onset, both parameters in wild type plants performed stronger than the mutant one. Wild type Col-0 and vte4 mutant obtained the values of 9.79 and 7.58 µmol/gFW rosette when total sugars concentration was considered, respectively (Table 9). Consistent with total sugars outcome, starch concentration in wild type Col-0 plant was higher with 50.68 µmol/gFW rosette while mutant Arabidopsis scored 48.11 µmol/gFW rosette by day 11 of salt stress initiation (Table 9).
Chlorophyll Content Alteration in Response to Different Abiotic Stresses

To investigate the effect of water stress on physiological aspect of *Arabidopsis thaliana*, chlorophyll content was studied (Figure 1). When plant type was regarded, wild type plant scored higher than *vte4* mutant *Arabidopsis* (Figure 1 A). Wild type gained the value of 37.56 in which the value for mutant plant was 36.11. After 20 days of water stress onset, different water regimes demonstrated varied results in terms of chlorophyll content (Figure 1 B). The highest value was observed for control condition (100% field capacity) with value of 39.33. The lowest score belonged to 50% field capacity with the value of 33.84 while moderate-stressed plant (75% field capacity) scored 37.41 (Figure 1 B).

**Table 9:** Impact of plant types (wild type and *vte4* mutant) on secondary metabolites parameters of *Arabidopsis thaliana* after 11 days of salt stress onset.

<table>
<thead>
<tr>
<th>Parameters</th>
<th><strong>Arabidopsis thaliana plant type</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild type</td>
</tr>
<tr>
<td>Proline (µmol/g FW rosette)</td>
<td>43.55&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total Protein (µmol/g FW rosette)</td>
<td>40.89&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total Amino acids (µmol/g FW rosette)</td>
<td>18.78&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Total Sugars (µmol/g FW rosette)</td>
<td>9.79&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Starch (µmol/g FW rosette)</td>
<td>50.68&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
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Means in the same row followed by different letters indicate a significant difference according to DMRT (P<0.05).

Figure 1. Effect of water stress on physiological aspect A. plant type, B. water treatment
To determine the impact of prolonged water deficiency on photosynthetic apparatus of *Arabidopsis thaliana*, the same study was conducted (Figure 2). After 24 days of water deficit initiation, different water frequency indicated non-similar results regarding chlorophyll content (Figure 2 A). The highest value went to control condition with 39.17. The lowest value belonged to 8 days of water withholding where it scored 34. In addition, moderate-stressed plant (4 days of water withholding) scored 37.18 (Figure 2 A). When plant type was considered, wild type Col-0 *A.thaliana* gained higher value compared to *vte4* mutant plants (Figure 2 B). Wild type obtained the value of 37.68 while the value for *vte4* mutant plant was 35.89.

![Figure 2. Impact of prolonged water deficiency on photosynthetic apparatus](image)

In addition, the effect of salinity on chlorophyll content of wild type Col-0 and *vte4* mutant *Arabidopsis thaliana* was investigated (Figure 3). Regarding plant type in *Arabidopsis thaliana*, wild type plant recorded higher value compared to *vte4* mutant plants (Figure 3 A). Wild type indicated the value of 33.92 while the value for *vte4* mutant plant was 31.88. By day 11 of salt stress onset, NaCl concentrations resulted in different outcomes in terms of chlorophyll content (Figure 3 B). The highest value belonged to control condition with 39. The lowest value went to 100mM NaCl concentration (severe stress) in which it scored the value of 26.7 whereas moderate-stressed plant (50mM NaCl concentration) scored 33 after 11 days of stress onset (Figure 3 B).
Studying the central metabolism of plants can provide a complementary approach to unfold the impact of different oxidative stresses, which may have been neglected in studies of physiological and morphological responses. This study investigates the accumulation level of five major metabolites and their alteration including total sugars, starch, total amino acids, proline and total protein in wild type Col-0 and vte4 mutant Arabidopsis thaliana. The plants were grown under water stress, salt stress and prolonged water deficiency to characterize the metabolism responses and total chlorophyll content as a part of photosynthetic apparatus. The rationale behind employing various abiotic stresses in this study was to exploit specific stress patterns.

The results indicated that both vte4 mutant and wild type A.thaliana plants obtained the highest value in terms of major secondary metabolites as the intensity of all stress types escalated. This result was in agreement with another study where starch and sucrose level accumulated in A.thaliana plants at the end of photoperiod (HUMMEL et al., 2010).

The primary question addressed in this study was whether water stress and salinity result in oxidative stress due to diminished photosynthesis or other changes in central metabolism.

Figure 3. Effect of salinity on chlorophyll content A. plant type B. salt treatment
metabolism. Numbers of evidence imply that under imposed conditions oxidative stress was experienced by both wild type and vte4 mutant A.thaliana. When 50% of field capacity was adopted, the accumulation of proline and total protein was provoked in which they recorded the value of 58.66 µmol/g FW rosette and 48.67 µmol/g FW rosette, respectively. The same trend was observed for total amino acids, total sugars and starch for both plant types. This outcome was in accordance with another study where the enhancement of main metabolites was reported for 24 different accession of Arabidopsis thaliana (CROSS et al., 2006).

The impacts of abiotic stresses on different aspects of plant physiological responses are interrelated and complicated. It has been suggested that cellular water content mainly controls stomatal aperture. Meanwhile, stomatal conductance has a direct effect on CO2 photosynthetic carbon fixation and diffusion which ultimately affects metabolic functions. Photosynthesis plays a crucial role in terms of plant productivity and is directly affected by salinity and water stress. It is believed that photosynthetic rates of crop leaves diminish as leaf water potential, the relative water content and chlorophyll content reduced (LAWLOR and CORNIC, 2002). The effects of abiotic stress on photosynthesis are complex. In addition, this complexity is consists of combination of the inhibition of central metabolic processes and stomatal closure, including adenosine triphosphate synthesis and ribulose bisphosphate synthesis (CHAVES, 1991; CORNIC, 1994). When total chlorophyll content was determined in our study, the results indicated that under all three stresses the chlorophyll content reduced as both plant types experienced the highest level of stress intensity. Under prolonged water deficiency, when 8 days of water withdrawing was imposed to plants, the value of 34 was obtained after 24 days of water deficit onset. Arabidopsis plants were exposed to different NaCl concentrations in order to investigate the effect of salinity on total chlorophyll content in which 100mM NaCl seemed to have the strongest impact with the value of 26.7 and this result was obtained only after 11 days of salt stress initiation. We have already reported the impact of salinity on growth and development of A.thaliana with the distinctive alterations in both plant types (KHALATBARI et al., 2013).

It is noteworthy that wild type Col-0 A.thaliana scored higher values in all 5 major metabolites compared to vte4 mutant in which γ-TMT gene has been silenced (absence of γ-tocopherol methyltransferase enzyme activity). Mutants with reduced γ-TMT activity have been isolated from Synechocystis (shr0089), sunflower (Helianthus annuus), and Arabidopsis (vte4) with accumulated γ-tocopherol instead of α-tocopherol (SHINTANI and DELLAPENNA, 1998; BERGUMLLER et al., 2003; HASS et al., 2006). It should be noted that our result was not in agreement with previous study where Arabidopsis vte4 mutant was tested for other types of abiotic stress such as heat, high light, and cold tolerance and did not indicate any different stress response in comparison with wild type Col-0 (BERGUMLLER et al., 2003). Recent studies have demonstrated that vitamin E-deficient Arabidopsis thaliana mutants known as vte have lacked the protective function of tocopherol which plays a key role in chloroplasts as an antioxidant. Tocopherols can protect plants from oxidative stresses such as salinity, drought and water deficit and are able to scavenge lipid peroxyl radicals which prevent the propagation of lipid peroxidation, thus tocopherols are considered as excellent quenchers and scavengers of singlet oxygen controlling its levels. The vte4 mutant A.thaliana lacks α-tocopherol due to absence of γ-tocopherol methyltransferase enzyme activity. Therefore, this mutant indicated more susceptibility toward salinity and water stress compared with wild type Col-0 implying this fact that an alteration in tocopherol composition of vte4 mutant plant affects the biochemical and physiological responses of this A.thaliana type to different abiotic stresses.
In our study, we have provided this evidence that α-tocopherol exerts an exclusive function protecting plants from different abiotic stresses such as salinity and water stress in terms of major secondary metabolites and total chlorophyll content. Our outcomes raise a wealth of new questions on the roles of α-tocopherol. Further attempts will be encompassed to unfold the specific functions of tocopherol derivatives in their specific antioxidant activities, applying transgenic A. thaliana.

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REFERENCES


ANALIZA SEKUNDARNIH METABOLITA I UKUPNOG SADRŽAJA HLOOROFILA OMOGUĆAVA NOVI UVID U ULOGU ALFA-TOKOFEROLA KOD DIVLJEG TIPIA I vte4 MUTANTA Arabidopsis thaliana U USLOVIMA RAZLIČITOG ABIOTIČKOG STRESA

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Izvod
Biljke su izložene u prirodnim uslovima gajenja različitim abiotičkim stresu uključujući zaslanjenost, deficit vode, niske temperature i jaku svetlost. Reakcija biljaka može da bude na nivou fizioloških i biohemijskih procesa. Ova hipoteza je testirana poređenjem divljeg genotipa Col-0 i vte4 mutanta Arabidopsis thaliana merenjem glavnih sekundarnih metabolita, i ukupnog hlorofila u uslovima zaslanjenosti, vodnog stresa i produženog vodnog stresa. Ispitivanja su vršena u odvojenim eksperimentima. Na osnovu dobijenih rezultata zaključeno je da su biljke divljeg genotipa Col-0 Arabidopsis pokazale jače osobine kod svih metabolita u poređenju sa vte4 mutantom. Smanjenje α-tokoferola u biljkama vte4 mutanta je dovelo do akumulacije proline, ukupnih proteina i ukupnih aminokiselina kao i skroba i ukupnih šećera u poređenju sa divljim genotipom A.thaliana. Dobijeni rezultati ukazuju da gubitak alfa-tokoferola kod vte4 mutanta A.thaliana u različitim uslovima abiotičkog stresa utiče na efikasnost i stabilnost centralnog metabolizma i fotosintetskog aparata.

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